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SYNTHESIS OF BIODIESEL BY DIRECT TRANSESTERIFICATION OF THE BOTRYOCOCCUS BRAUNII MICROALGAE BIOMASS IN A SYSTEM OF SIMULTANEOUS OIL EXTRACTION AND TRANSESTERIFICATION REACTION

DOCTORAL THESIS IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR IN SCIENCES OF NATURAL RESOURCES

PAMELA ALEJANDRA HIDALGO OPORTO

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"SYNTHESIS OF BIODIESEL BY DIRECT TRANSESTERIFICATION OF THE BOTRYOCOCCUS BRAUNII MICROALGAE BIOMASS IN A SYSTEM OF SIMULTANEOUS OIL EXTRACTION AND TRANSESTERIFICATION REACTION"

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PAMELA ALEJANDRA HIDALGO OPORTO

....
**Director Programa de Postgrado
Doctorado en Ciencias de
Recursos Naturales**

....
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Dra. Maria Elena Lienqueo

....
Dr. Germán Aroca

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....
Dr. Gustavo Ciudad

....
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Universidad De La Frontera

                                                                       ....
                                                                       Dr. Raúl Muñóz T

… para Sebastián

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Summary and outline of this thesis

The *in-situ* or direct transesterification is a technique of great value, because it is able to extract and convert triglycerides into alkyl esters of fatty acid (or biodiesel) in a single step, bypassing the use of large quantities of organic solvents of lipid extraction stage.

The outline of this thesis begins with a general introduction. In Chapter 1, we address the general objectives of our thesis with regards to the development of a technology for the production of biodiesel by *in-situ* transesterification from the biomass of the microalga of *Botryococcus brauni.* The hypothesis raised was, that a continuous extraction system of microalgae biomass using a mixture of methanol/co-solvent and acid catalyst could promote the oil extraction, esterification and transesterification reactions simultaneously to reach higher productivity compared to a conventional biodiesel production process.

In Chapter 2, we made a literature review of advances in direct transesterification of microalgal biomass for biodiesel production, including: different catalyst, critical parameters and novel approach for this process. Microalgae biomass is an interesting raw material to produce biodiesel. However, there are several drawbacks that must be solved before their industrial application in biodiesel production by using transesterification as conversion process. The main problems are related with the high water content of the biomass (over 80%) and the several process necessaries for its production such as: drying, cell disruption, oils extraction, transesterification and biodiesel refining. In comparison with traditional method (extraction and transesterification), the use of direct transesterification could be a cheaper alternative since the cell disruption, lipid extraction and transesterification are carried out in one step, with a direct reaction of oil-bearing biomass to biodiesel.

In Chapter 3, we show different operational strategies for biodiesel production by direct transesterification of microalgal biomass (*Botryococcus braunii*). These operational strategies were performed in two reaction systems: a conventional stirred batch reactor (SBR) and a reflux extraction reactor (RER). This evaluation included the use of different acyl acceptors, also of the application of different acid catalysts and solvent mixtures. The highest biodiesel production yields (80.6%) were obtained in the RER, using methanol as acyl acceptor and H_2SO_4 as catalyst. On the opposite, the lowest biodiesel production yield (64.5% wt) was observed in the SBR system using methanol

In Chapter 4, we observed an improvement in the FAME yield of *in situ* transesterification from microalgal biomass through particle size reduction in a stirred batch reactor. In this study, three particle size fractions (Size 1: <150 µm; Size 2: 150 µm< D< 500 µm; Size 3: >500 µm) were studied. According to the obtained results, the best FAME yield was obtained when the particle size decreased due to increment of the specific surface area. It is suggested that the decrease in the particle size increases mass transfer and therefore lipids extraction performance. In addition, mechanical grinding could cause cell wall disruption, enhancing solvent permeability into the cell during to reaction.

In the Chapter 5, we show the feasibility of FAME production from *in-situ* transesterification of biomass microalgae with an acyl acceptor maintained to *continuous reflux*. In this system, lipids could be extracted and then esterified in presence of higher solvent volume, hence favoring product formation. Although in this system does not use any application of shear stress to produce microalgae cell wall disruption, the highest FAME yield were obtained in this configuration due to the fact that the main lipids extraction mechanism was the diffusion. In this configuration, 80% wt of FAME yield was reached, but with the incorporation of a co-solvent into reaction (47% v/v of hexane), this

FAME yield increased to 94% wt. However, at upper levels of co-solvent a decrease on FAME yield was observed. It is suggested a decrease of the selectivity to polar lipid of microalga membranes with the increase co-solvent dosage.

In Chapter 6, we show the feasibility of FAME production from wet microalgal biomass using a SBR, besides addressing the evaluation of the reaction kinetics. We observed that the reaction was tolerant to a moisture content of the biomass lower to 30% wt. Regarding reaction kinetics, the proposed model of FFA esterification and acylglycerols transesterification was suitable.

Finally, in Chapter 7 we present a general discussion and conclusion of this work, where the highest FAME yield was obtained in the reflux extraction reactor. However, in this system it is very difficult to calculate the real solvent ratio. Besides, it requires of large reaction time to reach the FAME extraction complete. Instead, in a stirred batch reactor, where the solvent is in direct contact with the biomass, the control of solvent volume is simple.

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General introduction

General Introduction

1.1 Introduction

Today energy is the most important resource for mankind and its sustainable development is becoming one of the global problems confronting the world due to the energy crisis. Major energy resources come from fuels, due to their energy content with significant amounts. Nowadays, there is a strong dependence of our life on fossil fuels such as petrol oil, coal and natural gas, since more than 80% of the world's energy needs are from fossil fuels in the industrial production sector, domestic uses or in the transportation sector. The problem is mainly that the population growth is not covered by domestic crude oil production and its derivatives. In addition, the formation of fossil fuels requires millions of years, hence the petrol fuels are non-renewable as well as the change of the crude oil prices leads to global and international conflicts especially in the developing countries. Renewable energy is considered as one of the most important resources in many countries around the world, which accounts for about 10% of the world's energy consumption and can be converted into other usable forms of energy as biofuels. Liquid biofuels have become a green important alternative fuel that offers several advantages including its renewability, high energy content and low emission profile of carbon dioxide.

Biodiesel is a renewable non-toxic, biodegradable fuel that has a high cetane number and calorific value, low sulfur and aromatic, apart from a high flash point and lubricity (Demirbas, 2007; Ma & Hanna, 1999). In its burning emissions unburned hydrocarbons by over 90% and among 75-90% of polycyclic aromatic hydrocarbons are reduced. In addition, the emissions of particulate matter and carbon monoxide compared with

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petroleum diesel are reduced, although nitrogen oxide emissions are slightly ncreased(Demirbas, 2007).

Biodiesel has been produced mainly from oilseed intended to food consumption, such as rapeseed or soybeans (first-generation fuels), which has limited its development, because it competes directly with food. With the development of fuel of second and third generation, both microalgae and other non-conventional raw materials such as oil palm, jatropha and waste oil, have generated great interest (Berchmans & Hirata, 2008b; Lozada et al., 2010).

Microalgae have several advantages such as a higher rate of CO_2 sequestration (up to 6,24 Kg m^{-3} day^{-1}), contrasting traditional crops (De Schamphelaire & Verstraete, 2009). The highest yields in the production of lipids that the traditional crops. In relation to the volumes of water required for the cultivation of microalgae, they are up to 8 times lower compared with a rapeseed crop, and similar to a corn crop, but up to 10 times higher than switchgrass crops (Dismukes et al., 2008); microalgae can grow even in brackish water such as *Dunaliella salina* or non-arable land (Mata et al., 2010) or in wastewater with high organic matter content, as derived from agricultural waste (Mata et al., 2010; Wang et al., 2008a). Instead, oilseed crops only in fresh water (Dismukes et al., 2008); microalgae also require minor extensions for their cultivation and during the exponential growth, they can duplicate their biomass content in 3.5 h with an oil content between 20% and 50% (on basis of dry weight biomass); The production of different types of lipids and hydrocarbons depending on the species of microalgae is another advantage that they have (Chisti, 2007; Mata et al., 2010)

Since its inception, biodiesel production based on traditional crops has been limited by the high cost of raw material. It is estimated that the cost of oil corresponds to a number near 80% of the total cost of biodiesel production (Shi and Bao, 2008). To reduce this limitation, the research has been focused on finding cheaper feedstocks such as waste oil or non-traditional crops e.g. jatropha or castor, among others.

The research has been focused on the reduction of stages of the process such an alternative for mitigating the high cost of feedstock, or supercritical and direct or *in-situ* transesterifications.

In transesterification with supercritical fluids (or non-catalytic transesterification) steps of removal of the catalyst and of saponified products are eliminated but their high costs limit their use (Cao et al., 2005; Kasteren et al., 2007). In contrast, the *in-situ* transesterification, a direct conversion of biomass to monoester is possible, thereby eliminating the steps of extraction and purification of raw material (Ehimen et al., 2010b; Harrington & D'Arcy-Evans, 1985; Harrington & D'Arcy-Evans, 1985b; Ozgul-Yucel & Turkay, 2002; Özgül & Türkay, 1993; Revellame et al., 2010; Shiu et al., 2010). Higher conversions into biodiesel have been obtained with *in-situ* transesterification compared with the conventional method (Harrington & D'Arcy-Evans, 1985; Harrington & D'Arcy-Evans, 1985b; Kildiran et al., 1996b; Ozgul-Yucel & Turkay, 2002; Özgül & Türkay, 1993; Siler-Marinkovic & Tomasevic, 1998; Yi-Hsu & Shaik, 2005; Zeng et al., 2009b).

Only a few reported studies regarding the production of biodiesel have focused on the production of biodiesel from microalgae, the majority has been limited to the increase of lipid productivity and culturing algae on a large scale (Krohn et al., 2011).

Besides, the *in-situ* transesterification has yielded high conversions to biodiesel in microalgae. However, eliminating the oil extraction step is one of the main challengers in the industrial production of biodiesel from microalgae. Lipid extraction from microalgae is performed by solvent extraction and not by conventional physical methods as expeller due to difficulties in breaking the cell wall, which is composed mainly by algaenan (Ehimen et al., 2010b)

The enormous potential to develop a process of *in-situ* transesterification of biomass to biodiesel is interesting to be evaluated. It could simplify the process and get a more efficient way

1.2 Hypothesis and research objectives

1.2.1 Hypothesis of the thesis:

The mixture of solvents and acid catalyst in a system of simultaneous oil extraction and transesterification promotes oil extraction, increasing the kinetics of esterification and transesterification of lipids, because it is possible to maintain a higher stoichiometric ratio methanol-oil during the reaction.

1.2.2 Research objectives

1.2.2.1 General objective

To improve the productivity in the production of biodiesel by direct transesterification of the *Botryococcus braunii* microalgae biomass in a simultaneous system of oil extraction, esterification and transesterification using a homogeneous acid catalyst.

1.2.2.2 Specific objectives of the thesis

1. To determine the influence of the solvent mixture in lipids extraction yield of *Botryococcus braunii* microalga biomass in a continuous.extraction system

2. To determine the influence of the solvent mixture in the biodiesel production of *Botryococcus braunii* microalga biomass in a system of simultaneous oil extraction and transesterification using a homogeneous acid catalyst.

3. Implementing of a continuous extraction system for oil extraction and biodiesel production from *Botryococcus braunii* microalga biomass using a homogeneous acid catalyst.

Chapter 2

Advances in direct transesterification of microalgal biomass for biodiesel production

Advances in direct transesterification of microalgal biomass for biodiesel production

Hidalgo, P.[1], Toro, C.[1], Ciudad, C.1, Navia, R. [1,2]

[1]Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]Departamento de Ingeniería Química, Universidad de La Frontera, Casilla 54-D, Temuco, Chile[3]

1. Introduction

Increasing attention has been focused on the utilization of microalgae biomass as non-conventional feedstock for biodiesel production. Compared to first generation biodiesel, microalgae strains can be cultivated on non-arable land using both, saline or wastewater with high organic matter content (Mata et al., 2010; Tredici et al., 1992; Wang et al., 2008b; Widjaja et al., 2009). In addition, microalgae are characterized by a high biomass productivity, rapid lipid accumulation and a high CO_2 sequestration rate (up to 6.24 Kg m^{-3} day^{-1}) compared to traditional crops (De Schamphelaire & Verstraete, 2009; Halim et al., 2011; Tramper et al., 2003). According to several reports, lipids content of microalgae can vary between 15 and 75% in dry matter (Chisti, 2007; Mata et al., 2010). Lipids composition depends on the species and culture conditions, as are mainly composed by two fractions: i) saturated and monounsaturated fatty acids and acylglycerols which are suitable for biodiesel production (neutral lipids) and ii) waxes, sterols, ketones, hydrocarbons, carotenes and chlorophylls or unsaponifiable matter not suitable for biodiesel production. This latter fraction interferes in biodiesel production because of its foaming properties, which difficults the separation of the reaction products (Vera et al., 2011). This unsaponifiable matter is not affected during biodiesel preparation and it has no harmful effects in engines, except for a change in the crystallization onset temperature caused by polar sterols (Van Gerpen et al., 1996).

Although there are several research works published in the field of transesterification of lipids derived from oilseeds, only few studies focused on the conversion of microalgae lipids by transesterification are available in the scientific literature. In fact, the main topics already published on biofuels production from microalgae deal with microalgae cultivation

conditions and the selection of most adequate strains, rather than the downstream process (Krohn et al., 2011; Mutanda et al., 2011).

The processes to obtain biodiesel from microalgae by transesterification have several bottlenecks that difficult its industrial production. In fact, there are two main bottlenecks that directly affect the reaction yield: i) to extract internal lipids is energetically demanding, as the cell wall of some species of microalgae can strongly modulate any extraction process and ii) lipids extraction yield is negatively affected in wet biomass. These key issues indicate the necessity of several process steps including biomass drying, cell disruption, lipids extraction, lipids separation, transesterification, and biodiesel purification.

In this sense, direct transesterification of wet microalgae biomass may be considered as a promissory alternative that could simplify the biodiesel production process from microalgae by reducing the number of steps necessary to obtain the biofuel. In this scheme, extraction and transesterification are carried out in one step, with a direct reaction of oil-bearing biomass to biodiesel, avoiding the steps of cell disruption and lipids extraction from the feedstock.

Therefore, the aim of this work was to review the different alternatives to produce biodiesel from microalgae, including the use of the whole biomass for biodiesel production, the use of different catalysts, besides a critical analysis of operational parameters and a novel approach for this process.

2. Microalgae as feedstock biodiesel production

Microalgae are photosynthetic eukaryotic organisms which can produce high-added-value compounds such as hydrocarbons, pigments, carbohydrates, proteins and lipids (Banerjee et

al., 2002; Chisti, 2007; Tran et al., 2009). These microorganisms can accumulate important quantities of lipids (Balat & Balat, 2010), and additionally, they present a fast growth and high productivity compared to agricultural crops (Chisti, 2007; Mata et al., 2010). Therefore, microalgae appear as an important feedstock for producing different types of biofuels (Sim et al., 2001) such as methane, bioethanol and biodiesel (Banerjee et al., 2002; Chisti, 2007; Khan et al., 2009; Melis & Happe, 2001; Spolaore et al., 2006). Besides, from exhausted microalgae biomass (before or after lipid extraction), a series of by-products of high added value can be produced, including nutraceuticals, biopolymers and fertilizers thus advancing in the microalgae biorefinery concept (Dismukes et al., 2008; Loera-Quezada & Olguín, 2010).

Additionally, from an ecological and environmental point of view, microalgae cultures have a smaller ecological footprint because the land area needed for its production is 1-2 orders of magnitude lower than conventional crops (as shown in Table 1). For instance, conventional crops such as soybean can produce approximately 636 L lipids/ha whereas microalgae could produce up to 58,700 L lipids/ha based on 30% lipids content in dried biomass.

Table 1. Comparison of different feedstocks for biodiesel production

Oil crop	Oil yield (L oil/Ha)	Land area (m^2 year/kg biodiesel)	Oil content (% oil by wt in biomass)	Prices (USD/ton)	References
First generation feedstock (Edible oils)					
Soybean	636	18	18	684	(Mata et al., 2010)
Rapeseed	974	12	37-50	683	(Mata et al., 2010)
Palm	5,366	2	36	478	(Mata et al., 2010)
Second generation feedstock					
Jatropha	741	15	Seed: 35-40 Kernel: 50-60	739	(Gui et al., 2008a; Mata et al., 2010; Tomomatsu & Swallow, 2007)
Castor	1307	9	48	1025	(CastorOil, 2012; Mata et al., 2010)
Waste cooking oil	-	-	-	224	(Balat, 2011)
Yellow grease	-	-	-	374	(Balat, 2011)
Third generation feedstock					
Microalgae	58,700-136,900	0.1-0.2	30-70	-	(Mata et al., 2010)

Depending on the specie and the culture conditions the lipids content in microalgae biomass can vary between 15% and 75% w/w, being the most common range between 20 and 30% (Table 2). There are several reports in the literature related to the selection of the best microalgae strain for biodiesel production; however no clear selection criteria has been already establish. Rodolfi *et al.* (2009) found that *Nannochloropsis* is one of the best candidates for lipids production between 30 screened strains of microalgae, due its high lipids content. Lee *et al.* (2010) found that *B. braunii* is the best candidate for biodiesel production due its high lipids content in relation to *C. vulgaris* and *Scenedesmus sp*. However, *B. braunii* grows on fresh water, limiting its application compared to saline microalgae. Other researchers have focused on *Chlorella* sp, because it is readily available

and can be easily cultured at laboratory scale (Ahmad et al., 2011; Miao & Wu, 2006; Rodolfi et al., 2009; Xu et al., 2006).

Table 2. Oil content of some microalgae (Chisti, 2007; Khan et al., 2009; Sakthivel et al., 2011; Taher et al., 2010)

Algal group	Microalgae	Oil content (% dry wt)
Green algae	*Botryococcus braunii*	29-75
	Dunaliella primolecta	23
	Tetraselmis sueica	15-23
Diatoms	*Chlorella sp*	28-32
	Phaeodactylum tricomutum	20-30
	Cylindrotheca sp	16-37
Eustigmatophytes	*Nannochloris sp*	20-35
	Nannochloropsis sp	31-68
	Isochrysis sp.	25-33

The main lipids-producing microalgae species have a similar lipids profile, generally equivalent to vegetable oil from plants suitable for biodiesel production (Xu et al., 2006), as shown in Table 3. A typical fatty acids profile is composed by oleic (18:1), palmitic (16:0), stearic (18:0), iso-margaric 17:0, and linoleic (18:2) acid (Demirbas, 2009b; Demirbas & Demirbas, 2011). Table 3 shows the fatty acids composition of different vegetable species, where the high proportion of saturated and monounsaturated fatty acids are considered optimal to meet biodiesel quality standards (Dayananda et al., 2005; Demirbas, 2009b). However, not all lipids accumulated in microalgae can be transformed into biodiesel, indicating that neutral lipids is most important than total lipids content. The fatty acids profile is also affected by environmental factors and cultivation conditions, and may vary during different growth phases. Table 4 shows that the fatty acids composition of *B. braunii* can vary with different growth temperatures. In addition, it has been reported that nitrogen deficiency and salt stress could induce the accumulation of C18:1. Besides, it has been

shown that the presence of iron could stimulate the accumulation neutral lipids in *Chlorella* microalgae due to the modification of the metabolic pathways related to lipids accumulation (Liu et al., 2008; Mata et al., 2010; Sayadi et al., 2011; Sharma et al., 2012). Despite the several advantages of using microalgae biomass for lipids production compared to oil crops, the high production costs, around 4-10 times more expensive than petroleum-derived fuels or first generation biodiesel (Groom et al., 2008) suggest that processing microalgae in a biorefinery concept could be a possible solution to overcome the economical problem in the near future.

Table 3. Properties of feedstock used for biodiesel [*) Waste cooking oils]

Oils / Properties	Kinematic viscosity (cSt)	Density (Kg/m³)	Acid value (mg KOH/g oil)	Cloud point (°C)	Pour point (°C)	Iodine value (gI₂/100g)	Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)	Ricinoleic (C18:1)	γ-linolenic acid	Others components	Reference
First generation feedstock															
Soybean	32.6	914	-	-3.9	-12.2	117-143	10.7	3.9	22.8	50.8	6.8	-	-	0.5	(Barnwal & Sharma, 2005; Ganesan et al., 2009a)
Rapeseed	37	911	-	-3.9	-32	94-120	6.1	2.3	56.0	24.2	0.5	-	-	0.7	(Barnwal & Sharma, 2005; Ganesan et al., 2009a)
Sunflower	39.9	916	-	7.2	-15	110-143	6.7	4.5	17-20	68.8	-	-	-	-	(Barnwal & Sharma, 2005; Ganesan et al., 2009a)
Palm	41.67	-	0.15	11	8	35-61	42.8	4.5	40.5	10.1	0.2	-	-	1.1	(Ma & Hanna, 1999; Moser, 2009)
Second generation feedstock															
Jatropha	35.4	918	0.24	-	-6	101	14.2	6.9	43.1	34.3		-	-	1.4	(Balat, 2011; Gui et al., 2008a)
Camelina	28.28	-	2.06	-10	-17	-	6.8	2.7	18.6	19.6	32.6	-	-		(Moser & Vaughn, 2010)
Castor	240	960	4.9	-12	-32	39.5	1.3	1.2	3.6	5.5	0.5	86	-	-	(Chakrabarti & Ahmad, 2008; Gui et al., 2008b; Martín et al., 2010)
WCO[*]	36.4	-	-	-	11	141.5	20.4	4.8	52.9	13.5	0.8	-	-	4.6	(Balat & Balat, 2010)
Third generation feedstock															
L. starkeyi	-	-	-	-	-	-	33.0	4.7	55.1	1.6	-	-	-	4.8	(Li et al., 2008)
R. turoloides	-	-	-	-	-	-	24.3	7.7	54.1	2.1	-	-	-	1.1	(Li et al., 2008)
M. isabellina	-	-	-	-	-	-	24–35	3.5–8.0	49–54	2–11	-	-	0.4–2	-	(Subramaniam et al., 2010)
M. rouxii	-	-	-	-	-	-	20.1	9.6	22	17		-	18.1		(Jeennor et al., 2006)
E. coli	-	-	-	-	-	-	25.0	-	31.8	-	-	-	-	47.61	(Shaw & Ingraham, 1965)
F. chlorophenolica	-	-	-	-	-	-	53.4	-	2.43	-	-	-	-	-	(El-Naggar & El-Aaser, 2004)
Microalgae	-	-	-	-	-	-	15	11	36	-	-	-	-	15.8	(Demirbas & Demirbas, 2011)

Table 4. Fatty acid composition of some algae (% total fatty acids)

Fatty acid	Nannochloropsis sp [1]	Chlorella Sorokiniana [1]	Chorella vulgaris [1]	Isochrysis galbana [1]	Spirulina platensis [1]	Brotryococcus Braunii [2,3]	Brotryococcus Braunii [2,4]	Brotryococcus Braunii [2,5]
C12:0						0.10	0.27	0.55
C14:0	6.90			23.10	29-34	1.00	1.49	3.40
C14:1						0.09	0.16	0.41
C15:0						0.71	0.68	1.62
C16:0	19.90	40.00	18.00	14.00		26.24	24.68	28.34
C16:1ω5					5.00-7.00			
C16:1ω6						0.37	0.62	0.43
C16:1ω7	27.40	4.00	5.00	2.00		0.47	2.76	4.01
C16:1ω9				1.00	36-39			
C16:1ω13						0.07	0.33	0.51
C16:2 ω7		11.00	12.00	1.00				
C16:3		17.00	2.10					
C18:0				1.10	1.00-2.00	2.52	3.05	3.22
C18:1 ω7				1.00		2.47	1.52	1.25
C18:1 ω9	1.70	5.00	9.20	13.00	1-2	0.14	14.66	3.58
C18:2	3.50	36.00	43.00	5.00	1.00-2.00	8.28	6.03	8.87
C18:3						40.31	25.65	23.67
C20:0						0.28	0.43	0.37
C20:4						0.18		
C20:5	34.90			5.00		0.25		
20:3						0.16		
22:0						0.22	0.21	0.12
24:0						0,32	0,29	0,19

[1](Hu et al., 2008), [2](Kalacheva et al., 2002)[3] cultivation temperature of 18°C for 6 days, [4] cultivation temperature of 25°C for 6 days [5] cultivation temperature of 32°C for 6 days.

3. Transesterification for the synthesis of biodiesel

Biodiesel is normally produced by transesterification of lipids (mainly triglycerides, TGs) using an alcohol as acyl acceptor and a catalyst (Khan et al., 2009; Meher et al., 2006; Mittelbach, 1996). Either primary or secondary short-chain alcohols are often used, such as methanol, ethanol or isopropanol (Ghadge & Raheman, 2006; Ma & Hanna, 1999).

The production of biodiesel from microalgae as well as first generation biodiesel has been mainly performed in two stages, the first stage consisting in lipids extraction and the second one in biodiesel production (Figure 1). Lipids extraction from microalgal biomass is an important step in the overall process of biodiesel production. Extracting cellular internal lipids is energetically demanding (Brennan & Owende, 2010; Golueke et al., 1957), being necessary to apply cell disruption methods (Lee et al., 2010; Mendes-Pinto et al., 2001).

The extraction of microalgae lipids is usually performed using solvents (Ehimen et al., 2010a). Soxhlet extraction with hexane and the Blight and Dyer extraction method using a mixture of solvents (chloroform/methanol) are the most used techniques for lipids extraction from microalgae (Lee et al., 2010), where the extraction efficiency decreases with the increment of water content in the biomass. Other available techniques are supercritical fluid extraction, microwaves, ultrasound-assisted extraction and heat reflux extraction (Koberg et al., 2011). Supercritical carbon dioxide extraction has some advantages compared to other used techniques, including favorable mass transfer rates and production of solvent-free extracts, but the high costs associated to this process (equipment and operation) are the main disadvantages (Amaro et al., 2011; Koberg et al., 2011).

Extraction is the most important process before transesterification of lipids takes place, as its efficiency is directly related to the overall process efficiency in biodiesel production.

Therefore, it is necessary to develop cheaper and efficient extraction processes to reach industrial biodiesel production using wet microalgae at appropriate costs.
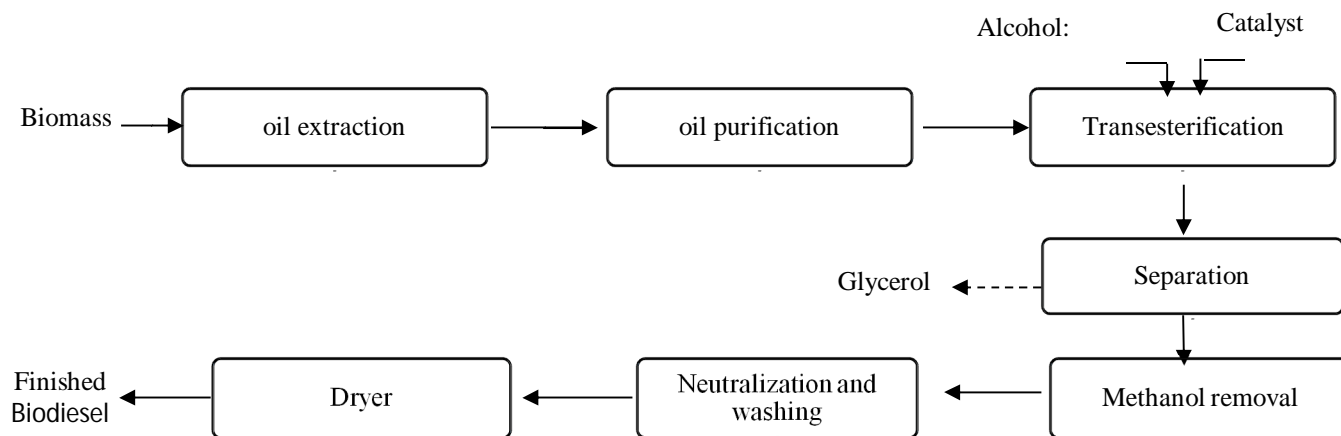
Figure 1. Conventional process of producing biodiesel

3.1 Catalysts used in microalgae lipids transesterification

Transesterification of lipids for biodiesel production from microalgae has been carried out by both homogenous and heterogeneous catalysis (Nagle & Lemke, 1990b; Vijayaraghavan & Hemanathan, 2009). The main advantages and disadvantages of both catalysis types are shown in Table 5. In addition, Table 6 shows a summary with the most used catalysts for microalgae lipids transesterification.

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Table 5. Advantages and disadvantages of the main types of catalysts used for transesterification

Catalyst	Advantages	Disadvantages	References
Homogeneous Alcaline	(a)Higher reaction rate and higher conversion (> 90%). (b) Low cost and widely available.	(a) Formation of soaps for saponification in presence of FFA (>1%) and water (>0.06%). (b) Necessity of a stage for removing the catalyst. (c) Wastewater production for washing the biodiesel to removal glycerol and rest of catalyst	(Antolín et al., 2002; Huang et al., 2010; Ma & Hanna, 1999; Meher et al., 2006; Vicente et al., 2004)
Heterogeneous alkaline	(a) Higher reaction rate than acid catalyst. (b) Easy separation of catalyst from product. (c) Possibility to reuse and regenerate the catalyst	(a) Sensitive to FFA content in oil with of soap formation (b) Leaching of catalyst during the reaction	(!!! INVALID CITATION !!!)
Homogeneous acid	(a) It does not affected by water content of raw material and FFA feedstock (b) Simultaneous esterification and transesterification	(a)Low reaction rate (4000 times slower than the alkali-catalyzed transesterification). (b) Necessity for catalyst neutralization (c) Necessity of a stage for removing the catalyst.	(Ghadge & Raheman, 2006)
Heterogeneous acid	(a)It does not affected by water content of raw material and FFA feedstock (b) Simultaneously esterification and transesterification (c)Easy separation of catalyst from product. (d)Possibility to reuse and regenerate the catalyst	(a) Leaching of catalyst during the reaction (b)High reaction temperature, high molar ratio alcohol/oil and high reaction time are required	(!!! INVALID CITATION !!!)

Table 6 Transesterification of microalgae oils

Microalgae	Reaction conditions					Physicochemical properties of biodiesel							References
	Catalyst	Algae: Catalyst (wt or vol. /wt or vol.)	T (°C)	oil: alcohol (wt or mol or vol/vol. or mol)	Time (min)	Density (Kg/m^3)	Viscosity (cSt)	Flash point (°C)	Pour point (°C)	Calorific value (MJ/Kg)	Acid value (mgKOH/g)	Alkyl esters (%)	
Homogeneous catalyst													
Schizochytrium limacimun	H_2SO_4	1:0.6 (vol oil/vol)	90	1:3.4 (wt /ml methanol)	40	-	-	-	-	-	-	66.37[1]5 2.66[2]	(Johnson & Wen, 2009)
Chlorella protothecoides	H_2SO_4	1:1 (vol oil/vol)	30	1:56 (mol /mol methanol)	240	864	5.2	115	-12	41	0.37	80	(Xu et al., 2006)
Chlorella protothecoides	H_2SO_4	1:0.60 (vol oil/vol)	50	1:30 mol /mol methanol	300	862	-	-	-	-	-	>70	(Miao & Wu, 2006)
Tetraselmis suecica	H_2SO_4	1:0.018 (vol oil/vol)	80	1:20 (wt /ml methanol)	20	-	-	-	-	-	-	78	(Wahlen et al., 2011a)
Freshwater algae	KOH	1:0.0125 (wt oil/wt)	-	10:3 (wt triglyceride/ml ethanol)	540	801	-	98	-14	40	0.4	-	(Vijayaraghavan & Hemanathan, 2009)
Oedigonium sp.	NaOH	1:0.083 (wt oil/wt)		1:8 (vol /vol methanol)	-	-	-	-	-	-	-	>90	(Hossain et al., 2008)
Heterogeneous catalyst													
Nannochloropsis oculata	CaO/Al_2O_3	1:0.02 (wt /wt)	50	1:30 (mol/mol)	240	-	-	-	-	-	-	97.5[3]	(Umdu et al., 2009)
Enzymatic catalyst													
Chlorella protothecoides	*candidiasis* sp.	1:0.75 (wt/wt)	-	1:3(mol oil/mol methanol)	720	-	-	-	-	-	-	98	(Li et al., 2007)

[1] With addition of Chloroform (4ml) and dry algae. [2] With addition of Chloroform (4ml) and Wet algae [3]80% loading CaO in Al_2O_3

3.1.1 Homogeneous catalysis

Homogeneous alkaline catalysis

Homogeneous catalysis includes alkaline and acid catalysts (Ganesan et al., 2009a; Lam et al., 2010). Homogeneous alkaline catalysis has been the most used route for biodiesel production from oils and fats, as it catalyzes the reaction at low temperature and atmospheric pressure. In addition, high conversion yield can be achieved in short times (minutes), being the most economical way to catalyze the transesterification reaction (Freedman et al., 1984; Fukuda et al., 2001; Ganesan et al., 2009a; Hossain et al., 2008; Lam et al., 2010). NaOH is widely used in homogeneous alkaline industrial catalysis (Meher et al., 2006), as it promotes high reaction biodiesel productivities and has a low cost (Ma & Hanna, 1999). After transesterification, the catalyst, glycerol and other impurities such as soap must be removed. Saponification compounds containing alkaline metals may increase biodiesel ash content and particulate matter emissions. In addition, alkaline metals may generate some problems in the engines, such as corrosion of motor components and deactivation of catalytic converters (Cooke et al., 2009; Meher et al., 2006).

Biodiesel production from microalgae lipids has been performed using both acid and alkaline homogeneous catalysis (Nagle & Lemke, 1990b; Vijayaraghavan & Hemanathan, 2009). However, due to the high FFA content in microalgae lipids alkaline catalysts are not suitable for biodiesel production (Ehimen et al., 2010a; Miao & Wu, 2006). Nagle and Lemke (1990) reported that the use of acid catalysts produced higher biodiesel conversion yield from microalgae lipids compared to alkaline catalysts under the same reaction conditions. In addition, Hossain et al. (2008) reported the possible conversion of lipids

from *Spirogyra sp.* and *Oedogonium sp* with sodium hydroxide as catalyst into biodiesel, however using hexane as co-solvent in the transesterification process.

Homogeneous acid catalysis

Acid catalysts are used when the free fatty acids (FFA) content in lipids is higher than 1% wt. (Lam et al., 2010; Ma & Hanna, 1999; Schuchardt et al., 1998). Therefore, liquid acid catalysts have been proposed to overcome the limitations of high FFA content. The most used acid catalysts in the transesterification process are H_2SO_4 and HCl. Both have the advantage of promoting high conversion yields from feedstocks with high acidity, due to FFA esterification. However, they require larger response times unlike alkaline catalysts (Ganesan et al., 2009b; Krohn et al., 2011; Nagle & Lemke, 1990b).

The use of acid catalysts can promote both transesterification and esterification reaction of microalgae lipids. Miao and Wu (2006) reported biodiesel production yields higher than 70% wt. in 5 h with H_2SO_4 as catalyst using the microalgae *Chlorella protothecoides* with a lipids content of 55% wt. (Table 6). Moreover, Johnson and Wen (2009) reported conversions over 50% wt. using the microalgae *Schizochytrium limacinum* with a shorter reaction time (40 min) due to the addition of chloroform in the reaction.

Two-step reaction using homogeneous catalysis

Some studies have proposed a combination of both catalysts acid and alkaline to produce biodiesel from lipids with a high FFA content (Francisco et al., 2010; Lam et al., 2010). Initially, an acid catalyst is used to convert FFA into esters through esterification. When FFA content in the lipids is reduced to less than 1 % wt., a second transesterification step of lipids can be performed by using an alkaline catalyst (Canakci, 2007; Canakci & Van Gerpen, 2003; Felizardo et al., 2006). Canakci and Van Gerpen (2003) reported the

production of biodiesel from feedstocks with high FFA content via a two-step method. The feedstock was first treated with H_2SO_4 to reduce FFA level to less than 1 % wt., followed by transesterification catalyzed by KOH.

Francisco et al. (2010) reported the biodiesel production from microalgae lipids in two steps, where they found a saponification followed by esterification using H_2SO_4. According to Canakci (2007) the efficiency of the two-step catalysis method of lipids with a high FFA content could be higher than 90%. However, high concentrations of the alkaline catalysts are necessary, due to the neutralization produced by the presence of the acid catalyst from the first step, increasing the operational costs (Kulkarni & Dalai, 2006; Lam et al., 2010).

Despite the high conversion yields reached by homogeneous catalysis, there is always a catalyst loss after the reaction. The catalyst may remain in the biodiesel phase, and therefore a biodiesel refining or washing step is needed. In this sense, the use of heterogeneous catalysis in biodiesel production will play a relevant role in the near future.

3.1.2 Heterogeneous catalysis

Different solid catalysts have been developed for biodiesel production, such as zeolites, oxides, hydrotalcites, and exchange resins, among others. Heterogeneous catalysis has several advantages as it is a non-corrosive process, environmentally friendly and presents fewer disposal problems. Solid catalysts can also be designed to give higher activity, selectivity and longer catalyst lifetimes, but the energy requirements are higher (Liu et al., 2007). Heterogeneous catalysis includes alkaline and acid solid catalysts.

Heterogeneous alkaline catalysis

Heterogeneous alkaline catalysis is widely used in the transesterification process, as it promotes a faster reaction rate and an easily separation of the final product (Lam et al., 2010; Liu et al., 2007). Calcium oxide has been the most used solid alkaline catalyst in transesterification of lipids or fats. The use of CaO for biodiesel production has drawn much attention due to its high basic strength and low solubility in methanol, being synthesized from cheap sources like limestone and calcium hydroxide (Zabeti et al., 2009). However, CaO can be consumed during transesterification as it reacts with glycerol forming calcium diglyceroxide (Kouzu et al., 2008a; Kouzu et al., 2009). In addition, CaO is rapidly hydrated and carbonated in air presence, deteriorating its catalytic performance. The reduction of its catalytic activity is produced by the adsorption of CO_2 and H_2O on the solid surface in the form of carbonates and hydroxyl groups (Granados et al., 2007; Hattori, 1995). Nevertheless, the catalytic activity of CaO can be regenerated by means of thermal treatment at 700°C (Lam et al., 2010).

Heterogeneous alkaline catalysis has been reported in the process of lipids and fats transesterification including MgO, SrO, BaO, and mixed Mg-Al_2O_3, among other catalysts (Lam et al., 2010; Liu et al., 2007). However, the use of solid alkaline catalysts can result in low biodiesel conversion yields when high FFA content is present in lipids, promoting soap formation. For instance, CaO and MgO can promote the formation of calcium and magnesium soap, being their use restricted in the case of microalgae oil transesterification (Kouzu et al., 2008b; Liu et al., 2007; Umdu et al., 2009). Moreover BaO has been suggested as an effective catalyst that promotes the reaction due to its high activity or high strength of basic sites compared to other oxides. The number of basic sites per weight unit for this type of catalysts increases in the order MgO < CaO < SrO < BaO (Hattori, 1995; Zhang et al., 1988). However, BaO has been also reported as an unsuitable catalyst for this

process, as it dissolves in methanol and has a low surface area (Lam et al., 2010; Patil et al., 2011c). In addition, SrO has been found to be suitable for the transesterification of vegetable lipids with a conversion yield higher than 95%, maintaining its activity even after 10 repeated cycles (Liu et al., 2007; Zhang et al., 1988).

Umdu et al (2009) reported the transesterification of lipids from the microalga *Nannochloropsis oculata* by using CaO and MgO supported on alumina. They reported that pure CaO and MgO were not active and CaO/Al_2O_3 catalyst showed the highest activity, due to high basicity (number of basic sites per square meter) and basic strength. The highest yield (97.5%) was obtained when loading CaO on Al_2O_3 (80-20% wt.) using a methanol/lipid molar ratio of 30:1.

Other important catalyst used in the transesterification of lipids is K_3PO_4, which has shown a high catalytic activity in the reaction. K_3PO_4 can be hydrolyzed in the presence of water, forming HPO_4^{2-}, $H_2PO_4^-$ and OH^- ions in the reaction solution, being the resulting reaction mixture strongly alkaline (Thanh et al., 2012). Guan et al (2009) found a yield of 97.3% in the transesterification of waste lipids using a catalyst concentration of K_3PO_4 of 4 wt.% at 60 °C for 120 min.

Heterogeneous acid catalysis

Solid acid catalysts have a strong potential to replace liquid acid catalysts in biodiesel produced from microalgae lipids (Ganesan et al., 2009a). The most used catalysts in heterogeneous acid catalysis for lipids transesterification are zirconium oxide (ZrO_2), titanium oxide (TiO_2), zeolites, ion exchange resins and heteropolyacids.

ZrO_2 has been used as a solid acid catalyst for transesterification of different feedstocks due to its strong surface acidity. This acidity can be enhanced by coating the surface with

anions like sulfate, tungstate and alumina. The combination of Al_2O_3 or tungsten oxide (WO_3) with ZrO_2 enhances the acidity and mechanical strength of the catalyst. On the opposite, the mixture of ZrO_2 with sulfuric acid (H_2SO_4) is not effective due to sulfate leaching during transesterification (Lam et al., 2010).

In biodiesel production from microalgae, the Mcgyan® Process has been reported. The Macgyan® process uses porous metallic oxides composed by ZrO, TiO and Al_2O_3 that simultaneously esterified and transesterified FFA and triglycerides (TG) to biodiesel under supercritical conditions, with residence times of only a few seconds (Krohn et al., 2011; McNeff et al., 2008).

The main advantages of acid heterogeneous catalysis for the transesterification of microalgae lipids are the possible catalyst recovery and reutilization, and higher efficiency. However, more research is necessary specifically in the re-activation process for their suitable reutilization.

3.1.3 Enzymatic catalysis

Biological catalysts can be used to transform lipids with high FFA content, such as microalgae lipids, which is a limitation for homogeneous alkaline catalysis. FFA can be esterified to alkyl esters in the reaction using lipases. Lipases do not require excessive energy expenditures and if the catalyst is immobilized, it facilitates its recovery and reuse (Azócar et al., 2011; Ciudad et al., 2011; Fukuda et al., 2001). However, the lower productivity due to higher reaction times added to the high cost of biological catalysts limits its development.

In relation to microalgae biodiesel production, there are only few reports related to transesterification with lipases. Li et al (2007) reported 98% yield of biodiesel for the

transesterificación of microalgae lipids of *Chlorella protothecoides* using 75% of lipase *candidiasis sp* (12,000 U g^{-1}, based on lipids quantity) and a 3:1 methanol to lipids molar ratio for a reaction time of 12 h. The main problem of using lipases as catalyst is related to the lipids composition and the presence of possible inhibitors that could affect the performance of the biological catalyst (Liu et al., 2010).

Several steps are necessary to extract the lipids from microalgae biomass. It is necessary to dry the biomass to increase the lipids extraction yield and to use solvents for the extraction process. In addition, a solvent recovery stage including biodiesel refining is needed. In this sense, the exploration of other alternatives such as the direct use of biomass in an in-situ transesterificaction process may be also attractive.

3.2 Direct transesterification of microalgae biomass

The production of biodiesel is mainly based on the use of refined lipids as feedstock, which contribute up to 70% of the total biodiesel costs (Haas et al., 2006). Therefore, reducing the lipids extraction and purification steps could be a useful way to decrease biodiesel production costs from microalgae (Amaro et al., 2011).

An alternative to the conventional process (Figure 2) is the direct transesterification, where the lipid extraction and transesterification are carried out in one step, with a direct reaction of oil-bearing biomass to biodiesel (Amaro et al., 2011; Ehimen et al., 2010a; Haas et al., 2006).

The first research of direct transesterification was published by Harrington and D'Arcy-Evans (1985). In fact, using sunflower seeds authors obtained more biodiesel (up to 20%) by direct transesterification compared to the conventional lipid extraction and

transesterification process (Harrington & D'Arcy-Evans, 1985a). This method could be advantageous when using microalgae biomass, as the lipid extraction process from microalgae is usually performed by a specific solvent (Ehimen et al., 2010a). A solvent that could be simultaneously used for the extraction process and as acyl acceptor for transesterification would be desirable for decreasing total biodiesel production costs. In addition, Wahlen et al. (2011) reported that biodiesel production yield could be incremented by the extraction of fatty acids from membrane phospholipids of microalgae cells. Table 7 shows a summary of different studies on direct transesterification for biodiesel production from microalgae biomass. This method has a potential application in biodiesel production at industrial level using different raw materials. According to different reports, the most important parameters influencing in the process are: a) alcohol/lipids molar ratio, b) catalyst dosage, c) reaction time, d) temperature, e) the use of a co-solvent and f) the water content of the biomass (Ehimen et al., 2010a; Georgogianni et al., 2008; Griffiths et al., 2010; Haas et al., 2004; Harrington & D'Arcy-Evans, 1985).
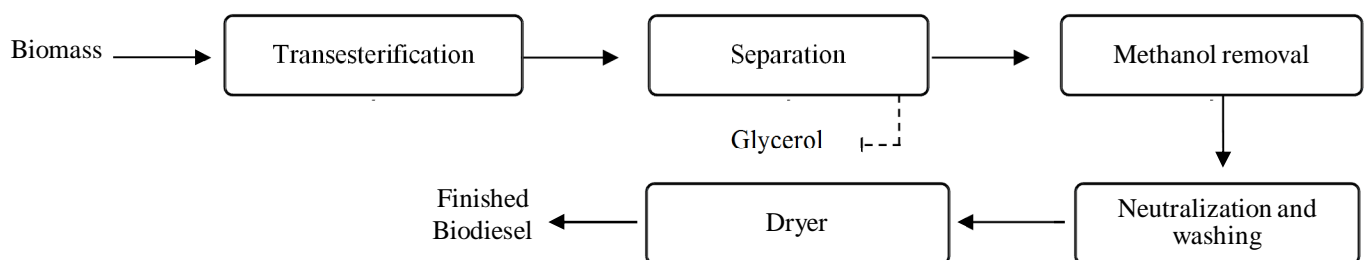
Figure 2. In-situ transesterification process of producing biodiesel

Table 7. Direct transesterification of microalgae biomass

Microalgae	Catalyst	Algae: Catalyst (wt or v. /wt or v.)	T (°C)	Algae or oil: alcohol (wt or mol/v. or mol)	Time (min)	Density (Kg/m^3)	Viscosity (cSt)	Flash point (°C)	Alkyl Esters (%)	References
Conventional direct transesterification										
Schizochytrium limacimun	H_2SO_4	1:0.6 (wt dry algae/ml)	90	1:3.4 (wt/ml methanol)	40	-	3.87	204	63.47[1]	(Johnson & Wen, 2009)
Schizochytrium limacimun	H_2SO_4	1:0.6 (wt wet algae/ml)	90	1:3.4 (wt wet algae/ml methanol)					7.76[2]	(Johnson & Wen, 2009)
Chrorella	H_2SO_4	1:1 (wt dry algae /wt)	90	1:4 (wt/ml methanol)	480	883	-	-	-	(Ehimen et al., 2010a)
Chaetoceros gracilis	H_2SO_4	1:2.2 (v oil/ml)	80	1:20 (wt/ ml methanol)	10	-	-	-	32.9	(Wahlen et al., 2011a)
Nannochloropsis sp.	SrO	1:0.3 (wt /wt)	60		5	-	-	-	2.9	(Koberg et al., 2011)
Assisted direct transesterification										
Supercritical methanol										
Nannochloropsis sp.	-	-	255	1:9 (wt wet algae /ml methanol)	25	-	-	-	90	(Patil et al., 2011b)
Microwave										
Nannochloropsis sp.	KOH	1:0.02 (wt dry algae /wt)	-	1:12 (wt dry algae /ml methanol)	4	-	-	-	>77	(Patil et al., 2011a)
Nannochloropsis sp.	SrO	1:0.3 (wt /wt)	60	1:1 (wt dry algae /ml methanol)	5	-	-	-	37.1	(Koberg et al., 2011)
Ultrasound radiation										
Nannochloropsis sp.	SrO	1:0.3 (wt /wt)	60	1:1 (wt dry algae /ml methanol)	5	-	-	-	20.9	(Koberg et al., 2011)

[1] Chloroform as solvents use (4ml) and dry algae. [2] Chloroform as solvents use (4ml) and wet algae.

3.2.1 Critical parameters in the direct transesterification of microalgae biomass

3.2.1.1Alcohol type and alcohol/lipids molar ratio

In the direct transesterification process, alcohol performs a vital role in the reaction, acting as both solvent (extracting the lipids from the cells) and as acyl acceptor (converting lipids to fatty acid alkyl esters) (Georgogianni et al., 2008; Wahlen et al., 2011a).

Methanol has been the most used alcohol in the direct transesterification process, although it can extract less TG from microalgae biomass compared to other alcohols in a direct transesterification process (Georgogianni et al., 2008; Kildiran et al., 1996b). In this type of reactions, the effect of alcohol is complementary to the type of catalyst. A homogenous catalysis (alkaline or acid), facilitates the lipids extraction with an increase in the production to biodiesel by direct transesterification of biomass.

In direct transesterification, higher efficiencies in the extraction of TG have been reported for primary alcohols of longer chains such as ethanol, 1-propanol, 1-butanol, 2-methyl-1-propanol, and 3-methyl-1-butanol, among others (Kildiran et al., 1996b; Wahlen et al., 2011a).

Wahlen et al (2011) reported the use of primary alcohols of longer chains in the direct transesterification of the microalgae *Chaetoceros gracilis*. They found TG higher extraction yields with primary alcohols of longer chains, rather using than methanol. However, when H_2SO_4 was used as catalyst, alcohol type presented no significant effect in fatty acid alkyl esters production yield, being similar to those obtained with methanol.

Normally, the methanol/lipids molar ratios used for direct transesterification are much higher than the stoichiometric value to favor products formation as transesterification is an equilibrium reaction. For the direct transesterification of sunflower lipids, methanol/lipids

molar ratios of 532:1 (Harrington & D'Arcy-Evans, 1985a), 300:1 (Siler-Marinkovic & Tomasevic, 1998), and 543:1(Haas et al., 2004) have been reported. For direct transesterification of rapeseed lipids values of 275:1 (Haagenson et al., 2010) have been reported, while for soybean oil values of 226:1 (Haas et al., 2004).

Ehimen et al (2010) used a methanol/lipids molar ratio between 105:1 and 524:1 for the direct transesterification of *Chlorella biomass*. They observed a reduction of specific gravity with the increase in the methanol/lipids molar ratio from 105:1 to 315:1 and with increasing temperature from 23°C to 90°C. However, no significant trends were observed when a higher methanol/lipids molar ratio was used (higher than 315:1). Alcohol excess plays also a role as extraction solvent, providing access of alcohol and catalyst to the substrate, altering the permeability of the solid substrate (Haas & Scott, 2007). In the reaction, methanol excess is responsible for breaking linkages between glycerin and fatty acids, being its presence essential in the process (Al-Widyan & Al-Shyoukh, 2002). However, a high methanol excess can provoke a decrease in the separation yield between ester and glycerin phases (Miao & Wu, 2006).

Although direct transesterification may need a higher solvent volume, it could be an alternative for industrial microalgae biodiesel production, as the solvent could be recovered and reused in the process in a closed loop, reducing thus the negatively impact of the solvents in the whole process costs

3.2.1.2 Catalysts role

The production of biodiesel by direct transesterification has been mainly performed by using acid catalysis, due to high FFA content of microalgae lipids. Here, sulfuric acid has

been the most used catalyst as it converts both TAG and FFA into biodiesel (Ehimen et al., 2010a; Wahlen et al., 2011a).

Regarding the dosage of catalyst used in transesterificacion of biomass, values between 20 and 100% (based on lipids content in biomass) have been reported. In this process, the catalyst might facilitate the lipids extraction due to the cell wall rupture, complementing the alcohol role in the lipids extraction.

Miao and Wu (2006) used the microalgae *Chlorella protothecoides* and applied concentrations ranging from 25% to 100% wt. (based on lipids content) of acid catalyst. They reported an increment in biodiesel yield with catalyst concentrations up to 60%. Although higher catalyst concentrations could reduce reaction time (Al-Widyan & Al-Shyoukh, 2002; Siler-Marinkovic & Tomasevic, 1998), they reported that higher concentrations of the acid catalyst reduced the reaction yield, probably due to lipids destruction in such acidic conditions.

Although acid catalyst concentration is a very important parameter in transesterification efficiency (Demirbas, 2007; Meher et al., 2006; Schuchardt et al., 1998), in the case of direct transesterification this parameter however does not show a clear effect in biodiesel production yield.

3.2.1.3 Temperature and reaction time

Previous studies of conventional and direct transesterification have demonstrated that when increasing the temperature the time necessary to reach maximum biodiesel yield decreases (Ozgul-Yucel & Turkay, 2002; Wahlen et al., 2011a). This effect has been mainly reported for acid catalyzed transesterification reactions (Canakci & Van Gerpen, 2003; Ozgul-Yucel & Turkay, 2002; Wahlen et al., 2008). This increment can be attributed to an enhanced

miscibility between the reacting species (Ehimen et al., 2010a; Ozgul-Yucel & Turkay, 2002). Temperature is fundamental in the transesterification process, and to a positive effect in lipids extraction yield due to an increment in lipids solubility. In addition, high temperatures may increase biodiesel production yield, but also producing a possible lipids degradation (Canakci and Van Gerpen 2003)

3.2.1.4 Co-solvents

In direct transesterification of microalgal biomass, the selected alcohol must fulfill two simultaneous roles: to extract the lipids and to participate as acyl acceptor in the transesterification reaction. Due to the low performance of aliphatic alcohols such as methanol as lipids extractants from biomass, the incorporation of a co-solvent in the reaction medium has been evaluated. The use of co-solvents could improve the performance of direct transesterification systems. In fact, an appropriate co-solvent can improve mass transfer and accelerate lipids extraction, even reducing the extraction time (Johnson & Wen, 2009; Zeng et al., 2009a).

Zeng et al. (2009) evaluated the lipids extraction yield at 25°C for 60 min with different solvents, detecting that the highest extraction yield was obtained for diethoxymethane (96%), followed by hexane (93%), tetrahydrofuran (93%) and methanol (< 9%). This low lipids extraction yield obtained with methanol indicates that this solvent is not suitable to perform direct transesterification of microalgal biomass; however, lipids extraction could be increased by incorporating a suitable co-solvent in the reaction medium.

Different co-solvents have been used for the direct transesterification of oilseed biomass, such as: tetrahydrofuran (THF), diethoxymethane (DEM), hexane, benzene, toluene, chloroform and petroleum ether (Carrapiso & García, 2000; Johnson & Wen, 2009; Zeng et

al., 2009a). Benzene and toluene lipids extraction yield is higher compared to chloroform, but benzene is toxic. Several researchers have used toluene as co-solvent, however, toluene can overlap the peaks of short-chain fatty acid methyl esters, interfering the measurement and quantification of biodiesel content (Carrapiso & García, 2000; Sukhija & Palmquist, 1988).

Zeng et al (2009) found that DEM was a suitable co-solvent in the direct transesterification of sunflower seed with methanol. In addition, Johnson and Wen (2009) found that chloroform was a more suitable co-solvent in the direct transesterification of microalgae *Schizochytrium limacinum* at 90°C and for 40 min. Johnson and Wen (2009) reported a biodiesel yield of 63.47% using chloroform as co-solvent. The lowest biodiesel yields were obtained with hexane (9.15%) and petroleum ether (9.71%).

3.2.1.5 Direct transesterification using wet biomass

The influence of microalgae moisture levels is relevant regarding biofuel production costs. According to several reports, high moisture content in the biomass has a negative effect on lipids conversion yield into biodiesel as hydrolysis reactions of lipids with the formation of fatty acids are promoted (Ehimen et al., 2010a; Johnson & Wen, 2009; Wahlen et al., 2011a). Ehimen et al (2010) reported a reduction in lipids conversion yield to biodiesel with an increment in water content for direct transesterification using *Chlorella* biomass. When the biomass moisture level reached 0.7% wt, a conversion yield of 81.7% wt was observed, however, for *Chlorella* biomass with 73% wt water content only a 19.5% wt conversion yield into biodiesel was obtained.

An alternative to overcome the effect of water content in biomass for the direct transesterification process is the increase in methanol dosage and the use of supercritical processes.

a) Increasing methanol excess

The negative effect of high water content can be partially compensated by using more methanol in the reaction (Wahlen et al., 2011a). Wahlen et al (2011) observed that an increase in water content, provoked a reduction from 100% to 50% in biodiesel production yield when using wet algae biomass for direct transesterification. However, when increasing the methanol/biomass wt. ratio from 25 to 53, the biodiesel production yield was improved from 50% to 84% using an equal content of water and biomass.

Increasing the methanol volume in direct transesterification reaction is an alternative to reduce costs associated to biomass drying process, although it remains uncertain whether these savings will offset the cost of the increased methanol volume.

b) Supercritical processes

A catalyst-free method for biodiesel production has been recently developed by employing supercritical methanol (Demirbas, 2009a). This process is carried out at high reaction temperature and pressure (> 240 °C and > 8 MPa, respectively) (Kusdiana & Saka, 2004). Supercritical methanol can form a single phase with lipids, in contrast to the two phases at the normal conditions. This can be achieved because the decrease in the dielectric constant of methanol at supercritical state. In such a supercritical process, the reaction was completed in a very short time within 2 to 4 min, and no feedstock pretreatment (reduction of FFA and water) and post-reaction processes (washing) are needed (Song et al., 2008).

This method has been mainly used for biodiesel production from lipids, where FFA and triglycerides can be simultaneously esterified and transesterified (Kusdiana & Saka, 2004). Although the high costs and energy requirements have limited its development, Kusdiana and Saka (2004) reported optimum transesterification conditions of rapeseed oil to biodiesel at 350°C, 43 MPa and 4 min with an oil/methanol molar ratio of 1:42.

Supercritical processes have been also used in the direct transesterification of microalgal biomass, as alcohol in supercritical state (methanol) can simultaneously extract and transesterify the lipids (Patil et al., 2011b).

Supercritical transesterification can be considered as an alternative process to reduce costs associated with drying microalgal biomass for biodiesel production. High moisture content does not interfere in the reaction unlike a standard alkaline catalyzed transesterification.

Patil et al. (2011b) reported biodiesel production using wet algal biomass containing about 90% water under supercritical methanol conditions. They reached 90 % biodiesel yield applying a   wet microalgal biomass/methanol ratio of 1:9 wt/v, a reaction time of 25 min and reaction temperature of 255°C, when performing direct transesterification of *Nannochloropsis*.

Recently, a process that combines two processing steps of microalgal biomass, including lipids hydrolysis and further transesterificaction has been reported (Levine et al. 2010). In the first step, wet microalgal biomass reacts under subcritical water conditions to hydrolyze intracellular lipids, to conglomerate cells into an easily filterable solid that retains the lipids, and produces a sterile, potentially nutrient-rich aqueous phase. In the second step, the wet FFA rich solids are subjected to supercritical direct transesterification with ethanol to produce biodiesel in the form of fatty acid ethyl esters (FAEE).

3.3 Novel approaches in direct transesterification of microalgal biomass

To improve the direct transesterification process, different technologies have been applied mainly including microwave and ultrasonic technology.

3.3.1 Microwave technology

Transesterification can be performed using different heating systems, also including microwave radiation. Microwave radiation is a non-ionizing radiation that influences molecular motions such as ion migration or dipole rotation, not altering the molecular structure. A molecule possessing a dipole moment is sensitive to external electric fields. Therefore, when it is exposed to microwave radiation, its dipole rotation will attempt be aligned with the applied electric field (Refaat, 2010).

In microwave-assisted transesterification, methanol absorbs microwave radiation, redirecting its dipole quickly. This rearrangement allows the destruction of the methanol-lipids interface extracted from the dry algae (Patil et al., 2011a)

Transesterification reaction carried out under microwave radiation is efficiently accelerated to short reaction times. Besides, a drastic reduction in glycerol production is observed (Azcan & Danisman, 2008; Barnard et al., 2007; Hernando et al., 2006; Rathana et al., 2010). The microwaves transfer energy in an electromagnetic form, and the oscillating microwave field tends to move continuously to polar ends of molecules or ions (Azcan & Danisman, 2008). Consequently, collisions and friction between the moving molecules generate heat (Marra et al., 2010). Heat is transferred directly into the reaction media with a rapid temperature increase throughout the sample (Lam et al., 2010).

In microwave assisted transesterification of lipids mainly alkaline catalysts have been used. Hernando et al. (2006) reported 92% biodiesel yield from rapeseed lipids with 1.3% wt.

NaOH after 5 min, while Azcan and Danisman (2008) reported 93.7% biodiesel yield from rapeseed lipids with 1% wt. KOH in 5 min. Moreover, research concerning the use of microwaves in transesterification of feedstocks with a high FFA content has been already published. Rathana et al. (2010) reported a two-step process assisted by microwave irradiation with kenaf seed lipids. In the first stage FFA levels decreased from 11% wt. to 0.312% wt. using sulfuric acid as catalyst, while in the second step, lipids were methylated under microwave radiation, reporting 94% biodiesel yield with 0.55% wt. KOH in 6.5 min. Barnard et al. (2007) reported the production of biodiesel from waste cooking oil in only one step, under continuous flow. In that study 97.9% biodiesel yield, after 10 min with 1% wt. KOH and flow rate of 2 L/min was observed.

Microwave irradiation has been used in the past to extract lipids from biomass, soils and vegetable feedstock (Pan et al., 2002). Microwaves can penetrate through the cell wall structure with an efficient recovery of oils, and currently microwaves are a tool for simultaneous extraction and transesterification of biomass for biodiesel production, making their use in microalgal biomass feasible (Patil et al., 2011a).

Microwave-assisted transesterification reaction could be performed to produce biodiesel without catalysts. However in this process uses higher temperatures, larger volumes of solvents and longer reaction times (Geuens et al., 2008).

The microwave-assisted transesterification process requires suitable dry algae-methanol ratio for favoring simultaneous extraction and transesterification. According to Patil et al. (2011a), higher biomass to methanol ratios could shift the transestefication forward perhaps due to increased contact area between methanol and lipids, resulting in higher biodiesel yields. Regarding catalysts, it has been reported that homogeneous catalysis using KOH is

more suitable for microwave irradiation unlike solid catalysts (Patil et al., 2011a; Refaat, 2010)

In the microwave-assisted transesterification of microalgae biomass a more efficient process has been achieved when using homogeneous alkaline catalysis. The lower efficiency of the heterogeneous catalysis is related to the few centimeters of penetration depth of microwave radiation (Andrade et al., 2011). Koberg *et al.* (2011) reported a FAME yield of 37.1% with SrO (30% wt.) using a methanol–chloroform mixture (1:2 v/v) after 5 min. On the opposite, Patil et al. (2011a) reported a yield over 70% wt. using KOH (2% wt.) as catalyst, dry algae to methanol (wt/v) ratio of about 1:12 and reaction time of 4 min (Table 7).

3.3.2 Ultrasonic technology

Ultrasonic technology is an effective method to enhance mass transfer rate between immiscible phases (Georgogianni et al., 2006; Ji et al., 2006; Pan et al., 2002). This high frequency sound wave can compress and stretch the molecular spacing of media in which it passes through. Thus, these molecules could remain continuously vibrating with the formation of fine micro-bubbles or micro-cavities, and energy generation (Ji et al., 2006; Lam et al., 2010).

Ultrasonic technology applied in transesterification has proven to be an efficient mixing technique which provides sufficient activation energy to initiate the reaction. Ji et al. (2006) reported that, a biodiesel yield of 99% in 5 min using ultrasonic technology is possible, in comparison to 1 h using stirred batch reactor systems. Besides, the molar ratio of alcohol to lipid can minimize and reduce energy consumption compared to the conventional method (mechanical stirring) (Lam et al., 2010).

Microwave and ultrasonic technology can accelerate the microalgae cells disruption, and as a result an easier release of oil can be observed. Both techniques can be used in the direct transesterificaction of microalgal biomass with reaction times lower than conventional catalysis (Demirbas, 2007; Meher et al., 2006; Schuchardt et al., 1998).

In relation to microalgae, a recent work of Koberg et al. (2011) reported a FAME yield of 20.9%, much higher than the control (2.9%), for the direct production of biodiesel from *Nannochloropsis* using ultrasonic technique.

4. Conclusion

Biodiesel production from microalgae biomass is still one of the most important topics under research, mainly because of the high energy demand involved in biomass pretreatment, lipids extraction and biodiesel refining. Therefore the application of direct transesterification could be an alternative to reduce the critical steps to produce biodiesel, avoiding the lipid extraction process and simultaneously reducing the necessary equipment. In addition, direct transesterification could be used using the same extraction solvent or could be even improved by adding a co-solvent. Moreover, some important issues in the future will be related to the purification of biodiesel from microalgae.

The most important parameter in direct transesterification seems to be the biomass water content, decreasing the efficiency of the system with an increase in water content. Therefore, future research should be focused in this aspect to directly handle microalgae biomass with its characteristic high water content.

The incorporation of promising technologies for lipids transesterification and biodiesel production, such as supercritical process, microwave and ultrasonication, might enhance

mass transfer rates between the immiscible phases diminishing the reaction time. However, it is still necessary to decrease the costs of these technologies so they can be suitable alternatives in future industrial applications.

Chapter 3

Evaluation of different operational strategies for biodiesel production by direct transesterification of microalgal biomass

Evaluation of different operational strategies for biodiesel production by direct transesterification of microalgal biomass

Pamela Hidalgo[1], Claudio Toro[2], Gustavo Ciudad[1,4], Sigurd Schober[3], Martin Mittelbach[3] & *Rodrigo Navia[1,4]

[1]Scientific and Technological Bioresources Nucleus, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]Centro de Investigación en Polímeros Avanzados (CIPA), Beltrán Mathieu 224 piso 2, Concepción, Chile

[3]Institute of Chemistry, Working Group Chemistry and Technology of Renewable Resources, University of Graz, Heinrichstraße 28, A-8010 Graz, Austria.

[4]Departament of Chemical Engineering, Universidad de La Frontera, Casilla 54-D, Temuco, Chile

1. Introduction

Microalgae are receiving increasing attention worldwide as an alternative and renewable source for energy production. Microalgae have higher lipids production yields, which have been reported between 58000 L/ha and 136000 L/ha, besides they have much faster growth-rates than terrestrial crops. (Demirbas, 2011) Instead, oil from oilseeds such as rapeseed or soybean present oil yields of 1190 L/ha and 446 L/ha, respectively. Besides, microalgae can grow in wastewater with high organic matter content, as a wastewater of carpet mill effluents; (Chinnasamy *et al.*, 2010; Park & Craggs, 2010) or even in brackish water and use non-arable land, also requiring less land extensions for their cultivation. Additionally, microalgae can produce different types of lipids and hydrocarbons depending on the species of microalgae.

Despite the several advantages of using microalgae for biofuels production compared to oil crops, they production at industrial scale still faces relevant problems, mainly due to the high costs of biomass production and fuel conversion routes. Microalgal biofuels are 4-10 times more expensive than petroleum-derived fuels or first generation biodiesel (Groom et al., 2008). Key technologies for biofuels production are culture conditions for high oil productivity, development of effective and economical microalgae cultivation systems, as well as separation and harvesting of microalgal biomass. Besides, cost-effective routes for biofuel production including biomass drying, lipids extraction and added-value products recovery (e.g. proteins, carbohydrates and pigments) as well as biodiesel production and refining processes should be also optimized (Chen *et al.*, 2011). The main critical points are biomass drying, with an energy consumption near 80% and lipids transesterification, with a 10% of the total energy consumption of biodiesel production from microalgae (Lardon *et al.*, 2009). The reduction of the energy consumption in these limiting steps is fundamental

for a possible industrial scale up. With the purpose of eliminating the biomass drying process, the implementation of high temperature and pressure transesterification of wet biomass has been tested. In this process, direct conversion under supercritical methanol condition appears as a great alternative, because it is a one-step process for direct liquefaction and conversion of wet algal biomass into biodiesel. This one-step process enables simultaneous extraction and transesterification of lipids (Patil et al., 2011b).

Patil et al (Patil et al., 2011b) reached 90% wt of biodiesel yield obtained by supercritical transesterification using a wet microalgal biomass/methanol ratio of 1:9 wt/v, a reaction time of 25 min and a temperature of 255 °C. Moreover, Levine et al (Levine *et al.*, 2010) reported a process that combines two steps, lipids hydrolysis (at 250°C) using subcritical water and wet biomass, where an easily filterable cells conglomerate (hydrochar) retains the lipids, followed by a supercritical transesterification of the hydrochar (at temperatures between 275 and 325 °C).

On the other hand, high conversion yields have been achieved in conventional direct transesterification processes at moderate temperatures (< 100°C) with relatively low cost equipment. Such developments could be implemented at industrial level, although a large alcohol excess is required and the process is highly sensitive to water.

Other limiting step in using microalgae for biodiesel production is lipids extraction. In fact, lipids extraction from microalgae is mainly performed by cell wall disruption methods and solvent extraction and not by conventional physical methods, such a screw press, due to difficulties in breaking the cell wall. (Pieber et al., 2012a)

In this sense, in-situ transesterification could be a way to reduce biodiesel production costs by eliminating the lipids extraction step. In-situ transesterification differs from the conventional reaction as biomass (oil-bearing materials) is directly treated with a mixture of

alcohol and acid or base, resulting in cell wall disruption and the extraction of lipids as fatty acid alkyl esters (typically fatty acid methyl esters, or FAME). Extraction and transesterification steps occur in one step, with the alcohol acting as both an extraction solvent and an acyl acceptor (Hidalgo et al., 2013a).

The main factors influencing biodiesel production yield in direct transesterification are the acyl acceptor and catalyst type. The acyl acceptor has a dual role, as lipids extraction solvent and as esterification reagent, also facilitating the access of the catalyst to the substrate, due an alteration of the cell wall permeability(Haas & Scott, 2007).

Due to FFA content, acid catalysts have been used in lipids transesterification from microalgae. The use of alkaline catalysts would not be suitable due to saponification of FFA from microalgae lipids, leading to soap formation and hindering the separation and purification of biodiesel (Wahlen et al., 2011b). Acid catalysts can perform both transesterification and esterification of microalgae lipids and may additionally play an important role as cell disruption agent (Hidalgo et al., 2013a).

However, they require larger reaction time and need more solvent volume and higher temperature unlike alkaline catalysts.

Direct transesterification of biomass has been mainly carried out in stirred batch reactor (SBR), where the solvent is in direct contact with the biomass(Haas & Wagner, 2011). This system is of easy implementation, but it has some disadvantages such as a low lipids extraction yield due to a limited diffusion and the necessity of a subsequent step for separating the biomass from the reaction product.

The traditional system used in lipids extraction of oilseeds by solvent could be implemented in the direct transesterification of biomass as a reflux extraction system,

where the sample is in continuous contact with fresh solvent, facilitating mass transfer, also increasing the lipids extraction yield (Wang *et al.*, 2010).

This system has not been applied in the direct transesterification of microalgal biomass yet, its evaluation being of high interest. Besides, reflux extraction reactor (RER) system could be an interesting option to diminish the inhibitory effect of water concentration during the esterification of FFA.

Therefore, in this work, different operational strategies for biodiesel production by direct transesterification of microalgal biomass were tested. These strategies were applied in two different systems: Stirred batch reactor (SBR) and RER. The strategies include the evaluation of different acyl acceptors, catalysts and the effect of the solvent mixture in FAAE yield.

B. *Braunii* was used in this study. While this microalgae is characterized for synthesizing and accumulating high amounts of hydrocarbons and ether lipids (Banerjee *et al.*, 2002; Metzger & Largeau, 2005), its high content of saturated and monounsaturated fatty acids makes this microalga suitable for FAME production (Kalacheva *et al.*, 2002; Kalacheva *et al.*, 2001).

2. Experimental section

2.1 Materials

2.1.1 Biomass

Microalgae *Botryococcus Braunii* used in this work was donated by Desert Bioenergy S.A., Chile.The algae were cultivated in the Microbial Ecology Laboratory of the University of Antofagasta in northern Chile. The microalga was flocculated with ferric chloride (FeCl$_3$)

in a dosage of 15 mg/L (Martínez *et al.*, 2014). The chemical composition of microalgal biomass is shown in Table 1. To obtain a homogeneous material for performing the experiments, wet biomass with 80% water content was dried in a tunnel dryer at 60°C for 48 h until reaching 10% moisture. Finally, the biomass was milled obtaining a fine powder (of less than 500 µm), which was refrigerated for further use in the following 30 days. Although samples storing at 4°C in amber glass bottles does not prevent lipids degradation, peroxidation rate severely declines in such conditions, as observed by Belarbi *et al.* (2000) (Belarbi *et al.*, 2000).

2.1.2 Reactives

All the solvents used in the reaction were of analytical grade. Methanol and ethanol were used as acyl acceptors in the reaction. Petroleum ether and chloroform were used as co-solvents. Sulfuric acid, hydrochloric acid and Amberlyst-15 (SIGMA Aldrich, acid resin) were used as catalysts. Heptadecanoic acid methyl ester of chromatographic purity was used as internal standard for chromatographic analyses.

2.2 Experimental set up

In this work the direct transesterification of biomass was performed in two different systems (Figure 1)
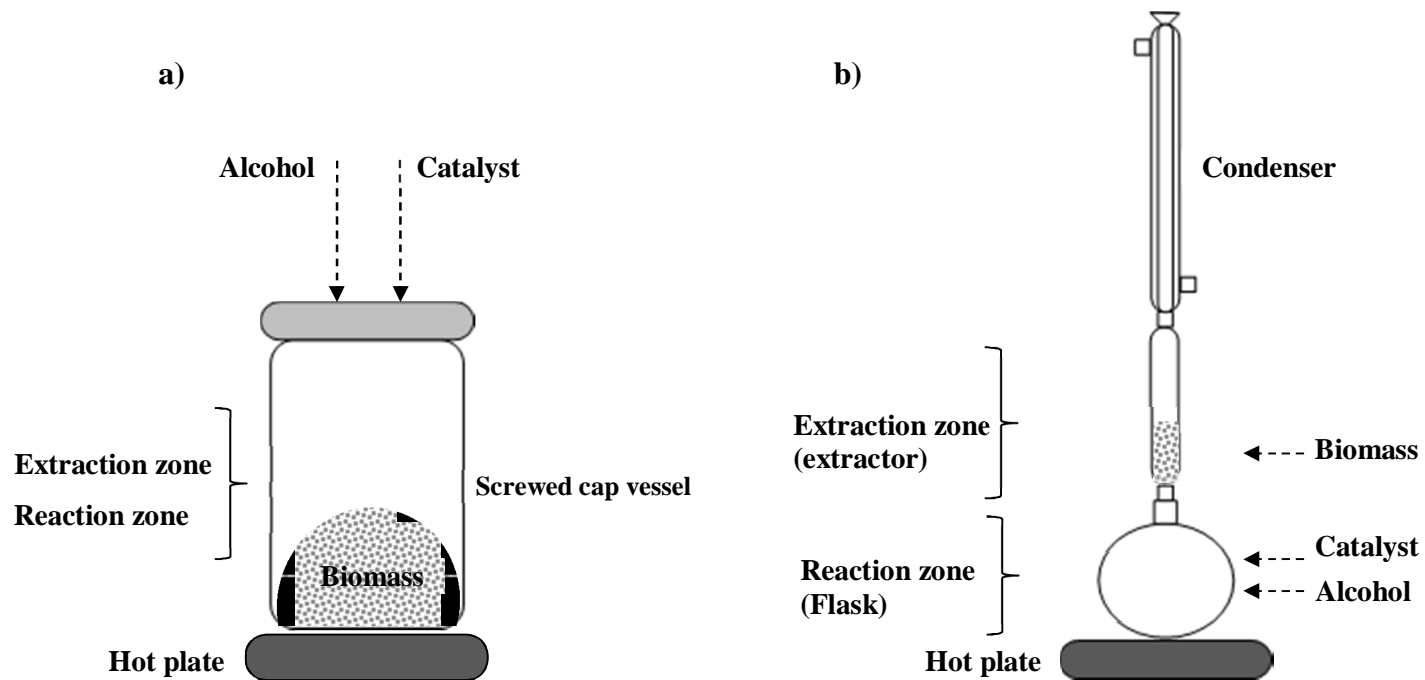
Figure 1. Systems used for microalgal biomass direct transesterification. a) Stirred batch reactor (SBR) and b) Reflux extraction reactor (RER)

2.2.1 Stirred batch reactor (SBR)

The SBR consists on a screwed cap vessels (15 mL), where the biomass, solvent (acyl acceptor) and catalyst are reacting together. In this process the catalyst has two functions: as acid for cell wall disruption and as catalyst for promoting transesterification/esterification reactions. The temperature and agitation was controlled in an incubator shaker at 55°C and 250 rpm, respectively. An acyl acceptor dosage of 7 mL/g was used. This corresponds to a methanol/fatty acid molar ratio of 293:1 and an ethanol/fatty acid molar ratio of 204:1. The solvent volume added in SBR was the necessary to allow a homogenous mixture between biomass and catalyst.

2.2.2 Reflux extraction reactor (RER)

The RER was divided in an extraction and a reaction zone. The extraction zone consists of a butt extractor tube coupled with a condenser, and the reaction zone consists of a round flask with flat bottom and heating plate to allow temperature control. In the RER system, the lipids extraction is carry out separately from the reaction by a direct contact of the biomass with the condensed solvent in the extraction zone. Then, the condensed solvent with the extracted lipids flows down to the reaction zone, reacting with the catalyst. A methanol/fatty acid molar ratio of 840:1 and an ethanol/fatty acid molar ratio of 582:1 were used. The solvent volume was calculated as the minimum volume to allow a continuous contact with biomass.

To increase FAAE production yield in direct transesterification of microalgal biomass, different strategies involving acyl acceptor type, catalyst type and solvent mixtures were evaluated and studied in both systems. The operational conditions of the performed

experiments are shown in Table 2. The reaction time was established in 5 h according to previous experiments (data not shown). In all cases, an acid catalyst concentration of 100% wt based on the total fatty acids (TFA) content was used.

Table 1. Chemical composition of *B. Braunii* microalgae expressed on a dry matter basis (% wt).

	Content (% wt)
Microalgae	
Moisture	10.1±2.5
Total lipids content	21.5±1.3
Proteins	25.3±1.9
Ash	18.1±1.2
Other components	25.1±1.5
Total lipids content	
Saponifiable lipids (*)	77.0±0.5
Unsaponifiable lipids	23.0±1.2

(*) Saponifiable lipids are transformed to FAAE

FAAE produced were separated by adding petroleum ether to the sample and evaluated gravimetrically. In this purpose, the complete separation of FAAE from the biomass, the sample was centrifuged at 10.000 rpm for complete separation of the organic phase from the biomass. Finally, the supernatant was put into a rotary evaporator to remove the solvent and was weighed and used for FAAE content determination by GC-MS

2.3 Evaluation of acyl acceptor type

In lipids transesterification, methanol has been the most used acyl acceptor, mainly due to its low cost; so methanol was used in the reaction. Besides, ethanol was also evaluated as acyl acceptor in the reaction, because in the lipids extraction from microalgae, primary alcohols of longer chains (compared to methanol) are able to extract lipids more efficiently.

(Wahlen et al., 2011b).The operational conditions of these experiments are shown in Table 2.

2.4 Evaluation of catalyst type

In the direct transesterification process, acid catalysts are used due to the high FFA content in microalgae lipids. (Wahlen et al., 2011b) In B. *Braunii* FAA content accounted for 56.4% of total lipids (as shown in Table 1). Acid catalysts convert both FFA and triglycerides (TG) into FAAE. For this purpose, sulfuric acid, hydrochloric acid and Amberlyst-15 were used as catalysts.

Methanol was used as solvent for further experiments due to a higher FAAE yield obtained in section 2.3. Sulfuric and hydrochloric acid have been mainly used as catalysts in the esterification and transesterification of lipids with high FFA content (Berchmans & Hirata, 2008a; Demirbas, 2009a). Moreover, Amberlyst-15 has been used with high efficiency as acid catalyst for the conversion of lipids with high FFA content (Park *et al.*, 2010). The experiments were performed in both systems according to the operational conditions summarized in Table 2.

Table 2. Operational conditions used in the direct transesterification of microalgal biomass trials

System	Temperature (°C)	Solvent (mL/g dry biomass)	Agitation (rpm)	Catalyst (% on the base of the microalgae oil mass content)
RER	Solvent boiling point	40	without mixing	Liquid catalyst: 100% Solid catalyst: 20%
SBR	55	7	200	Liquid catalyst: 100% Solid catalyst : 20%

2.5 Evaluation of solvents mixture

Increased lipids extraction yield has been achieved by using organic solvents combination, such as chloroform-methanol and hexane-isopropanol, among other mixtures (Lee et al., 2010).

In direct transesterification of lipids, the addition of a co-solvent mixture can improve the mass transfer, increasing the reaction yield (Johnson & Wen, 2009). Besides, solvents mixtures use can increase the extraction of structural lipids from membrane cells, which are forming complex molecules with phospholipids and proteins (Halim *et al.*, 2012). Therefore, in this process the effect of solvent mixtures was evaluated on FAAE yield. Experiments were performed with a solvent/co-solvent ratio of 1:1 v/v. Methanol and ethanol were used as acyl acceptors, petroleum ether and chloroform as co-solvents; and sulfuric acid, chlorhydric acid and Amberlyst-15 were used as acid catalysts. In addition, the incorporation of different proportions of co-solvent (1:3; 1:1 and 3:1 v/v alcohol: co-solvent ratio) in the reaction was evaluated. The experiments were carried according to the conditions of temperature, solvent volume, agitation and catalyst dosage shown in Table 2. All trials were performed in triplicate.

2.6 Analytical methods

2.6.1 Total fatty acids content

Total fatty acids (TFA) content, which corresponds to the saponifiable fraction that can be converted into FAAE, was determined by lipids extraction of microalgae using a chloroform/methanol mixture (Bligh & Dyer, 1959b), where 0.2 g dry microalgae sample was sonicated in 0.8 g distilled water for approximately 20 min. Then, the sample was

mixed with 3.75 mL chloroform/methanol (1:2) for 1 min in a vortex and later centrifuged at 10000 rpm to allow the phases separation. The extracted organic fraction was put into a rotary evaporator to remove solvents and the product was weighed and gravimetrically quantified as total lipid content (TL% wt in equation 1). After this, the final product was hydrolyzed and esterified for FAAE quantification by GC-MS, according to Araujo method (Araújo, 1995). TFA was calculated using equation (1):

$$TFA\ (\%wt) = \frac{FAAE\ (\%wt) \times TL(\%wt)}{100}$$
eq. (1)

FAAE (%wt.): Fatty acid alkyl esters

TL (% wt.): Total lipid content (in dry microalgae basis)

Additionally, ash content was determined according to ASTM D 3174(ASTM, D3174) and protein content was determined using Kjeldahl method (Dupont *et al.*, 2011).

2.6.2 Chromatographic methods

FAAE identification and quantification were carried out using a Clarus 600 chromatograph coupled to a Clarus 500T mass spectrometer from Perkin Elmer (GC-MS). An Elite-5ms capillary column 30 m long, 0.1 µm thick and internal diameter of 0.25 mm was used. The vials were prepared by adding 10µL sample to 230µL methyl heptadecanoate (2030 mg/mL) as internal standard. The following temperature program was used: 150°C for 3.5 min and then increasing temperature at a rate of 1.1°C/min up to 240°C. Both the injector and detector temperature were 250°C and helium (He) was used as the carrier gas.

2.6.3 FAAE yield

FAAE yield used for determining the reaction yield was calculated using equation (2) and equation (3):

$$Total\ FAAE\ (\%wt) = \frac{total\ weight\ of\ FAAE\ (g)}{Dry\ biomass\ (g)} \, x100 \qquad\qquad \text{eq. (2)}$$

Where total FAAE (% wt) corresponds to the FAAE gravimetric yield and was evaluated by GC-MS.

$$FAAE\ yield\ (\%\ wt): \frac{FAAE(\%wt)xTotal\ FAAE(\%wt)}{TFA(\%wt)} \qquad\qquad \text{eq. (3)}$$

2.6.4 Statistical analysis

Differences between FAAE yields means of SBR and RER were analyzed using two-way ANOVA with the software JMP-8 and Sigma Stat 3.5. Two-way ANOVA was used to detect interaction between strategies. Initially, normality and homogeneity test were assessed in all variables and checked by residual plots. Then, analysis of variance (two-way ANOVA between groups) and Tukey's test were performed, P-value below 0.05 was considered significant.

3. Results and discussion

3.1 Effect of acyl acceptors type in SBR and RER systems for FAAE production

Prior studies of direct transesterification have been performed in a solvent-biomass direct contact system or SBR, where alcohol has an important role in the reaction, acting as both solvent for lipids extraction from biomass and as the reactant, converting the lipids to FAAE. To determine which alcohol would perform better in FAAE production using

Botryococcus *Braunii* microalgae, methanol and ethanol were tested as acyl acceptors in SBR and RER systems. The results indicate that the reaction *yield* was affected by both the acyl acceptor type and the used systems (Fig. 2). The acyl acceptors presented an effect in both the lipids extraction and FAAE conversion yield, showing a significant yield increment when methanol was used in both systems, where a higher FAAE yield was observed in RER system. In fact, a FAAE conversion yield of 80.1% wt was observed in RER system while only 64.5% wt was obtained in SBR system after 5 h, when methanol was used. On the opposite, FAAE conversion yields of 67.7% and 45.0% wt were obtained in RER and SBR systems, respectively, when ethanol was used as acyl acceptor.
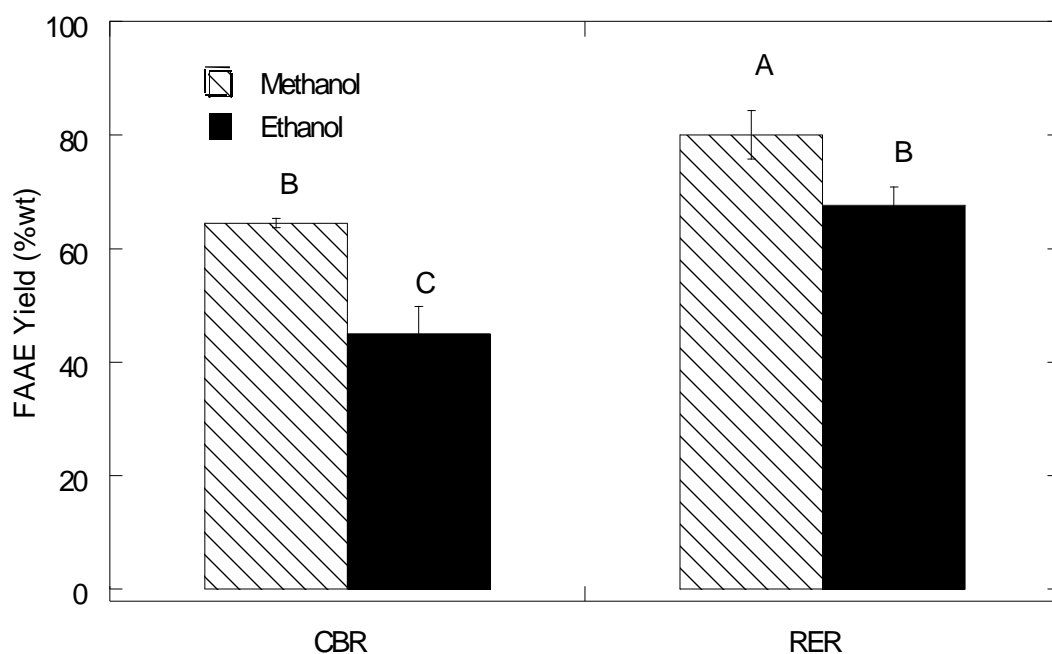
Figure 2. Effect of acyl acceptor type on FAAE yield in the direct transesterification of microalgal biomass for SBR and RER systems. Data represents the mean values of three replicates and the error bars show the standard deviations. The different letters indicate a significant difference at $P < 0.05$.

Lipids produced by microalgae are found both as cytosolic lipid bodies and as structural components of cell membranes. In the extraction of structural lipids the polarity of the

organic solvent is critical, as these lipids are found in the cytoplasm, forming complexes with polar lipids (such as phospholipids) and proteins (Halim *et al.*, 2012). Polar organic solvents (such as methanol and ethanol) are able to disrupt these associations by forming hydrogen bonds with phospholipids and proteins in the complex (Medina *et al.*, 1998).

Methanol and ethanol are polar solvents with a high dielectric constant (Mohsen-Nia & Amiri, 2013) normally used in the conventional transesterification of lipids, where methanol has been the most used acyl acceptor at industrial level, due to its lower price (USD 1.6/gal) compared to other alcohols.

Looking the results of this study, a high FAAE yield was obtained when methanol was used, probably due to the higher dielectric constant (more polar) compared to ethanol and the different miscibility of the alcohols in the non-polar oil/ester system. Moreover, during the reaction, emulsions are usually formed. In fact, in the methanolysis, these emulsions break down quickly and easily to form a lower glycerol rich layer and upper methyl ester rich layer. On the opposite, in the ethanolysis these emulsions are more stable and the separation and purification of esters can be extremely difficult (Ganesan et al., 2009c).

Related to the applied systems, in RER configuration higher FAAE yields were reached with both acyl acceptors compared to SBR configuration. In both systems the yield is limited by the extracted lipids which are transformed into FAAE by the action of acyl acceptors present in excess. Indeed, in the direct transesterification lipids pass through a lipid bilayer by simple diffusion following the concentration gradient, like in the extraction. As in the SBR system the alcohol molar ratio is lower than the RER system, lipids diffusion inside of cell could be limited, due to a decrease of the concentration gradient.

The systems are different in configuration. In the case of SBR, the solvent is the acyl acceptor and simultaneously helps to improve the mixture homogenization. In the case of

RER, the solvent is always in excess to move the reaction equilibrium of esterification to the products side. In the extraction zone, by the application of a reflux system, always fresh solvent is in contact with biomass, extracting the lipids for further reaction. In this system is very difficult to calculate the real solvent ratio, as it will depend on the design and configuration of the system. So, it is clear that the solvent role is different in both systems and therefore different solvent:fatty acids ratios were used to optimize the performance of each tested system.

The increase in alcohol: fatty acids molar ratio improves lipids conversion to FAAE. FAAE yield reached a maximum of 80.1% wt after 5 h with a solvent: fatty acids ratio of 840:1 in RER system. However, FAAE yield of only 64.5% wt was obtained in SBR system using a methanol/fatty acids molar ratio of 293:1. The reactants excess present in RER system is higher than that of SBR, favoring the mass transfer and the reaction yield. These reactions at moderate temperature are highly sensitive to water content and during the reaction, water is produced due to esterification of FFA. In CRB, the increase of water content by esterification of FFA can dilute the reaction mixture, thereby reducing reaction rates and provoking FAAE hydrolysis. (Levine *et al.*, 2013) Instead, RER system can be an option to avoid water presence in the reaction due to higher acyl-acceptor volume than CRB, diminishing the inhibitory concentration of water in the reaction. Besides, in RER system the biomass is in continuous contact with fresh solvent, enhancing mass transfer and lipids diffusion from inside the cell. In this system essentially involves percolation of the solvent through the biomass sample, is used in lipids extraction where the solvent is put in a flask and then evaporated, the vapors are cooled in the condenser located above the sample and the condensed solvent is trickled down through the biomass sample (Ranjan *et al.*, 2010).

FAAE yields obtained were higher than the maximum conversion of algal biomass obtained by Johnson and Wen (Johnson & Wen, 2009) of 66% wt, but lower than the 88% wt maximum conversion in 8 h reached by Ehimen *et al.* (Ehimen *et al.*, 2010a) using a 315:1 solvent molar ratio and the 95% wt obtained by Velasquez *et al.* (Velasquez-Orta et al., 2012b) in 24 h, using a solvent molar ratio of 600:1.

Moreover, using biomass with a moisture content of up to 10% will allow a decrease in biomass drying costs, while at moisture levels higher than 10%, water is more difficult to remove because it could be bound to pores or the cell wall, rather than free (Velasquez-Orta et al., 2013a).

3.2 Effect of catalyst type in SBR and RER systems for FAAE production

To determine which catalysts type would be more suitable to increase the direct transesterification yield, sulfuric acid, hydrochloric acid and Amberlyst-15 were studied as catalysts. According to the results, FAAE yield were higher in homogeneous rather than heterogeneous catalysis. In the case of the SBR system, the catalyst not only increases the reaction rate in the direct transesterification, but can also have an effect on microalgae cell wall disruption. Ozgul-Yucel and Turkay (Ozgul-Yucel & Turkay, 2002) reported an increment on the solution stickiness, due to the release of intracellular components in the direct transesterification of rice bran, when a liquid acid catalyst was used. Higher FAAE yields were observed in both reaction systems when H_2SO_4 was used as catalyst, with a FAAE yield of 64.5 and 80.1% for SBR and RER systems, respectively (Fig. 3).
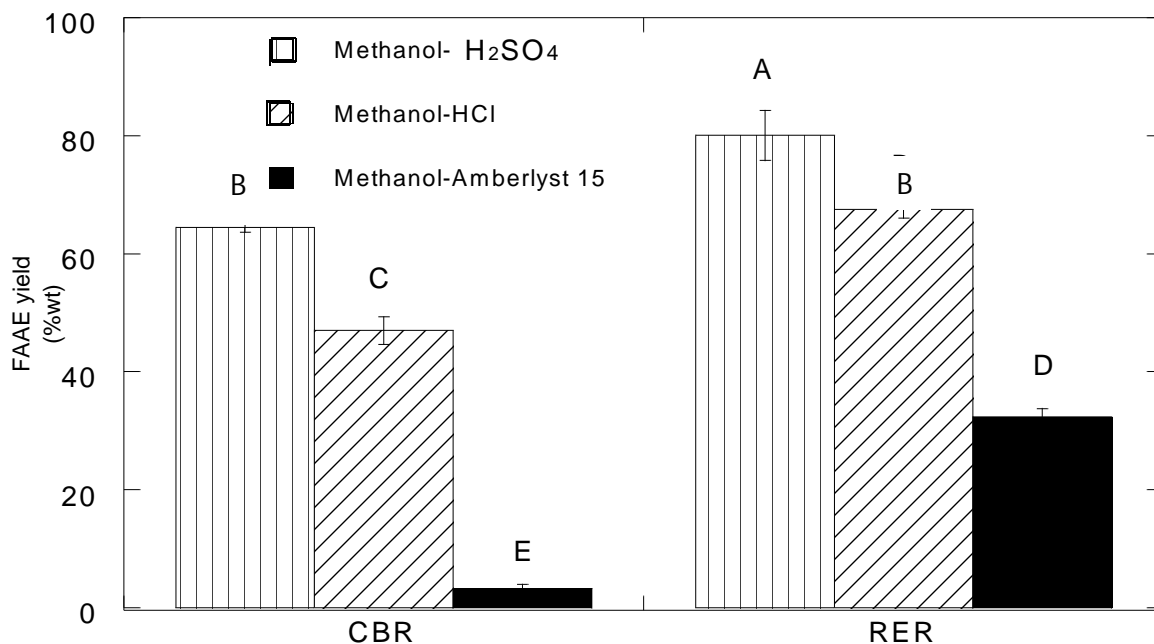
Figure 3. Effect of catalysts type in the direct transesterification of microalgal biomass for SBR and RER systems. Data represents the mean values of three replicates and error bars show the standard deviations. The different letters indicate a significant difference at P<0.05.

In the case of HCl, better results were obtained in RER system with a 67.6% FAAE yield. In the acid transesterification of lipids, H_2SO_4 has shown a superior catalytic activity compared to HCl (Al-Widyan & Al-Shyoukh, 2002) and in the direct transesterification of oleaginous microbial biomass, Liu and Zao (Liu & Zhao, 2007) have reported higher FAAE yields with H_2SO_4 than HCl. They reported FAAE yields close to 90%, when H_2SO_4 was used, and of 70% yield with HCl as catalyst.

In the case of the solid catalyst, the best results were observed in RER system. FAAE yields of 32.4% and 3.3 % wt in RER and SBR were obtained, respectively. Transesterification with Amberlyst-15 was evaluated with a dosage of 20% wt as higher concentrations would interfere with the solvent boiling performance in RER system.

The diminishment of FAAE yield comparing homogeneous catalysis with liquid acids with heterogeneous catalysis with solid Amberlyst-15 could be probably caused by a decrease in

the reaction rate due to mass transfer limitation.(Sharma *et al.*, 2011) Besides, in SBR, FAAE yield reduction could be coupled with a possible significant impact of the catalysts in cell disruption, releasing molecules different from the desired lipids. In general, when liquid acid catalysts were used in SBR, the obtained yield was lower than that of RER, possibly because some of the cell contents released in the liquid phase may be not lipids, but other cellular constituents such as carbohydrates and proteins, which can promote side reactions and a diminishment in FAAE yield. Although a significant diminishment in FAAE yield was observed with the heterogeneous catalyst, it has the advantage of being easily recovered and reused after activation.

3.3 Evaluation of solvents mixtures

Higher lipids extraction yields from different bioresources using solvent mixtures have been already reported. Normally, solvent mixtures composed by a non-polar and a polar organic solvent are used to assure complete lipids extraction from microalgal cells, both in the form of freestanding globules and in the form of membrane-associated complexes.(Halim *et al.*, 2012) Therefore, mixtures of petroleum ether-methanol, petroleum ether-ethanol, chloroform-methanol and chloroform-ethanol were evaluated in both reaction systems using a 1:1 v/v solvent/co-solvent ratio (Fig. 4).

Depending on the nature of the solvent mixture, different percentages of extracted lipids which are transformed into FAAE were obtained. Regarding the results shown in Figure 4, FAAE *yields* did not significantly increase by using solvent mixtures in a 1:1 ratio (P<0.05, Tukey Test). On the contrary, a reduction in FAAE yield, particularly in SBR system was observed.

However, with the variation of the solvent:co-solvent ratio (Figures 5 and 6) an increase in FAAE yield was detected. This effect was observed in treatments catalyzed by H_2SO_4 and HCl, where a co-solvent use of 25% v/v was incorporated. This means a 3:1 v/v methanol/co-solvent ratio in both systems.
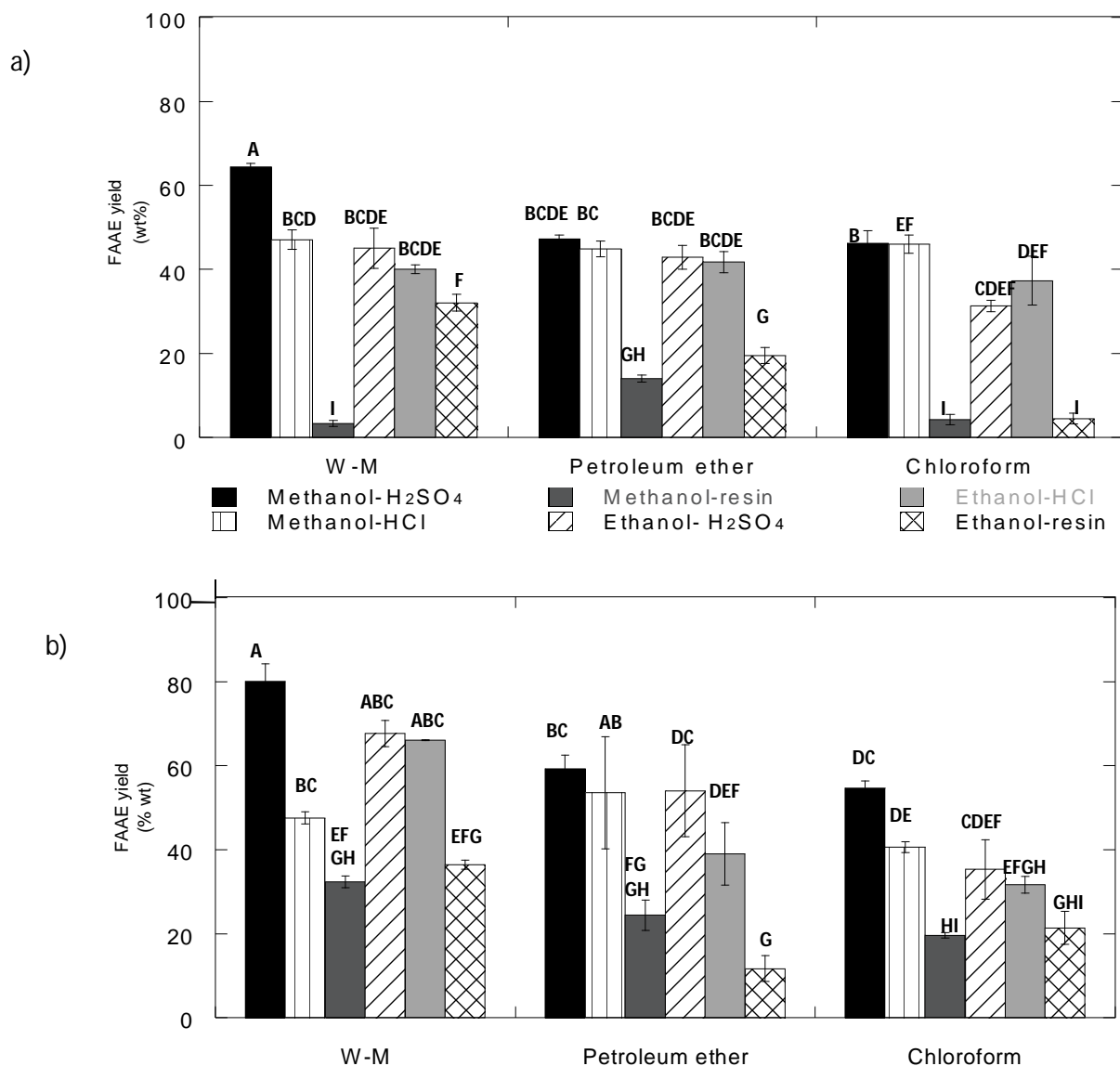
Figure 4. Effect of solvent mixture in the direct transesterification of microalgal biomass a) SBR b) RER. Data represents the mean values of three replicates and the error bars show the standard deviations. The different letters indicate a significant difference at P<0.05. *W-M: without mixture, only methanol or ethanol.
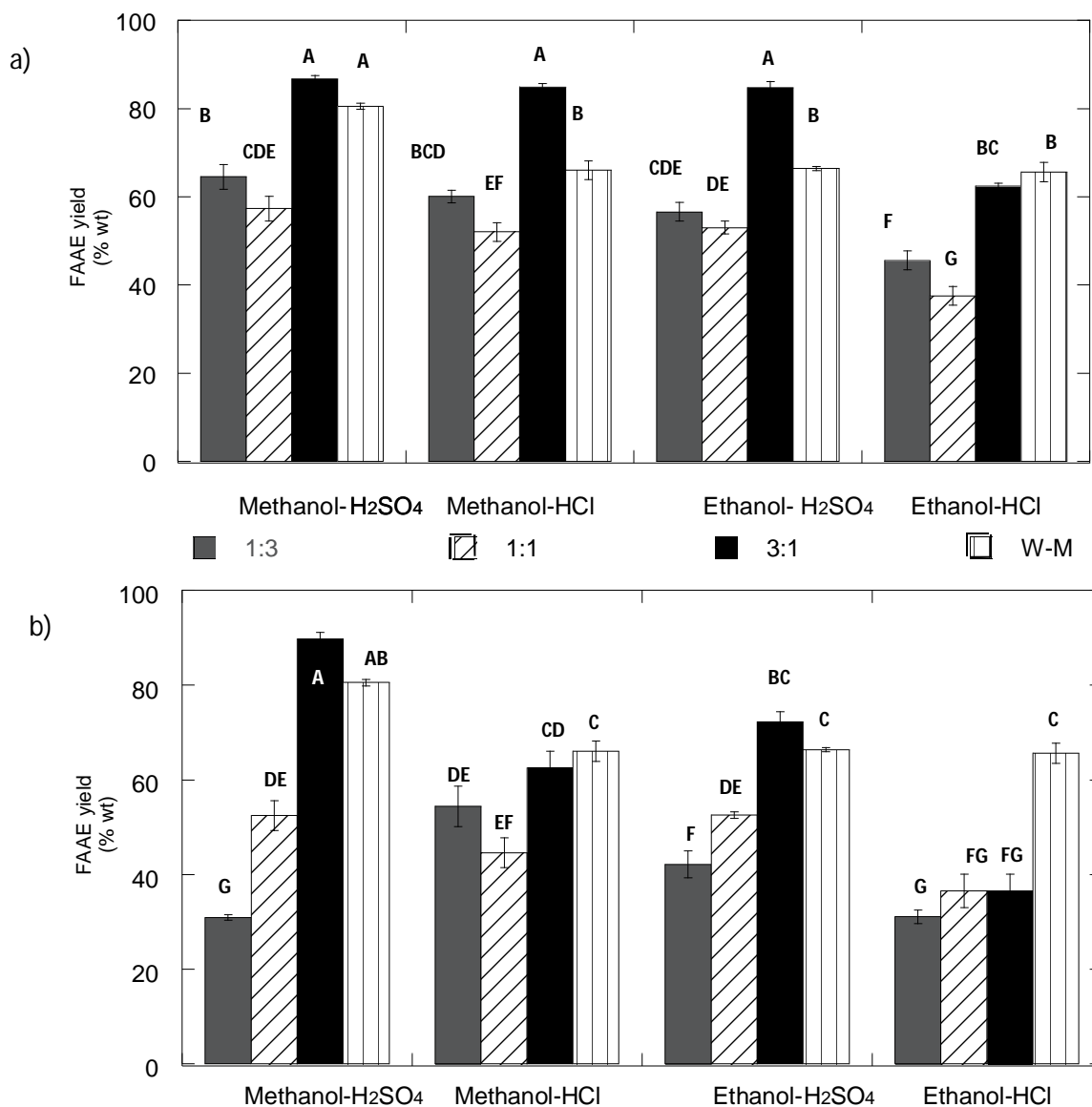
Figure 5. Effect of different solvent:co-colvent ratios in FAAE yield of the direct transesterification of microalgal biomass in RER a) solvent: Petroleum ether b) solvent: Chloroform. Data represents the mean values of three replicates and the error bars show the standard deviations. The different letters indicate a significant difference at P<0.05. *W-M: without mixture, only methanol or ethanol.
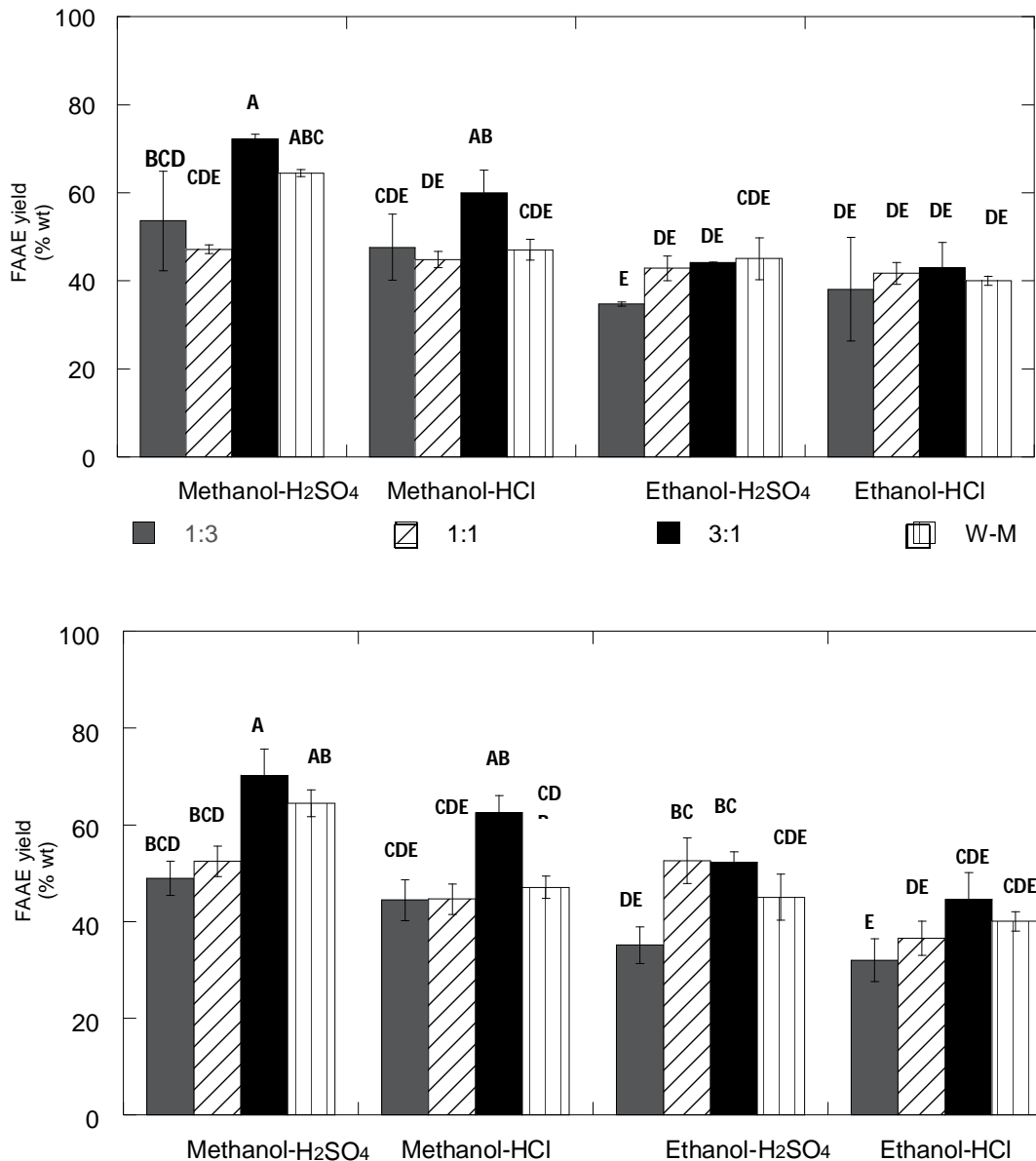
Figure 6. Effect of different solvent:co-colvent ratios in FAAE yield of the direct transesterification of microalgal biomass in SBR a) solvent: Petroleum ether b) solvent: Chloroform. Data represents the mean values of three replicates

The addition of a small volume of co-solvent increased the solubility of both the complex lipidic fraction and free lipids, which were converted to FAAE. The solvent mixtures contain polar and non-polar solvents, extracting a higher amount of lipids. In these cases, the polar solvent releases the lipids from their protein–lipid complexes, and the lipids

subsequently dissolve in the non-polar solvent.(Ryckebosch *et al.*, 2012) Lower solvents mixture polarity was reached by incorporating higher volumes of co-solvent, producing however lower FAAE yields according to the obtained results. Ryckebosch *et al.*(Ryckebosch *et al.*, 2012), observed lower lipids extraction yields from *C. vulgaris* with the decrease of solvents mixture polarity. The highest lipids extraction yield was obtained with a chloroform–methanol mixture of 1:1 (v/v). Instead, with a chloroform–methanol mixture of 2:1 (v/v) only 76.5% of the lipids extracted with the 1:1 mixture were obtained. These results are in contrast with those of Lee *et al.*(1996) (Lee *et al.*, 1996) who stated that the non-polar/polar solvents ratio has no effect on lipids recovery from fish.

The application of ethanol/petroleum ether mixtures using a 3:1 v/v ratio catalyzed with HCl caused a decrease in FAAE yield in RER system. This reduction can be associated to the low solvents mixture polarity caused by the low polarity of ethanol. Besides, HCl has a lower catalytic activity compared to H_2SO_4 (Liu & Zhao, 2007).

 Higher co-solvent contents in the reaction decreased the yield to FAAE in both systems, due to the reduction of alcohol/lipids molar ratio. Acidic transesterification requires a high alcohol/lipids molar ratio for increasing FAAE yield (Ganesan et al., 2009c).

Besides, the reaction is highly sensitive to water content produced by esterification of FFA. Higher co-solvent concentrations may increase the inhibitory effect of water in the mixture. The higher FAAE yield in RER system can be related to an increase of lipids diffusion across of the cell. Moreover, during fatty acids esterification water is produced. Water molecules might hydrolyze the esters to fatty acids, provoking a decrease in FAAE yield. (Berchmans & Hirata, 2008a) Finally, in RER system the solvent mixture has a boiling temperature lower than that of each single component decreasing hydrolysis reaction of FAAE to fatty acids (Matsoukas, 2012).

The flocculation agent ($FeCl_3$) could act as a Lewis acid catalyst in lipids transesterification at high temperatures in the in situ lipids conversion process (Jin *et al.*, 2014). Besides, the flocculation agent can produce changes in unsaturated fatty acids however it could be beneficial providing an increased lipid stability (Martínez *et al.*, 2014).

4. Conclusions

Although the RER system does not use any application of shear stress to produce microalgae cell wall disruption, highest FAAE yield were obtained in this configuration. In RER system, the main lipids extraction mechanism is related to diffusion, as the percolation of fresh solvent through the sample does not limit the diffusion of lipids out of the cell. In this system a FAAE yield of 80.1% wt using methanol and H_2SO_4 was obtained, while in SBR system FAAE yield was only 64.5% wt.

Even though the methanol/fatty acid molar ratio used were different in both systems, in SBR a high solvent volume could produce the dilution of catalyst (according to shown in the subsequent chapters). The dilution of catalyst could affect its role in the disruption of cell wall.

Moreover, when a solid catalyst was used in both systems, lower FAAE yields were observed due mass transfer limitations in the tri-phasic (alcohol-lipid-catalyst) system.

Finally, the addition of a co-solvent in the reaction increased FAAE yield in both systems, but using a 3:1 (v/v) solvent/co-solvent ratio.

According to the obtained results, the product does not fully accomplish the Normative EN 14214, therefore we speak about an unrefined FAAE mixture.

Chapter 4

Improving the FAME yield of *in situ* transesterification from microalgal biomass through particle size reduction and co-solvent incorporation

Improving the FAME yield of *in situ* transesterification from microalgal biomass through particle size reduction and co-solvent incorporation

Pamela Hidalgo[1], Gustavo Ciudad[1,2], Sigurd Schober[3], Martin Mittelbach[3]& *Rodrigo Navia[1,2,4]

[1]Scientific and Technological Bioresources Nucleus, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]Departament of Chemical Engineering, Universidad de La Frontera, Casilla 54-D, Temuco, Chile

[3]Institute of Chemistry, Working Group Chemistry and Technology of Renewable Resources, University of Graz, Heinrichstraße 28, A-8010 Graz, Austria.

[4]Centre for Biotechnology and Bioengineering, Chile

1. Introduction

The use of microalgae as raw material for biodiesel production has several advantages over the production from terrestrial plant crops as microalgae can accumulate high quantities of triglycerides and free fatty acids (Huang et al., 2010; Umdu et al., 2009). Biodiesel production from microalgae does not compete with arable land, their cultivation can even use less water and have a small ecological footprint compared to traditional oilseed crops (Dismukes et al., 2008; Widjaja et al., 2009).

There are different technologies available for producing biodiesel from microalgae including conventional methods where lipids are extracted and then transformed into FAME via transesterification. The main limiting step of this process is however the complexity of lipids extraction from microbial cells such as microalgae. In fact, lipids extraction from microalgae is mainly performed by cell wall disruption methods and solvent extraction and not by conventional physical methods, due to difficulties in breaking the cell wall (Ehimen et al., 2010a; Grierson et al., 2001; Pieber et al., 2012b). An alternative to the conventional process is the *in situ* transesterification of biomass to biodiesel, where lipids extraction and transesterification are carried out in one step (Hidalgo et al., 2013b; Meher et al., 2006). This process eliminates the lipids extraction step and could positively impact on process costs for an industrial scaling.

As reported in several works, *in situ* transesterification of microalgae has lower FAME yield compared to the conventional process. Johnson and Wen (2009), reported a FAME yield of 63%wt applying *in situ* transesterification to *Schizochytrium limacinum* biomass. Hidalgo et al. (2014) reported 65% wt of FAME yield with *B. braunii* biomass, using a methanol:lipids molar ratio of 293:1. Moreover, Ehimen *et al.* (2010) reported 88% wt of

FAME yield using a methanol:lipids molar ratio of 315:1. Therefore, in order to implement this process at large scale it is necessary to improve the performance of the reaction.

According to several authors, particle size has a crucial role on *in situ* transesterification performance, showing an increment on FAME yield with the reduction of the particle size (Kasim & Harvey, 2011; Zakaria & Harvey, 2012). During microalgae processing, the drying step can cause the formation of microalgae conglomerates and of case-hardening on the drying surface (Velasquez-Orta et al., 2013b). Grinding the dried algae diminishes the particle size favoring lipids extraction from the cells, due to the increase of particles surface area in contact with the solvent. Moreover, there are some reports showing that the incorporation of a co-solvent can help to improve lipids extraction; therefore this strategy could be applied to increase FAME yield of *in situ* transesterification process.

The main goals of this study were to evaluate the effect on FAME yield of microalgae conglomerates particle size and co-solvent addition to the reaction mixture during *in situ* transesterification of microalgal biomass. Additionally, the effect of temperature and catalysts dosage by using a Box-Behnken experimental design were evaluated. Moreover, the effect of the reaction in the particle size as an indirect measure of transesterification performance was studied.

2. Experimental section

2.1 Microalgae

The microalga *Botryococcus braunii* used in this work was supplied by Desert Bioenergy S.A., (Chile). The biomass was dried at 60°C for 24 h, by spreading a 5 mm layer of wet microalgae (80% wt) in a convective dryer until reaching a moisture content near 10%.

After this, the dried biomass was milled and then fractioned by using sieves of different mesh sizes (150 µm and 500 µm), obtaining three particle size fractions (Size 1: <150 µm; Size 2: 150 µm< D< 500 µm; Size 3: >500 µm). Microalgae powder fractions were stored at 5°C before used in the experiments.

2.2 Experimental setup

A series of experiments were conducted to evaluate the effect on biodiesel yield of different parameters including particle size of dry microalgae, co-solvent volume in the reaction, temperature and catalyst dosage. Petroleum ether due to its low toxicity, low cost, and great affinity toward neutral lipid (Ryckebosch et al., 2012), was used as co-solvent in the reaction.

The experiments were performed in vessels with screwed cap (20 ml) containing the reaction mixtures. Methanol was used as acyl acceptor of the reaction. Due to a high free fatty acids content, sulfuric acid (H_2SO_4) was used as catalyst, as it can catalyze both esterification and transesterification reactions (Ehimen et al., 2010a). The reaction was maintained at 200 rpm during 2 h, using 1 g of microalgae powder. The reaction was stopped with the addition of hexane and distilled water (2:1 vol/vol), where two phases were observed. The upper phase (nonpolar or hexane phase) was separated and hexane was evaporated by distillation.

For the case of co-solvent use, hexane and co-solvent were separated from the product by distillation. The solvent free sample was gravimetrically quantified and stored at 4°C for its analysis of FAME content by gas chromatography. Heptadecanoic acid methyl ester (C17:0) of chromatographic purity was used as internal standard in the quantification.

2.2.1 Influence of the methanol: total fatty acids molar ratio on FAME yield recovery

The methanol:total fatty acids molar ratio effect was evaluated in the range between 23:1 and 468:1, simultaneously evaluating the three particle size fractions of microalgae. High methanol excess was used to ensure a complete submersion of the biomass, allowing a homogeneous mixture between biomass and catalyst (Ehimen et al., 2010a). In the experiments a catalyst dosage of 75% (based on the total fatty acids content of the biomass) was used. A regression analysis was performed related to FAME yield and methanol: total fatty acids molar ratio for the different particle sizes and the results were adjusted to a polynomial model. Regression analyses were conducted by nonlinear curve-fitting methods using Kaleida Graph version 4.0 (Synergy Software) for Windows.

2.2.2 Optimization of the *in situ* transesterification process

An experimental Box-Behnken design (BBD) available in JMP software (SAS Institute Inc., Cary, NC) was applied to determine the influence of the operational conditions in the *in situ* transesterification process.

The number of experiments (N) required for the development of BBD was defined by equation (1).

$$N = 2k(k - 1) + n \qquad\qquad \text{eq. (1)}$$

Where k is the number of factors and n is the number of central points. In this study, k and n were set at 4 and 5, respectively, which mean that 29 experiments were run. The independent variables used in this study were catalyst concentration (A), reaction temperature (B), particle size (C) and co-solvent proportion (D).

The independent variables and levels of the factorial design are shown in Table 1. FAME yield was used as response variable. The optimal methanol: total fatty acids molar ratio obtained was used in these experiments, but incorporating co-solvent according to Table 1.

Table 1. Independent variables and levels of the Box-Behnken experimental design

Independent variables	Levels		
	-1	0	1
Particle size (µm)	<150	150< D <500	>500
Temperature (°C)	50	60	70
Co-solvent (% vol/vol)	25 (3.85*)	50 (2.6*)	75(1.35*)
Catalyst (%wt)**	75	125	150

* Value in parentheses correspond to polarity index (PI) of the mixture evaluated (Snyder, 1978).**On the basis of total fatty acids

2.2.3 Effect of in-situ transesterification on microalgae particle size distribution

During the in-situ transesterification of biomass, side reactions such as hydrolysis of protein and carbohydrates could happen, producing a diminishment of microalgae cell size (Velasquez-Orta et al., 2013b). The hydrolysis of cell constituents could consume part of the catalyst originally available for the lipids transesterification. Thus, side reactions during transesterification were evaluated by determining surface area, pore volume, total organic content and particle size distribution before and after in-situ transesterification. In addition, residual lipids content was also determined.

The effect of *in-situ* transesterification in microalgae conglomerates was evaluated using a dosage of 75% and 150% wt of acid catalyst. A high catalyst dosage was used to facilitate lipids extraction due to the cell wall disruption, produced by of cell constituents hydrolysis such as proteins and carbohydrates during the in-situ transesterification (Ehimen et al.,

2010a; Velasquez-Orta et al., 2013b). The experiments were performed under optimal conditions of temperature and co-solvent proportion, according to the previous results.

The surface area and pore volume were determined through of the Brunauer Emmett Teller (BET) method. The determinations were tested using a nitrogen porosimeter (Quantachrome, NOVA, and model number 1000). The total organic carbon was measured with an organic carbon analyzer (TOC- VCPH, SHIMADZU).

The particle size distribution of microalgae powder was analyzed with a laser diffraction particle size analyzer SALD-3101 (Shimadzu, Japan). For this instrument, the refractive index was set at 1.45 and distilled water was used as a dispersing agent to prevent particle aggregation. The residual lipids content was determined as total fatty acids, as described below. All the results obtained were analyzed by JMP statistical software.

2.3 Analytical techniques

2.3.1 Lipids characterization

Lipids were extracted for their characterization in a soxhlet apparatus. The extraction was carried out using 5 g of dry microalga, placed inside the extractor. A methanol/chloroform (2:1 vol/vol) solvent mixture was used in the extraction, boiling during 6 hours. Then, the solvent and lipids were separated in a rotatory evaporator. Thereby, the extracted lipids were gravimetrically quantified as total lipids (TL). In TL both transesterifiable lipids (defined as total fatty acids (TFA)) and fatty acid distribution were determined. TFA were determined as saponifiable lipids. Saponifiable lipids (SLs) were evaluated by difference between TL and unsaponifiable lipids (USLs). USLs were obtained according to AOCS method (Cc-6a-53).

Total acyl-glycerides (tri-di and mono-glycerides) content was determined according to EN 14105 by gas chromatographic (GC) methods, using tricaprine as internal standard. The acid value as well as free fatty acids (FFA) were titrimetrically determined by using AOCS method (Cd 3d-63). Esters content we determined by GC-FID. Phosphatides content was evaluated using AOCS method (Ca 12-55).

Fatty acid distribution from *B. braunii* was determined by gas chromatography according to AOCS method (Ce 2-66). The lipids profile of fatty acids as FAME was prepared via saponification followed by boron trifluoride-methanol esterification (AOCS, 2012).

2.3.2 Microalgae biomass characterization

Moisture content (MC) was determined at 105° C for 2 h using an oven (ASTM, D3173); volatiles content (VC) was determined at 925°C for 7 min using a furnace (ASTM, D 3175) and ash content (AC) was measured according to ASTM D3176 (ASTM, D3176). In addition, fixed carbon (FC) was calculated using equation (2):

$$FC\ (\%) = \ 100\ -\ VC\ (\%) - \ AC\,(\%) \qquad\qquad \text{eq. (2)}$$

The high heating value (HHV) or heat of combustion was determined using an isothermal bomb calorimeter (Parr-Instruments-Co, model 1108) by combusting the sample (microalgae biomass pellet) with excess oxygen at a pressure of 20 atmospheres in a sealed vessel (ASTM, D5865).

2.3.3 FAME production yield

FAME production yield was calculated using equation (3):

$$FAME_{yield} \ (\%wt): \frac{FAME(\%wt)xFAME_{\,g}\,(\%wt)}{TFA\,(\%wt)} \qquad\qquad \text{eq. (3)}$$

Where, FAME (%wt) represents to quantification by GC-FID of fatty acid methyl esters and $FAME_g$ (% wt) corresponds to the gravimetric quantification of lipids converted to FAME. TFA (total fatty acid) correspond to the gravimetric quantification of transesterifiable lipids, evaluated according to the previously described methodology.

2.3.4 Chromatographic methods

FAME quantification

A Clarus 600 chromatograph coupled to a flame ionization detector (FID) from Perkin Elmer was used for FAME identification and quantification. A capillary column, Elite-5ms (30 m x 250μm x 0.1 μm) was used. The vials were prepared by adding 100 μL sample to 1000 μL methyl heptadecanoate (1000 mg/mL) as internal standard.

The following temperature program was used: 150°C for 3.5 min and then increasing temperature at a rate of 1.1°C/min up to 240°C. Both the injector and detector temperature were 250°C and He was used as the carrier gas.

Acyl-glycerol quantification of the lipids

A Hewlett Packard 6890 series GC system with FID and a polar capillary column (J&W 123-5711, 15 m x 320 um x 0.10 um) was used for acyl-glycerol analysis. Helium was used as carrier gas (1 ml min^{-1}). A temperature gradient of 15 °C min^{-1} from 50 to 180 °C, then 7

°C min^{-1} from 180 to 230°C and finally an increase at a rate of 10 °C min^{-1} to 370°C were applied.

3. Results and discussion

3.1. Microalgae biomass and lipids properties

The total lipids (TL) content of the microalgae evaluated by soxhlet extraction with methanol/chloroform mixture was 29% (wt of dry microalgae). This result is according to literature where the lipidic fraction of *B. braunii* is in the range between 25 and 70% (Hidalgo et al., 2013b; Mata et al., 2010).

From the extracted total lipids two fractions were obtained, SLs and USLs. USLs reached 35%wt (on the basis of total lipids), being this fraction mainly composed of phytols, sterols, hydrocarbons, ketones and pigments (Halim et al., 2012). A SLs content of 65% (on the basis of total lipids), corresponding to 19.1% wt of dry microalgae was obtained. This fraction is mainly composed by neutral lipids (acyl-glycerides, free fatty acids (FFA) and esters) and polar lipids (phospholipids and glycolipids).

Table 2 presents the characterization of biomass and lipids from *B. braunii* microalgae used in the present investigation. The different particle size fractions studied did not affect the characterization.

Saponifiable lipids of *B. braunii* showed both high phospholipids (17.5%) and high FFA content (37.2%). In general, microalgae contain primarily polar structural lipids such as phospholipids and glycolipids (Olofsson et al., 2012).

Moreover, a low fraction of acyl-glycerides and esters were found in the lipidic fraction (Table 2). This observation is according to available literature reports, where a low acyl-

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glycerides and esters content has been found in *B. braunni* (Metzger & Largeau, 2005; Metzger & Largeau, 1999).

The fatty acids profile of *B. braunii* lipids was mainly composed by palmitic acid (C16:0; 12.9 %wt), oleic acid (C18:1; 58.6 %wt) and stearic acid (C18:0; 5.1%wt). The polyunsaturated fatty acids content was 7.25 %wt, composed by linolenic acid (C18:3; 5.80%), eicosatetraenoic acid (C20:4, 0.51%) and eicosapentanoic acid (C20:5; 0.94%). According to these results, the lipids of this microalgae could be suitable for its use in biodiesel, since according to EN 14.214 the maximum level of linolenic acid methyl esters (C18:3) a 12%. Nevertheless the levels of polyunsaturated methyl esters (>= 4 double bonds) are slightly higher to 1% established in the specification, effecting the stability of the sample.

Table 2. Physicochemical properties of *B. braunii* microalgae biomass and lipid

Analysis	Value		
Microalgae biomass (%wt)[*]			
	Size 1	Size 2	Size 3
Fixed carbon	15.5±1.5	17.5±1.1	15.8±0.9
Volatiles	73.0±0.3	70.4±0.7	72.4±1.1
Ash	11.5±1.7	12.1±1.1	11.8±1.1
HHV(MJ kg^{-1})	19.7±1.4	18.7±1.1	19.5±1.2
Microalgae lipids (%) [**]			
Triglycerides	1.43±0.1		
Diglycerides	1.43±0.3		
Monoglycerides	0.17±0.7		
Free fatty acids	37.2±1.2		
Esters	3.5±1.1		
Phospholipids	17.51±1.2		
Other components	38.76±0.2		

[*]Dry basis (d.b.) [**]% vol/vol of saponifiable lipids.
HHV: High heating value (MJ kg^{-1})

3.2 *In situ* transesterification

3.2.1 Effect of methanol:fatty acids molar ratio and particle size fractions

The amount of alcohol in the reaction plays a major role, directly affecting FAME yield (Ehimen et al., 2010a; Wahlen et al., 2011a). As transesterification is an equilibrium reaction, the methanol: total fatty acids molar ratio used for *in situ* transesterification is normally higher than the stoichiometric value to favor products formation (Hidalgo et al., 2013b). In Figure 1, the effect of particle size and methanol: total fatty acids molar ratio on FAME yield is shown, where the increase in methanol: total fatty acids molar ratio produced an increase on FAME yield for all particles size fractions studied.

An analysis of regression was done to correlate FAME yield with methanol: total fatty acids molar ratio at different particles size ranges. A polynomial model of second order gave the best fit ($R^2 \geq 0.90$) between FAME yield and methanol: total fatty acids molar ratio for size 1 and size 2 (Figure 1b). According to this, the maximum FAME yield obtained from polynomial model for the particle size fraction <150 µm (size 1) was 58.12% (using a 280:1 methanol: total fatty acids molar ratio), while for particle size 2 (between 150 and 500 µm) was 53.10% using a 281:1 methanol: total fatty acids molar ratio. The values found by the polynomial models were close to empirical data (Figure 1 a). An increment in the methanol: total fatty acids molar ratio over this value provoked a diminishment in FAME yield. This behavior was observed only for size 1 and 2, probably caused by a dilution effect (Velasquez-Orta et al., 2012a), as according to different research works, the decrease in the particle size increases mass transfer and therefore lipids extraction performance (Porwal et al., 2012; Zakaria & Harvey, 2012).

For particle size 3, a polynomial model of third order gave the best fit ($R^2 \geq 0.90$). The maximum FAME yield obtained was 40.76% using a 177:1 methanol: total fatty acids molar ratio.
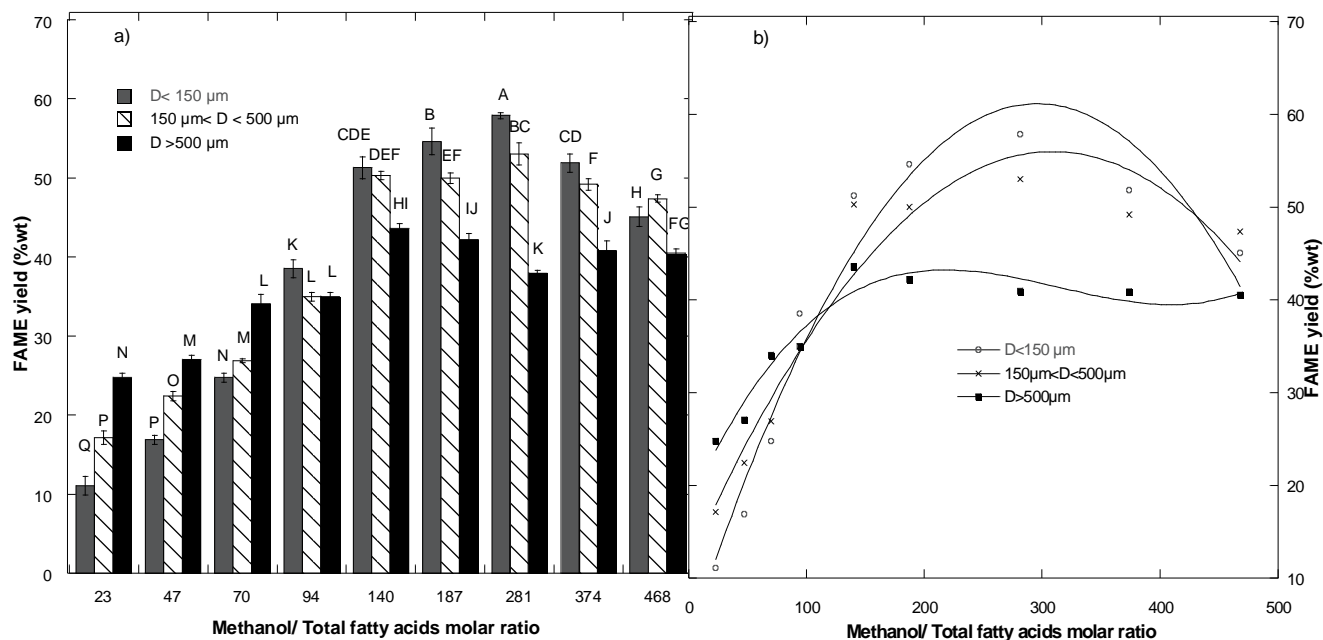
Figure 1. FAME yield at different particle sizes of microalgae powder and methanol: total fatty acids molar ratio (75% acid catalyst, 200 rpm, 2 h and 60°C). a) FAME yield where data represents the mean values in duplicate and the error bars show the standard deviations. The different letters indicate a significant difference at $P<0.05$. b) Fitted curve of polynomial model ($R^2 \geq 0.90$) for FAME yield.

3.2.2 Optimization of experimental design

A Box-Behnken experimental design was used for the evaluation of different operational conditions on FAME yield during *in situ* transesterification of *B. braunii*. The experiments matrix is shown in Table 3 and regression coefficients are shown in Table 4.

Table 3. Experimental matrix of the Box–Behnken design

Run	Catalyst (% wt)	Temperature (°C)	Particle size (μm)	Co-solvent (% vol/vol)	FAME yield (% wt)
1	112.5	60	150<D<500	50	48.22
2	112.5	70	150<D<500	75	38.69
3	75	60	D<150	50	56.23
4	112.5	50	150<D<500	25	51.65
5	150	60	150<D<500	75	61.86
6	112.5	70	150<D<500	25	75.45
7	150	60	150<D<500	25	56.13
8	112.5	70	D<150	50	68.47
9	112.5	50	150<D<500	75	39.05
10	112.5	70	D> 500	50	43.88
11	150	50	150<D<500	50	48.86
12	150	60	D<150	50	73.67
13	112.5	50	D> 500	50	43.58
14	112.5	50	D<150	50	61.13
15	150	60	D> 500	50	45.26
16	75	60	D> 500	50	35.01
17	75	70	150<D<500	50	47.70
18	150	70	150<D<500	50	58.69
19	112.5	60	150<D<500	50	60.19
20	112.5	60	D<150	75	49.00
21	112.5	60	150<D<500	50	55.03
22	75	60	150<D<500	75	32.63
23	112.5	60	D> 500	25	61.87
24	75	60	150<D<500	25	57.69
25	112.5	60	150<D<500	50	58.69
26	112.5	60	150<D<500	50	51.87
27	112.5	60	D> 500	75	43.12
28	112.5	60	D<150	25	73.78
29	75	50	150<D<500	50	41.09

Table 4. ANOVA of the polynomial model

Source	Coefficient estimate[a]	Sum of squares	df	Mean square	F-Value	P-value
Model		3270.68	14	233.62	7.90	0.0002**
Intercept	54.80					
A-Catalyst	6.18	457.88	1	457.88	15.49	0.0015**
B-Temperature	3.96	188.16	1	188.16	6.36	0.0244**
C-Particle size	-9.13	1000.26	1	1000.26	33.83	< 0.0001*
D-Co-solvent	-9.35	1049.24	1	1049.24	35.49	< 0.0001*
AB	0.80	2.59	1	2.59	0.09	0.7718
AC	-1.80	12.99	1	12.99	0.44	0.5183
AD	7.70	237.15	1	237.15	8.02	0.0133**
BC	-1.76	12.40	1	12.40	0.42	0.5278
BD	-6.04	145.87	1	145.87	4.93	0.0433**
CD	1.51	9.09	1	9.09	0.31	0.5881
A^2	-3.23	67.84	1	67.84	2.29	0.1521
B^2	-2.81	51.09	1	51.09	1.73	0.2098
C^2	1.79	20.76	1	20.76	0.70	0.4161
D^2	0.03	0.01	1	0.01	0.00	0.9897
Residual		413.92	14	29.57		
Lack of Fit		317.80	10	31.78	1.32	0.4234

[a]Coefficients refer to the given model. *Significant at level $p<0.001$. ** Significant at level $p< 0.05$

Using the coefficients determined using JMP software, a polynomial model was obtained. The model had a determination coefficient (R^2) of 0.89 and the lack of fit was not significant. Thereby the model was suitable to describe the effect of the independent variables on FAME yield. The polynomial model in terms of non-coded factors (p-value < 0.05) is shown in equation (4)

$$FAMEyield\ (\%\ wt) = 54.80 + 6.18A + 3.96B - 9.13C - 9.35D + 7.70AD - 6.04BD \quad \text{eq. (4)}$$

As is shown in Table 4, all regression coefficients of the linear terms had a significant effect on FAME yield (p-value <0.05). Besides, the interactions between the terms catalyst–co-solvent and temperature-co-solvent had statistical significance on FAME yield.

A positive coefficient value of the linear terms catalyst (A) and temperature (B) indicate a favorable effect on FAME yield. The increase of acid catalyst concentration improved FAME yield in the reaction. According to different authors, in *in situ* transesterification process, the catalyst has an important role in cell wall disruption (Carrapiso & García, 2000; Ozgul-Yucel & Turkay, 2002). In fact, H_2SO_4 can catalyze side reactions such as cell wall carbohydrate hydrolysis , producing low FAME yields (Velasquez-Orta et al., 2013b). Thus, high catalyst concentrations are needed to improve FAME yield.

Temperature affects both lipids diffusion and reaction rate. An increase in the reaction temperature improves the miscibility of reacting compounds, consequently increasing FAME yield (Zakaria & Harvey, 2012).

On the other hand, a negative coefficient value of the linear terms particle size (C) and co-solvent (D) indicate an unfavorable effect on FAME yield. The decreasing of particle size is an important factor to obtain higher FAME yields (Kildiran et al., 1996a; Yucel & Terzioglu, 2013). An extensive grinding to reduce the particle size increases the diffusion coefficient and lipid extraction performance (Kaul et al., 2010). Besides, during *in situ* transesterification a simultaneous lipids extraction and conversion to FAME occurs, and thus the lipid extraction performance can be improved by the reduction of particle size(Kaul et al., 2010; Kildiran et al., 1996a). In addition, mechanical grinding could cause cell wall disruption, enhancing solvent permeability into the cell.

Even though a pure acyl-acceptor such as methanol has a strong affinity with membrane-associated lipid complexes due to its ability to form hydrogen bonds, its polar nature is also a disadvantage as it limits the interaction with free-standing neutral lipid globules (Halim et al., 2012).The incorporation of a non-polar co-solvent has been used to ensure a complete lipids extraction from microalgae cells, forming a mixture with the polar acyl-acceptor.

Therefore, free lipids (freestanding globules) and lipids associated to the cell membrane as polar lipids (such as phospholipids and glycolipids) can be extracted(Halim et al., 2012; Hidalgo et al., 2014b).

Although, the use of a non-polar co-solvent has a positive effect in FAME yield (Table 5), high levels of non-polar co-solvent produced a decrease in FAME formation. This could be provoked by a decrease in the selectivity and affinity to polar lipids(Lee et al., 2010; Shahidi, 2005). A polar solvent has a higher affinity to polar lipids(Grima et al., 2013; Grima et al., 1994; Pieber et al., 2012b). A decrease in the polarity index (or relative polarity of the solvent mixture) was observed when a non-polar co-solvent was added. The polarity index diminished from 5.1 (pure methanol) to 1.35 (with 75% vol/vol petroleum ether in the reaction mixture). Besides, adding the non-polar co-solvent improved the SFA (saturated FAME) and MUFA (monounsaturated FAME) content, thus diminishing the unsaturation index of FAME, leading to a less vulnerable product to lipids peroxidation.

Table 5.Fatty acids composition of FAME with size 1 (expressed as a fraction of total fatty acids in FAME %)

Fatty acid	FAME composition			
	Methanol	25% of co-solvent	50% of co-solvent	75% of co-solvent
C16:0	10.6±0.1	12.60±0.1	13.70±1.4	15.10±0.4
C16:1	4.15±0.1	4.01±0.1	4.30±0.3	4.40±0.4
C18:0	4.05±1.2	5.20±0.1	5.30±0.3	6.70±0.8
C18:1	62.6±2.2	60.20±0.3	59.20±1.1	57.2±2.5
C18:2	3.95±0.2	3.10±0.1	2.90±0.4	2.00±0.1
C18:3	6.15±0.4	5.40±0.1	4.80±1.0	3.80±0.9
C20:4	0.85±0.4	0.50±0.1	0.20±0.5	0.10±0.1
C20:5	1.8±0.4	1.12±0.1	0.50±0.9	0.20±0.01
Other components	5.85±2.6	7.87±0.4	9.10±2.1	10.50±0.7
Unsaturation index*	1.06±0.1	0.94±0.1	0.87±0.1	0.78±0.0
MUFA	66.75±2.3	64.21±0.2	63.5±0.8	61.60±2.1
SFA	14.65 ±1.1	17.80±0.0	19.00±1.7	21.80±0.4
FAME yield	57.90±1.2	78.41±1.2	65.03±1.1	49.21±0.8

*According to Kates and Baxter (Kates & Baxter, 1962) Optimal reaction conditions: 125% wt acid catalyst, 200 rpm, 2 h, 67°C and using a particle size <150 µm.

The catalyst–co-solvent (AD) and temperature-co-solvent (BD) interactions presented statistical significance on FAME yield. The interaction profiles are shown in Figure 2, where a significant improvement of FAME yield was observed with the increase of temperature and the incorporation of the co-solvent (Figure 2a). The interaction catalyst–co-solvent (Figure 2b) presented a significant increase on FAME yield with the rise of acid catalyst concentration and co-solvent use. FAME yield improvement was observed for a catalyst dosage up to 100% wt and a co-solvent volume not higher than 30% vol/vol. At higher catalyst levels a decline on FAME yield was observed. Sulfuric acid is a strong oxidizing agent and an excessive concentration could lead to the destruction of polyunsaturated fatty acids or side reactions of cell constituents (Christie, 1993; Hidalgo et al., 2013b).

Moreover, particle size-co-solvent (CD) and catalyst-particle size (AC) interactions (Figure 2c and 2d) show a clear effect of particle size on FAME yield. Higher FAME yields were obtained for small particle size fractions.

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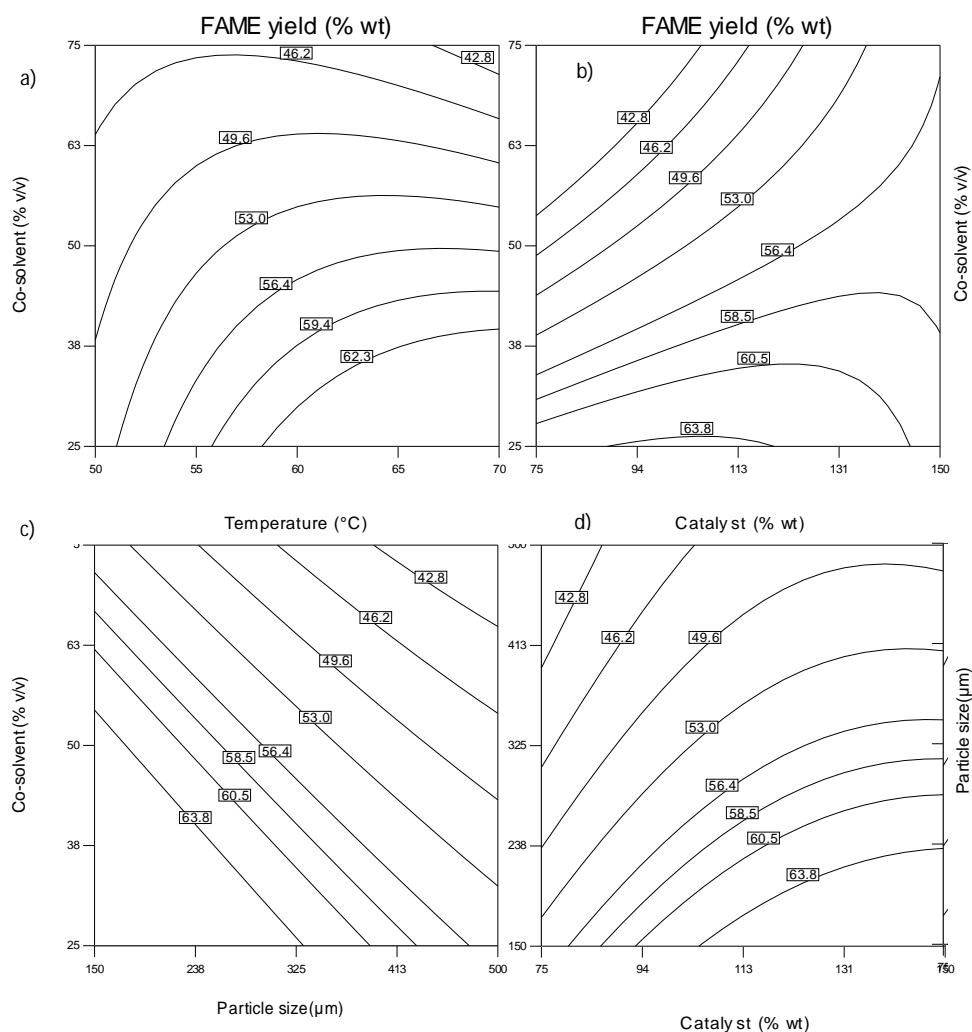
Figure 2. Contour plot of FAME yield for different variable interactions a) co-solvent-temperature b) co-solvent-catalyst c) co-solvent-particle size d) particle size-catalyst

A theoretical maximum FAME yield of 80.2% (78.3 ±1.2 %, experimental maximum) from the polynomial model was obtained. This maximum was reached using a particle size <150

µm, a catalyst dosage of 125%, reaction temperature of 67°C and co-solvent dose of 27% in the reaction mixture .

3.2.3 Effect of in situ transesterification on microalgae particle size

During *in situ* transesterification of biomass the removal of cell constituents such as proteins and carbohydrates is possible. At low catalyst concentration, low FAME yields were observed (Figure 2). This is due to the catalyzed hydrolysis of cell constituents of microalgae. Thus, increasing the catalyst concentration improves the FAME yield. Moreover, the hydrolysis of cell constituents by acid catalyst produces a decrease of microalgae conglomerates particle size (Velasquez-Orta et al., 2013b). As shown in Figure 3, the microalgae particle size diminished during the reaction. This effect was most significant when high catalyst dosage was used.

Statistical analysis of particle size distribution was performed for *B. braunii* before and after of *in situ* transesterification with high (150% wt) and low (75% wt) sulphuric acid dosage. Particle size did not show a normal distribution. Therefore, microalgae powder of different particle sizes were statistically compared using the Mann–Whitney test for non-parametric data. There were significant differences (p <0.05) for the different particle size fractions obtained among treatments. For microalgae powder with a size lower than 150 µm (size 1), the mean size of 130 µm diminished after reaction down to 105µm using a low catalyst dosage and down to 86 µm when using the highest catalyst level. Moreover, for the fraction with particle size between 150 and 500 µm (size 2) and mean size of 358 µm, after reaction the mean sizes of 286 µm and 125 µm were observed for disrupted particles with low and high catalyst dosage, respectively. According to these results, a large reduction of

the particles size after of *in situ* transesterification was obtained for particles of size 2. A mean size reduction of 20% (low catalyst dosage) and 65% (high catalyst dosage) was observed. Moreover, for particles with size higher than 500 µm (size 3), a mean size reduction close to size 2 was obtained. i.e., being 21% (low catalyst dosage) and 58% (high catalyst dosage). In fact, the observed differences for non-disrupted microalgae (mean size: 2357 µm of size 3) and microalgae after reaction treated with high catalyst dosage (mean size: 1011µm) were significant. A lower catalyst dosage in the reaction with microalgae of size 3 diminished the mean diameter down to 1849 µm.
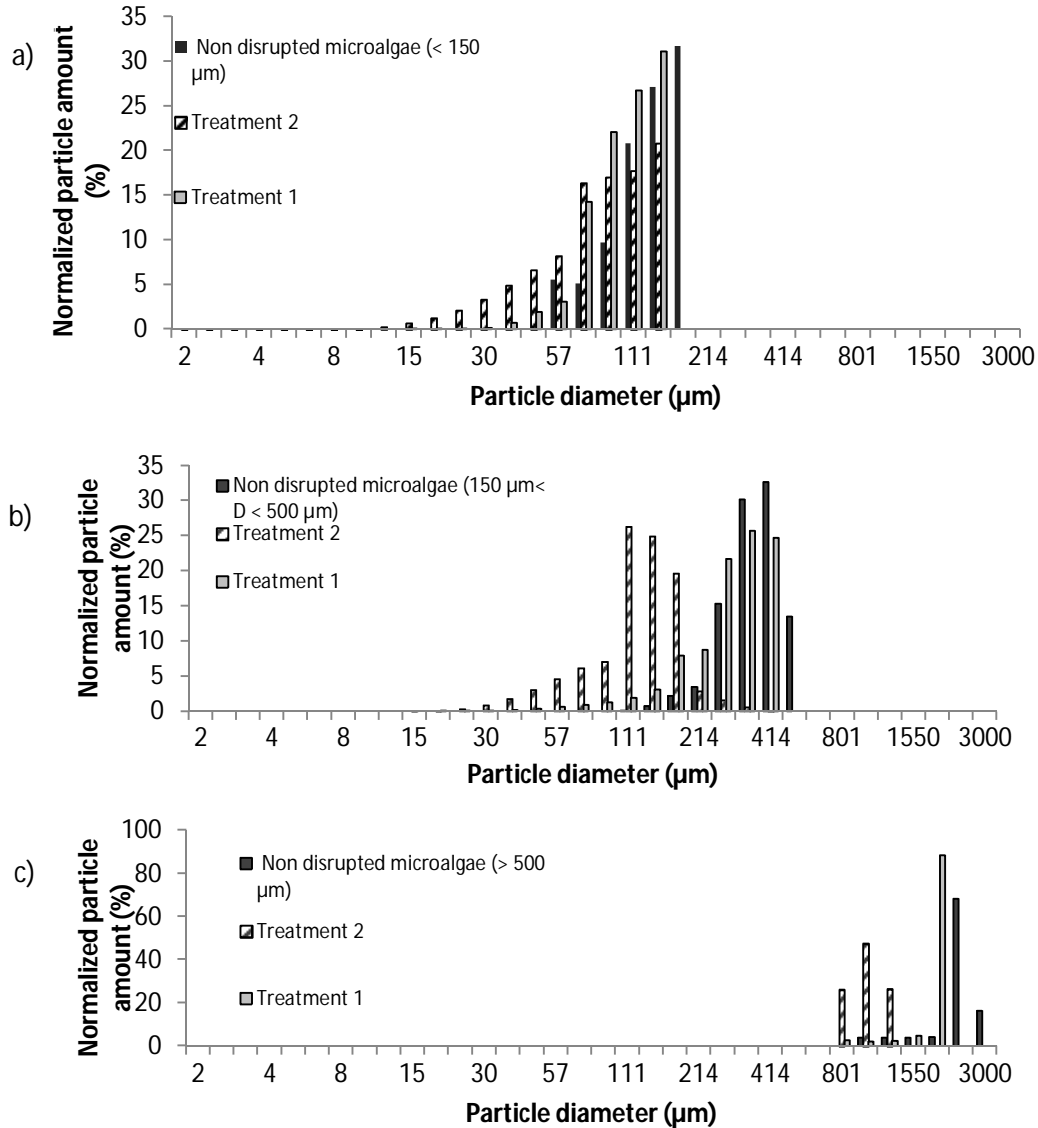
Figure 3. Particle size distribution of *B. braunii* before and after of *in-situ* transesterification (a) Size 1<150 µm (b) Size 2: 150 µm < D< 500 µm (c) Size 3 >500 µm. Treatment 1: Microalgae after *in-situ* transesterification using 75% acid catalyst; Treatment 2: Microalgae after *in-situ* transesterification using 150% acid catalyst.

Besides, due to acyl-acceptor and catalyst treatment, both the surface area and the pore volume increased in microalgae particles (Figure 4a and 4b).The more significant effect of the rise of surface area and pore volume was observed for the use of the highest catalyst dosage.

The reduction of fatty acid content in the microalgal biomass after *in situ* reaction was observed in all particle sizes of the microalga (Figure 4c). The remaining fatty acids content in microalgae after reaction was lower for the particle size fraction <150 um. This is coincident with the highest FAME yield obtained for this fraction.

Respect to total carbon content in the biomass, it also diminished after reaction (Figure 4d). The reaction reduced both the carbon content derived from fatty acids as well as other cell constituents such as polysaccharides, proteins and pigments (Rao et al., 2006). This carbon content decline in the biomass after *in situ* transesterification is due to both the hydrolysis of cell constituents catalyzed by H_2SO_4 and the extraction of polar pigments, such as chlorophylls and carotenoids by the acyl acceptor (Henriques et al., 2007; Velasquez-Orta et al., 2013b).
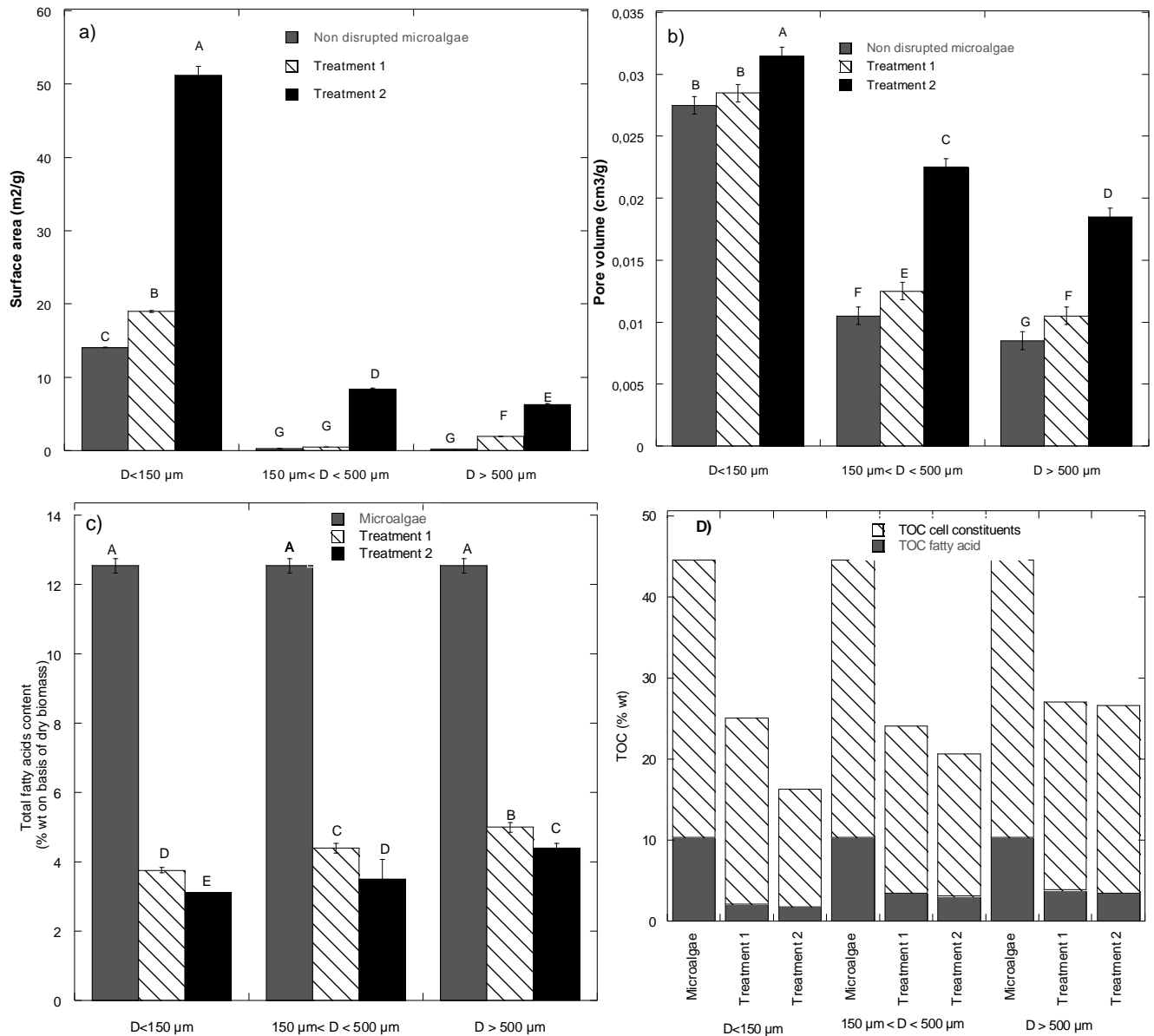
Figure 4. a) Surface area b) Pore volume c) Total fatty acids content and d) Total organic carbon content for the different particle sizes of microalgae before and after of *in-situ* transesterification. Treatment 1: Microalgae after *in-situ* transesterification using 75% acid catalyst; Treatment 2: Microalgae after *in-situ* transesterification using 150% acid catalyst. Data represents the mean values in duplicate and the error bars show the standard deviations. The different letters indicate a significant difference at p<0.05.

4. Conclusions

This study evaluated the essential process parameters of acid *in situ* transesterification of microalgae to produce FAME via an experimental design. Parameters such as temperature, catalyst dosage, particle size and co-solvent use were evaluated, where FAME yield was significantly affected by the investigated factors.

The diminishment of microalgae conglomerates particle size improved FAME yield due to an increment of the specific surface area of microalgae conglomerates. Moreover, a positive effect in FAME yield was observed by adding up to 30% vol/vol of a nonpolar co-solvent in the reaction due to an increase in the affinity to membrane lipids of microalgae. In addition, the incorporation of the co-solvent improved the stability of the product due to an increase in saturated and monounsaturated fatty acids.

A high dosage of catalyst was necessary to reduce catalyst loss during *in situ* transesterification, due to its consumption in parallel reactions such as hydrolysis of cell constituents.

The best result under optimized conditions was of 80.2% FAME yield using microalgae conglomerates particle size <150 µm, catalyst dosage of 125%, reaction temperature of 67°C and 27% vol/vol of petroleum ether as co-solvent addition to the reaction mixture . According to this result, the extraction and subsequent transesterification can be efficiently performed at a moderated temperature, which is favorable for the process economics.

Chapter 5

**Feasible FAME production from *in-situ*
transesterification of biomass microalgae with acyl
acceptor maintained to reflux**

Feasible FAME production from *in-situ* transesterification of biomass microalgae with acyl acceptor maintained to reflux

Pamela Hidalgo[1,2], Gustavo Ciudad[1,4], Sigurd Schober[3], Martin Mittelbach[3]& *Rodrigo Navia[1,4]

[1]Scientific and Technological Bioresources Nucleus, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]*Doctoral* Program in *Sciences* of *Natural Resources,* Universidad de La Frontera, Casilla 54-D, Temuco, Chile

[3]Institute of Chemistry, Working Group Chemistry and Technology of Renewable Resources, University of Graz, Heinrichstraße 28, A-8010 Graz, Austria.

[4]Departament of Chemical Engineering, Universidad de La Frontera, Casilla 54-D, Temuco, Chile

1. Introduction

Recent interest in the production of microalgae is mainly related to the use of microalgal biomass as renewable source for biofuels and bioproducts development. Microalgae have high lipid content which can be used for aquaculture, human nutrition or biofuel production. A lipid yield between 58000 L/ha and 136000 L/ha has been estimated. On the other side, oil from oilseeds such as rapeseed or soybean present oil yields of 1190 L/ha and 446 L/ha, respectively (Chen et al., 2011; Halim et al., 2010).

The lipid extraction process is one of the most limiting steps for the development of biodiesel production industry based in microalgae. In fact, lipid extraction from microalgae is mainly performed by organic solvents and not by conventional physical methods, due to difficulties in breaking the cell wall, making microalgae-based biodiesel production unfeasible at industrial scale (Ehimen et al., 2010a; Hidalgo et al., 2013b).

Simultaneous lipid extraction and esterification/transesterification is a technique of great value for biodiesel production from microalgae, as it allows extracting and converting fatty acids into fatty acid methyl esters (FAME) in a single step bypassing the use of large quantities of organic solvents used in lipid extraction (Jin et al., 2014; Wahlen et al., 2011b).

This process has been traditionally performed by direct contact between the biomass, the catalyst and the acyl-acceptor, where a high quantity of acyl acceptor is necessary to promote lipids diffusion from inside the cell (Griffiths et al., 2010). In this process,

lipids pass through a polar lipid bilayer by simple diffusion following the concentration gradient.

If the biomass is in direct contact with the acyl acceptor, lipids diffusion from cells to the acyl acceptor could be limited due to a decreasing concentration gradient in time. Thereby, high acyl acceptor volume is necessary to increase lipids extraction. However, a high acyl acceptor volume will decrease the acid strength of the catalyst and thus decrease the reaction yield (Hidalgo et al., 2014a). Additionally, the acid catalyst performs a dual role, as catalyst of the reaction and as a cell wall disruptor agent (Ozgul-Yucel & Turkay, 2002). Therefore, a reduction of acid strength of the catalyst due to the increase of acyl acceptor volume could have a negative impact on the total reaction yield.

The traditional configuration used in biomass direct transesterification corresponds to a closed reaction vessel containing the reaction mixture where the acyl acceptor is in direct contact with the biomass (Haas & Wagner, 2011). In this configuration both temperature and agitation are maintained during a specified period of time (Ehimen et al., 2010a; El-Shimi et al., 2013; Hidalgo et al., 2013b). This system is of easy implementation, but lipids extraction will be limited due to a decreasing concentration gradient of lipids (outside and inside the cell), requiring a subsequent step for separating the biomass from the reaction product (Hidalgo et al., 2014a). Normally, high acyl acceptor volume for increasing lipids diffusion and FAME yield has been used in biomass transesterification (Ehimen et al., 2010a). Acyl acceptor excess plays also a role as extraction solvent, improving the contact between catalyst and biomass, altering

the permeability of the solid substrate (Haas et al., 2007). Besides, acyl acceptor excess is responsible for breaking linkages between glycerin and fatty acids during the reaction (Hidalgo et al., 2013b). Siler-Marinkovic and Tomasevic (1998) used a wide range of methanol:lipid molar ratios, ranging between 100:1 and 300:1, for the transesterification of macerated sunflower seeds (Siler-Marinkovic & Tomasevic, 1998). Moreover, Ehimen *et al.* (2010) used a methanol:lipid molar ratio between 105:1 and 524:1 for the direct transesterification of *Chlorella* (Ehimen et al., 2010a).

Solvent reflux processes have been traditionally carried out for lipid extraction using a continuous or discontinuous (soxhlet) extractor. In this sense, the continuous process could have application in the production of biodiesel from microalgae and until today, only few studies tackle this issue in the literature. In the implementation of this system, microalgal biomass is contacted repeatedly with fresh portions of the acyl acceptor and in so, lipids can be extracted and esterified in the presence of a high acyl acceptor volume, hence favoring product formation. Although in this system no application of shear stress is used to produce microalgae cell wall disruption, highest FAME yields were obtained using this configuration, probably enhanced by high lipids extraction yields due to diffusion (Hidalgo et al., 2014a). According to our previous study, the use of solvent reflux increased FAME yield in direct transesterification of microalgal biomass. A FAME yield close to 80% was achieved using this configuration in contrast to the traditional configuration in batch reactor (FAME yield of only 60%) where the biomass is in direct contact with solvent and catalyst. Although better results were obtained in this system, only few studies related to the use of solvent reflux for the direct transesterification of microalgal biomass have been published yet. Therefore it

appears interesting to test this technique and its operational conditions when using *B. braunii* as a lipid source for microalgae-based biodiesel production.

Hence, the aim of this work was to carry out direct transesterification of microalgal biomass in a reflux extraction reactor (RER), a different configuration compared to the used techniques where lipid diffusion is limited. In RER, the microalgae sample is repeatedly contacted with pure solvent, thus increasing the lipid extraction and conversion yield to FAME. To optimize the operational conditions in this system, an experimental design using surface response methodology was developed to find out the influence of the operational variables and the interaction among them on FAME yield. The variables studied were co-solvent and catalyst concentration. Hexane was used as co-solvent in the reaction.

2. Materials and methods

2.1 Lipid quantification from *Botryococcus braunii*

The microalga used in this study was supplied by Desert Bioenergy S.A., Chile. The moisture content was 7.8 % wt and size distribution was < 150 µm. The lipids of *Botryococcus braunii* were extracted in a soxhlet apparatus for its quantification and characterization using a methanol:chloroform ratio of 2:1 v/v. The extracted lipids (Total lipids, TLs) were divided in two main fractions, saponifiable lipids (SLs) and unsaponifiable lipids (USLs). SLs were defined as total fatty acids (TFA) or transesterifiable lipids.

USLs were evaluated according to AOCS method (Cc-6a-53) and SLs by difference between TLs and USLs. The determination of USL profile was performed by gas chromatography coupled to a mass spectrometry detector (GC-MS) using derivatization through silylation with *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide(MSTFA) in pyridine.

The fatty acid distribution was determined according to AOCS method (Ce 2-66) (AOCS, 2012). The acid value as well as FFA were titrimetrically determined using AOCS method (Cd 3d-63). Phosphatides content was evaluated using AOCS method (Ca 12-55). Acyl-glyceride contents (tri-di and mono-glycerides) were determined by gas chromatography coupled to a flame ionization detector (GC-FID) using tricaprin as internal standard.

2.2 Experimental set up

The experiments were carried out in a reflux extraction reactor (RER). This system has been used for lipids extraction, where the biomass is repeatedly contacted with fresh portions of solvent. The system operates at the solvent´s boiling temperature for maintaining a constant reflux (Luque de Castro & Priego-Capote, 2010). In the experiments, the system was coupled to a continuous flow extractor, where the biomass was located. In RER, the extracted lipids were contacted with the catalyst and acyl-acceptor for its conversion to FAME during 5 h. Methanol was used as acyl-acceptor and sulfuric acid (H_2SO_4) was used as acid catalyst.

After direct transesterification, methanol was removed by distillation in a rotary evaporator and after that hexane (5 mL) was added for FAME extraction while the supernatant was removed. The supernatant was washed three times with distilled water (5 mL) to remove

traces of catalyst, methanol and hydrophilic components. Afterwards, the sample was centrifuged and the supernatant (or non-polar phase) was separated and distilled for its gravimetric quantification.

The solvent free sample was stored at 4°C for FAME determination by GC-FID. Heptadecanoic acid methyl ester (C17:0) of chromatographic purity was used as internal standard for the quantification.

FAME yield was calculated using equation (1):

$$FAME_{yield} \ (\%\,wt): \frac{FAME\,(\%wt)\,x\,FAME_{\,g}\,(\%wt)}{TFA\,(\%\,wt)} \qquad\qquad \text{eq. (1)}$$

Where, FAME (%wt) represent to quantification by GC-FID of fatty acid methyl esters and $FAME_g$ (% wt) corresponds to gravimetric quantification of lipids converted to FAME. TFA correspond to the gravimetric quantification of transesterifiable lipids.

2.2.1 Evaluation of the effect methanol: total fatty acids molar ratio on FAME yield

The evaluation was performed at five levels of methanol: total fatty acids molar ratios (36:1, 76:1, 151:1, 227:1 and 303:1). All the experiments were performed using 75% acid catalyst (on the basis of total fatty acids). Additionally, the RER system was compared with a control transesterification system (CTS) which consisted of a flask with a condenser and a heating plate with magnetic stirrer to maintain a homogeneous mixture during the reaction. Thereby in CTS, the biomass is directly contacted with the acyl- acceptor and catalyst.

2.2.2 Evaluation of efficiency of FAME extraction in RER

The removal of total organic carbon (TOC), protein, lipids and pigments of the biomass was evaluated before and after the reaction. The results of RER were compared with the CTS. The experiments were carried out using 75% acid catalyst (on basis of total fatty acids) and the methanol:total fatty acids molar ratio defined in 2.2.1.

TOC was evaluated in an organic carbon analyzer (TOC- VCPH, SHIMADZU). Protein content was determined using Kjeldahl method (Dupont et al., 2011). Lipid content of biomass after reaction was quantified by lipid extraction using methanol:chloroform according to the already described methodology. The estimation of carotenoids and chlorophylls were evaluated according to the procedure of Lichtenthaler (Lichtenthaler, 1987).

2.2.3 Optimization of direct transesterification in a reflux extraction system

An experimental design using surface response methodology was applied to find out the influence of the operational variables on FAME yield during direct transesterification of *B. braunii* biomass. The independent variables used in this study were co-solvent and catalyst concentration. Heaxane was selected as co-solvent. Although hexane is less efficient compared to other organic solvents in lipid extraction from microalgae (Nagle & Lemke, 1990a), it has minimal affinity towards polar lipids and hydrocarbons (Grima et al., 1994), and its use in mixture with methanol may extract a FAME fraction with higher purity, thus diminishing further purification steps.

The independent variables and levels of the experimental design are shown in Table 1. FAME yield was used as response variable. In these experiments, hexane was used as co-solvent in the reaction.

Table 1. Independent variables and levels of experimental design

Independent variable	Levels				
	-1.47	-1	0	1	1.47
Co-solvent (% v/v)	34	40	55	70	76
	(3.4)	(3.1)	(2.3)	(1.5)	(1.2)
Catalyst (% wt on the basis of Total fatty acids)*	59.6	75	112.5	150	165.4

* on the basis of total Total fatty acids ** Value in parentheses correspond to polarity index (PI) of mixture evaluated according to Snyder (1978) (Snyder, 1978)

2.3. Chromatographic methods

FAME quantification and distribution

An Agilent Technologies 7890A GC system with FID and a polar capillary column (J&W 122-7031, 30 m x 250 um x 0.15 um) were used for FAME identification and quantification. Helium was used as carrier gas (0.7 ml min^{-1}) and the sample was injected (1 μL) with a split ratio (ratio 100:1). The following temperature profile was used: 60°C for 2 min, then an increase of temperature up to 200°C at a rate of 10°C min^{-1}, finally a rise until 240°Cat a rate of 5 °C min^{-1}.

Acyl-glycerol quantification

An Hewlett Packard 6890 series GC system with FID and a polar capillary column (J&W 123-5711, 15 m x 320 um x 0.10 um) were used for acyl-glycerol analysis. Helium was used as carrier gas (1 ml min^{-1}). A temperature gradient of 15 °C min^{-1} from 50 to 180 °C,

then 7 °C min^{-1} from 180 to 230°C and finally an increase at a rate of 10 °C min^{-1} to 370°C were applied in this determination.

Lipid profile

An Hewlett Packard HP 6890 series GC system coupled to a Hewlett Packard 5973 mass selective detector was used in the identification and quantification of lipid profile. A capillary column (DB-5MS UI, 30 m x 250 um x 0.25 um) and helium (1 ml min^{-1}) were used. The sample was injected (1 μL) with split injection (ratio 50:1:1). The following temperature program was used: 50°C for 3 min and then increasing temperature at a rate of 10°C min^{-1} up to 300°C.

2.4 Statistical analysis

The analysis of variance and Tukey's test were performed were analyzed JMP-9 software (SAS Institute Inc., Cary, NC). A p-value below 0.05 was considered significant.

3. Results

A content of 26% of lipids (wt of dry microalgae) extracted by methanol/chloroform mixture using a soxhlet extractor was found for *B.braunii*. From the extracted lipids, a 58.8% corresponding to transesterifiable lipids defined as total fatty acids (TFA). TFA is composed by acyl-glycerols, free fatty acid, esters, phospholipids and glycolipids (Halim et al., 2012; Olofsson et al., 2012). A high content of free fatty acid (56.4% wt) and a low content of acyl-glycerol were found, according to shown in Table 2.

The fatty acid profile of *B. braunii* lipids was composed mainly by oleic acid (C18:1, 54.9%), followed by palmitic acid (C16:0, 12.2%), linolenic acid (C18:3, 5.5%), stearic

acid (C18:0, 3.9%) and linoleic acid (C18:2, 3.57%). A high saturated and monounsaturated fatty acids content are suitable for oxidative stability of the sample (Demirbas, 2009b). Moreover, a high content of C18:1 has been reported in this specie, due to role in the biosynthesis of a biopolymers derived of polymerization of long-chain fatty acids known as algaenan (Laureillard et al., 1988).

Of USLs, a high content was found (41.2% of total lipid extracted). In general a high content of USLs has been reported in the lipids from microalgae (Wang & Wang, 2012). USLs are mainly formed by sterols, phytols, fatty alcohols and hydrocarbons (Allard & Templier, 2000; Perry et al., 1978). Of lipid of *B. braunii*, the major components of USLs found were hydrocarbons (32.6%), alcohols (phytols: 25.3 %) and sterols (Campesterol and β-sitosterol: 19.6%).

Table 2. Composition of lipid of *B. braunii* microalgae.

Lipid composition	Content (%)
Saponifiable lipids *	
Triglycerides	1.7±0.3
Diglycerides	2.5±0.5
Monoglycerides	0.9±0.3
Free fatty acids	46.7±0.1
Esters	3.1±0.2
Phospholipids	16.3±0.2
Unidentified	28.8
Unsaponifiablelipids **	
Hydrocarbons	32.6±0.5
Sterols	19.6±1.9
Alcohols	25.3±1.2
Ketones	4.6±0.5
Unidentified	17.9

*% of saponifiable lipid ** % of unsaponifiable lipid

3.1 Effect methanol:total fatty acids molar ratio on FAME yield

Different experiments were performed using methanol: total fatty acids molar ratios ranging between 38 to 303. As shown in Figure 1, a significant increase of FAME yield up to 82 % was observed for RER compared to CTS and was directly proportional to the increase in the methanol: total fatty acids molar ratio until 151:1. At higher molar ratios, FAME yield remained constant. In this configuration, the main lipid extraction mechanism is diffusion, as it involves percolation of the solvent through the biomass, thereby allowing the diffusion of internal lipids out of the cell (Hidalgo et al., 2014a).

On the opposite, for CTS an increase of the methanol: total fatty acids molar ratio from 38:1 to 227:1 provoked an increment in FAME yield from 17% to 53% wt. However, an increase in the methanol: total fatty acids molar ratio above 227:1 produced a decrease in FAME yield. This reduction could be related to a dilution effect of the acid catalyst at high methanol: total fatty acids molar ratios (Velasquez-Orta et al., 2012a).
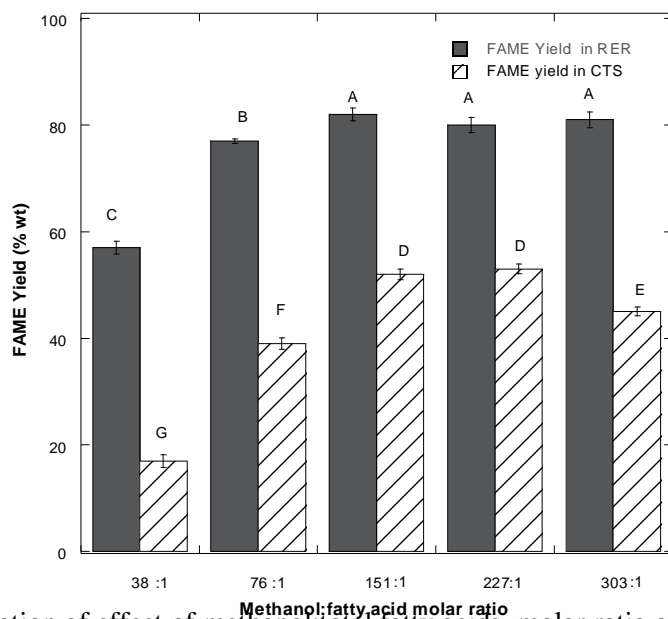
Figure 1. Evaluation of effect of methanol:total fatty acids molar ratio on FAME yield in the direct transesterification. Reaction condition: 75% acid catalyst (on basis of Total fatty acids), time reaction 5 h. Data represents the mean values of duplicates and error bars show the standard deviations. The different letters indicate a significant difference at P<0.05.

3.2 Spent microalgal biomass characteristics in RER

Higher FAME yields were observed in RER compared to CTS, suggesting a higher lipid extraction yield from biomass. After reaction, microalgal biomass diminished its total fatty acids, organic carbon, proteins and pigments content (as shown in Figure 2). A high total fatty acids extraction yield was observed in RER, as shown in Figure 2a. This result is coincident with the highest FAME yield obtained in RER. The lesser removal of total organic carbon in RER (Figure 2b) after direct transesterification can be related to a lower extraction yield of microalgae constituents such as protein, pigments and carbohydrates. In fact, methanol and sulfuric acid have a dual role during direct transesterification. Methanol acts as acyl acceptor as well as a polar nature cell constituents' solvent, while sulfuric acid acts as catalyst as well as hydrolyzing agent of cell constituents, process that is more relevant in CTS configuration as in RER the catalyst is not in contact with the biomass. Hydrophilic components such as pigments and proteins may form hydrogen bonds with methanol used as acyl-acceptor in the reaction (Halim et al., 2012; Henriques et al., 2007). Thereby, methanol plays a key role in the extraction of pigments, such as chlorophylls and carotenoids, as well as in the removal of proteins and polar lipids of cell wall. Methanol has been used in protein extraction replacing conventional membrane proteins extraction techniques (which include the use of detergents, chaotropic agents and organic acids) that require subsequent sample post-treatment, such as clean-up or pH adjustment (Zhang et al., 2007). From the obtained results, a high decrease of both pigments and proteins was observed in microalgal biomass after reaction in CTS (Figure 2c and 2d). The higher protein removal may be related to the affinity of the acyl acceptor to peptides present in the cell membrane. Besides, the catalyst can also promote side reactions, such as protein

hydrolysis (producing peptides), as well as carbohydrates and other cell constituents hydrolysis, producing a higher protein extraction yield in CTS compared to RER. Acid catalyst is efficient in the hydrolysis of hydrophobic peptide bonds of microalgae cell wall (Tsugita & Scheffler, 1982). In RER however, protein removal is related to protein affinity with methanol. Thereby in RER, the obtained FAME presents lower impurities content related to hydrolyzed proteins.

Moreover, when evaluating FAME yield, RER presented a higher conversion compared to CTS (Figure 3). Although RER is a suitable configuration to obtain high FAME yields, it requires long reaction times to reach a complete transesterification. Instead, in CTS a maximum FAME yield close to 60% was reached in 2 h and for a longer reaction time FAME yield remained constant.

The reduction of acyl-glycerols and FFA content was higher and faster in RER. It appears that in RER, once the lipids are extracted they are immediately transformed into FAME at higher levels of acyl-acceptor and catalyst than CTS. Since in CTS part of the catalyst participates in parallel reactions, the lower FAME yield may be caused by a loss of acid catalyst efficiency as it reacts with cell constituents provoking their hydrolysis (Carrapiso & García, 2000; Ozgul-Yucel & Turkay, 2002).

Besides, to increase the FAME yield in CTS at a level close to RER, higher solvent volumes and catalyst dosage would be required. According to Ehimen *et al.* (2010) a high FAME yield was obtained when using a methanol:lipids molar ratio higher than 350:1 in the direct transesterification of *Chlorella sp.* (Ehimen et al., 2010a). Moreover, Velasquez-Orta *et al.* (2012) obtained a 96.8% of FAME yield using a methanol:lipid molar ratio of 600:1 (Velasquez-Orta et al., 2012a)
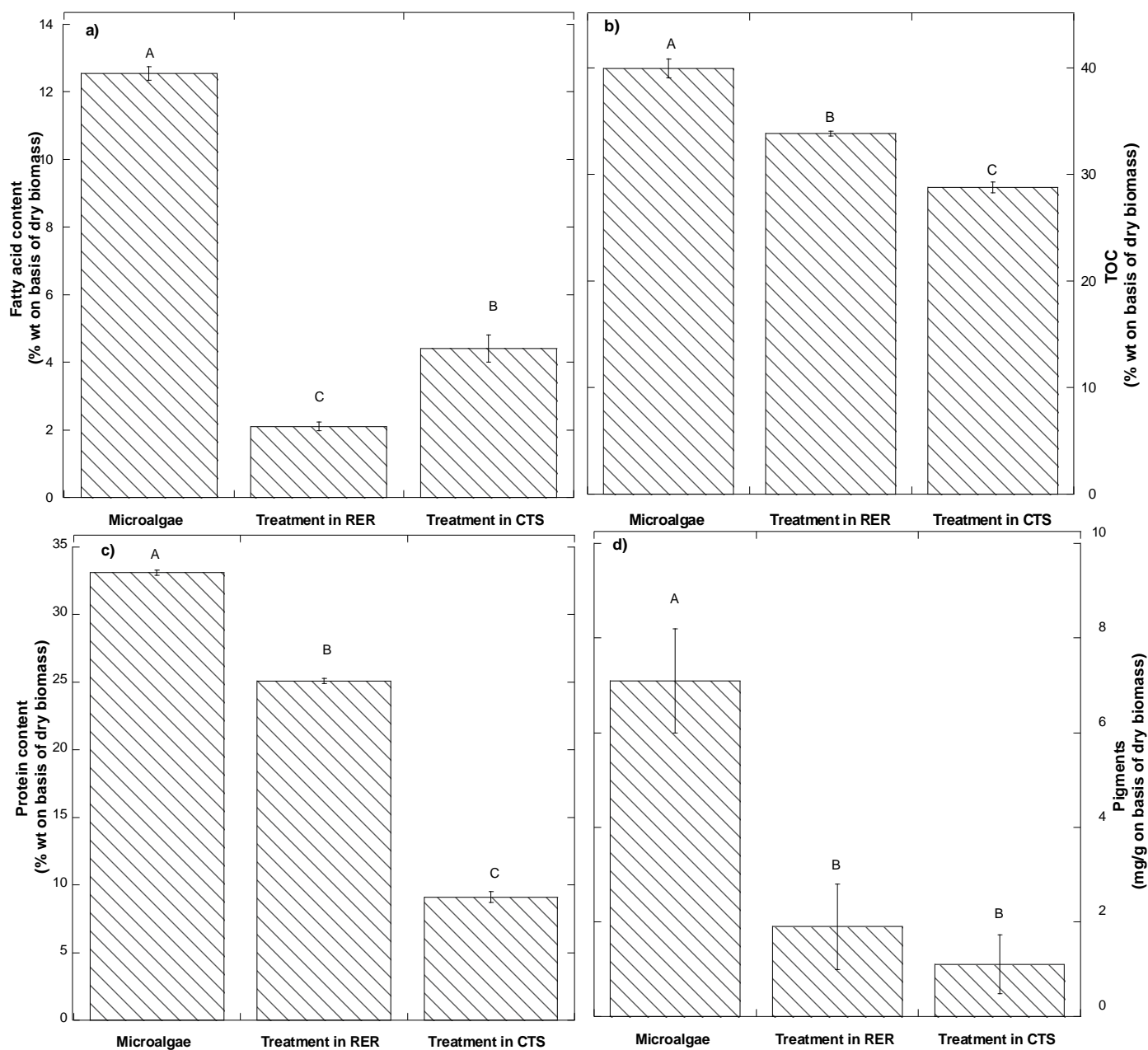
Figure 2. Removal of lipid components from microalga biomass before and after of *in-situ* transesterification in RER and CTS. A) Lipid content (as Total fatty acids) b) Carbon content c) Protein content d) Pigments (as carotenoid and Chlorophylls). Reaction condition: 75% acid catalyst (on basis of Total fatty acids), 151:1 methanol:total fatty acids molar ratio, time reaction 5 h. Data represents the mean values of two samples and the error bars show the standard deviations. The different letters indicate a significant difference at P<0.05.
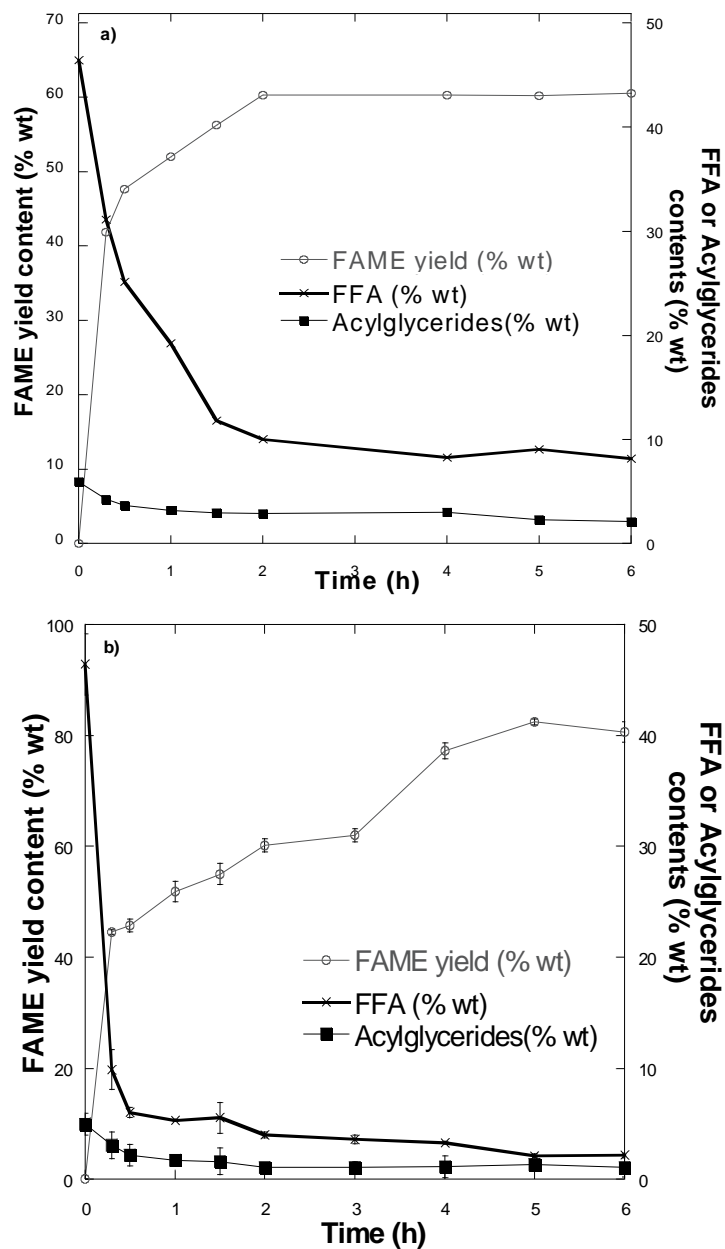
Figure 3. FAME formation and reduction of FFA and acyl-glycerides during the *in-situ* transesterification of microalgae biomass a) in CTS b) RER. Reaction condition: 75% acid catalyst (on basis of Total fatty acids), 151:1 methanol:Total fatty acids molar ratio, 6 h. Data represents the mean values of two samples and the error bars show the standard deviations.

3.3 Optimization of FAME yield in RER

An experimental design using surface response methodology was applied for the optimization of the direct transesterification using a RER. The reaction parameters evaluated were co-solvent and catalyst concentration and FAME yield as response variable. The experimental design matrix is shown in Table 3 and the regression coefficients determined using JMP software are shown in Table 4. Using the coefficients from Table 3, the polynomial model was determined. The full prediction model in terms of uncoded factors for FAME yield is:

$$FAME_{yield}(\%\,wt) = 90.8 - 12.1 \cdot A + 3.2 \cdot B + 1.9 \cdot AB - 9.9 \cdot A^2 - 2.5 \cdot B^2 \quad \text{Eq.(2)}$$

The determination coefficient (R^2) of the model was 0.963. This value indicates that the sample variation of 96.3% for FAME yield is attributed to the independent variables selected (catalyst and co-solvent) and 3.7% of the total variations are not explained by the model. Besides, lack of fit is not significant, thereby this is a suitable model to describe the effect of the independent variables on FAME yield.

Table 3. Experiment matrix for the factorial design and conversion values (experimental and predicted)[*,*] Value in parentheses correspond to codified terms

Run	Co-solvent (% v/v) A	Catalyst (% wt) B	FAME yield (% wt) experimental	FAME yield (% wt) predicted
1	55 (0)	165.4(1.41)	92.76	90.38
2	55(0)	112.5 (0)	90.42	90.82
3	55(0)	112.5(0)	93.52	90.82
4	40(-1)	150(1)	92.29	91.90
5	70(1)	75(-1)	61.03	61.37
6	40(-1)	75(-1)	93.02	89.22
7	55(0)	112.5 (0)	92.12	90.82
8	55(0)	112.5 (0)	88.52	90.82
9	70(1)	150(1)	67.70	71.44
10	34(-1.41)	112.5 (0)	85.30	88.26
11	55(0)	59.6(-1.41)	78.95	81.39
12	55(0)	112.5 (0)	89.52	90.82
13	76(1.41)	112.5 (0)	57.10	54.20

Tabla 4.ANOVA for the model

Source	Coefficient estimate[a]	Sum of squares	df	Mean square	F-Value	P-value
Model		1938.70	5	387.74	36.88	< 0.0001*
Constant	90.82					
A: Co-solvent	-12.08	1163.68	1	1163.68	110.67	< 0.0001*
B: Catalyst	3.19	80.96	1	80.96	7.70	0.0275**
AB	1.85	13.69	1	13.69	1.30	0.2914
A^2	-9.85	669.81	1	669.81	63.70	< 0.0001*
B^2	-2.48	42.53	1	42.53	4.04	0.0842
Residual		73.60	7	10.51		
Lack of Fit		57.48	3	19.16	4.75	0.0831

[a]Coefficients refer to the model given. *Significant at level $p<0.001$. ** Significant at level $p< 0.05$

The regression coefficients of the co-solvent (A) and catalyst (B) linear terms and the quadratic term A^2 have a significant effect on FAME yield (p-value < 0.05).

Negative values of the coefficients for linear and quadratic co-solvent terms indicate an unfavorable effect on FAME yield. Even though, the incorporation of a co-solvent increased the lipid extraction and FAME yield, also produced a decrease of the selectivity due to extracting sterols and hydrocarbons (Table 5).

A positive value of the catalyst linear term coefficient indicates a favorable effect on FAME yield. The increase of acid catalyst concentration improves FAME yield. The contour plot of Figure 4 shows that a significant increase on FAME yield was reached with the increase of acid catalyst concentration.

In Addition, of the results of polynomial model the incorporation of a 47% of co-solvent and adding a catalyst dosage of 104% wt produced an increase on FAME yield close to 15% (95% of theoretical maximum of FAME yield; 93 ±1.5 % of experimental maximum)
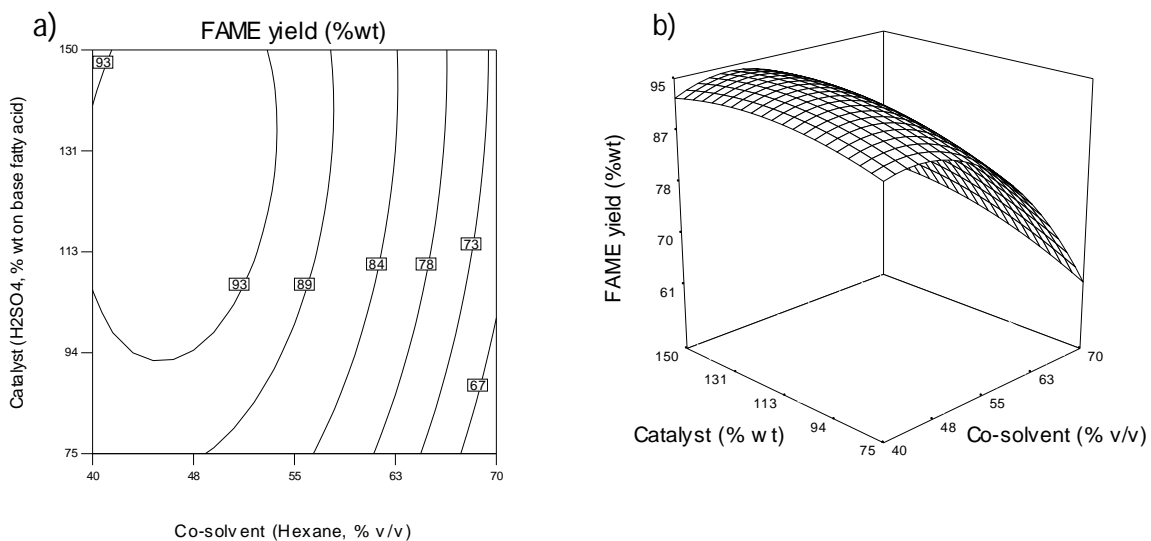
Figure 4.Response surface of FAME yield with respect to catalyst dosage and co-solvent volume.
a) Two-dimensional contour plot. b) Three-dimensional surface plot

Table 5.FAME composition and profile of main component of mixture unrefined

Contents (% wt)	Methanol	34% of co-solvent	40% of co-solvent	55% of co-solvent	70% of co-solvent	76% of co-solvent
FAME profile						
C10:0	0.1±0.1	-	-	-	-	-
C12:0	3.1±0.2	2.9±0.1	2.7±0.1	2.9±0.1	-	-
C12:1	0.2±0.1	0.5±0.1	0.4±0.2	0.5±0.1	-	-
C14:0	0.1±0.1	0.1±0.1	0.3±0.1	0.5±0.1	-	-
C14:1	1.2±0.2	1.1±0.2	0.9±0.1	0.9±0.2	0.7±0.1	-
C16:0	12.40±0.3	12.22±0.8	13.10±0.4	13.5±0.4	17.8±0.9	19.10±0.9
C16:1	4.01±0.2	3.96±0.2	3.50±0.2	3.2±0.4	3.1±0.5	3.50±0.1
C18:0	5.90±0.5	6.80±0.4	6.90±0.4	7.10±0.1	7.8±0.6	11.10±0.5
C18:1	50.10±0.6	48.10±0.3	48.70±0.8	44.50±0.7	45.1±0.8	45.10±0.9
C18:2	3.50±0.1	3.57±0.2	2.50±0.1	2.2±0.2	2.1±0.3	2.00±0.2
C18:3	5.50±0.2	5.52±0.2	4.90±0.2	4.5±0.5	4.1±0.2	5.10±0.1
C20:0	2.10±0.2	2.00±0.1	2.10±0.1	2.10±0.4	3.2±0.1	3.10±0.2
C20:1	1.20±0.1	1.10±0.4	1.10±0.1	2.30±0.3	2.2±0.1	-
C20:4	2.50±0.1	2.10±0.3	2.20±0.2	1.80±0.1	1.9±0.1	-
C20:5	1.10±0.1	1.07±0.2	0.50±0.2	0.3±0.2	-	-
C22:2	1.05±0.2	0.97±0.1	0.50±0.1	0.4±0.1	-	-
Unidentified	6.04	7.99	9.70	13.30	12.00	11.00
Unsaturation index*	1.58	0.94	0.87	0.79	0.75	0.68
FAME yield	82.2±1.2	85.30±1.1	93.2±1.2	90.42±2.1	65.3±1.0	57.10±0.9
Mixture profile						
FAME	70.2	65.1	60.2	65.2	63.2	62.5
Hydrocarbons	15.4	13.8	15.2	14.1	11.5	13.5
Sterols	-	4.3	4.4	4.5	5.1	5.8
Unidentified	14.4	16.8	20.2	16.2	20.2	18.2

*According to Kates and Baxter (Kates & Baxter, 1962). Experiment were made with 112.5% wt of catalyst dosage and 151:1 of methanol:Total fatty acids molar ratio.Data represents the mean values of two samples.

4. Conclusion

The *in-situ* transesterification *of Botryococcus braunii microalgae* with an acyl acceptor maintained to continuous reflux was evaluated. The reaction was carried up in a reflux extraction reactor (RER), where once lipids were extracted; they were contacted with catalyst and acyl acceptor. In this configuration there a physical separation of the zone of extraction and reaction that not limit the diffusion of lipid outside of the microalgae.

In RER the physic separation of lipid extraction zone, improved the FAME yield. In this system, the percolation of fresh solvent through the sample does not limit the diffusion of lipids outside of the cell. Accordingly, the use of a simultaneous system of extraction-transesterification in different spaces like in RER, improved the FAME yield.

FAME yield near 80% wt were obtained when was used RER. Opposite to control transesterification system (CTS) where the biomass is in direct contact with catalyst and acyl-acceptor, a 53% wt of FAME yield was obtained. Thereby, the use of a continuous flow extractor, that it separates the zone of lipid extraction and lipid conversion into FAME, it increases the FAME yield during *in-situ* transesterification of microalgae biomass.

Moreover in RER, the catalyst does not participate on side-reaction such as hydrolysis of cell constituents like in CTS. Because of this, there is not loss of catalytic capacity for the promoting transesterification/esterification reactions.

On the other hand, the incorporation of a co-solvent in the reaction for reach a complete extraction of lipid outside of cell had a positive effect on FAME yield. The incorporation of

co-solvent in the reaction, increased FAME yield from 80% (only methanol) to a maximum of 95% when a 47% v/v of hexane was incorporated. However, the selectivity towards non-saponifiable lipid as sterols was increased, affecting the quality of the sample.

Chapter 6

FAME production from microalgal biomass by *in-situ* transesterification process: reaction kinetics and optimization

FAME production from microalgal biomass by *in-situ*–transesterification process: reaction kinetics and optimization

Pamela Hidalgo[1], Gustavo Ciudad[1,2], Martin Mittelbach[3] & *Rodrigo Navia[1,2,4]

[1]Scientific and Technological Bioresources Nucleus, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]Departament of Chemical Engineering, Universidad de La Frontera, Casilla 54-D, Temuco, Chile

[3]Institute of Chemistry, Working Group Chemistry and Technology of Renewable Resources, University of Graz, Heinrichstraße 28, A-8010 Graz, Austria.

[4]Centre for Biotechnology and Bioengineering (CeBiB), Chile

1. Introduction

Microalgae offer several potential advantages as a non-food feedstock for biodiesel production as they can accumulate high quantities of lipids (Cheng et al., 2011). In general, microalgae contain between 2 to 40 %wt (based on dry weight) of lipids and the lipid profile depends on the type of microalgae (Balat & Balat, 2010). These lipids are composed by an unsaponifiable and a saponifiable fraction and in addition, some microalgae species are rich in neutral lipids compared to other species (Lv et al., 2010). Microalgae such as *Chlorella sp,* or *B. braunii* have a high fraction of unsaponifiable compounds mainly corresponding to hydrocarbons, phytols, sterols, ketones and pigments among other compounds (Hidalgo et al., 2014a; Velasquez-Orta et al., 2013b). Nevertheless, the high content of saturated and monounsaturated fatty acids makes them suitable for fatty acid methyl ester (FAME) production (Rasoul-Amini et al., 2011)

Although the high lipid contents of microalgae, the use of these lipids for biodiesel production has been limited due to the high energy demand needed in the extraction stage, as organic solvents have been used for lipids recovery. Here, traditional Soxhlet extraction method with hexane or Blight and Dyer extraction method have been the most used techniques for lipids extraction from microalgae at laboratory scale (Hidalgo et al., 2013b).

A method that has been developed for diminishing the high energy demand in biodiesel production is *in-situ* transesterification (Ehimen et al., 2010b; Mata et al., 2010). However, this process has been developed mainly using dry microalgal biomass, where 85% of the total energy consumption is needed for drying (Lardon et al., 2009). Therefore, if biomass drying could be totally or partially avoided, significant energy and cost savings should be expected. Furthermore, microalgal biomass drying can cause the formation of microalgae

aggregates and the formation of case-hardening on the drying surface (Velasquez-Orta et al., 2013b).

A significant amount of research work has been performed regarding transesterification kinetics for biodiesel production. The main focus of these research works has been the evaluation of biodiesel production kinetics from pure lipids. Uzun *et al*. (2012) investigated the effect of the reaction parameters for biodiesel production from waste frying oil and reported that the reaction follows a pseudo first order kinetic model (Uzun et al., 2012). Shahbazi *et al*. (2012) reported however that the reaction follows a second order kinetic model for biodiesel production when palm oil is used (Shahbazi et al., 2012). In addition, Kusdiana and Saka (2001) proposed that the reaction follows a first order kinetic model for biodiesel production using rapeseed oil (Kusdiana & Saka, 2001) and Kumar *et al*. (2011) reported that the reaction follows a second order kinetic model with respect to triglyceride concentration and a first order kinetic model with respect to methanol concentration for biodiesel production from *mahua* and jatropha oil (Kumar et al., 2011).

In the case of kinetic studies of biodiesel production from microalgae, there are only very few studies already published. In addition, limited research addresses the evaluation of the reaction kinetics using a single extraction and transesterification stage. Nautiyal *et al*. (2014) reported that the *in-situ* transesterification from *Spirulina platensis* followed a first order kinetic model, assuming that transesterification reaction is a function of FAME concentration. In this work, the effect of water formation due to free fatty acids (FFA) esterification was not discussed. However, microalgae lipids have a high FFA content (Ehimen et al., 2010b). Thus, studies where is considered the esterification of lipids could

result of interest for understand the kinetic mechanism of conversion of lipid with high FFA content to FAME during *in-situ* reaction of biomass.

Thereby, this work presents the kinetic study of FAME production from *in-situ* transesterification using partially dried microalgal biomass. The experimental parameters such as moisture content, methanol:petroleum ether molar ratio and catalyst concentration were investigated to optimize the process. For the optimization of the experimental parameters, a Box-Behnken experimental design was developed to find out the influence of the operational conditions and the interaction among them on FAME yield. Using the optimized conditions of the process, a kinetic study including esterification, transesterification and simultaneous transesterification and esterification was performed for the determination of the kinetic model involved in the conversion of lipid into FAME by i*n-situ* reaction.

2. Materials and methods

2.1. Materials

B. braunii microalgae used in this study were supplied by Desert Bioenergy S.A, Chile. The microalgae used in the evaluation of experimental parameters, was dried. For microalgae drying a thickness of 5 mm wet microalgae (with an initial moisture content of 80% wt) was spread in a glass plate. The plate was then placed into a convective dryer to reach moisture contents of 10% wt and 45% wt (based on dry biomass). After that, dry microalgal biomass was stored at 5°C. All reagents used for this study were of analytical grade.

2.2 Reaction in a single extraction–transesterification stage

The experiments were conducted using a vessel with screwed cap (20 ml) which contained the reaction mixtures. Methanol was used as acyl acceptor and sulfuric acid (H_2SO_4) was used as catalyst. The reaction was maintained at 200 rpm for 2 h at 60°C, using 1 g microalgae. The reaction was stopped with the addition of petroleum ether and distilled water (1:1 vol/vol) to the reaction mixture, where two phases were observed. The nonpolar upper phase was separated and evaporated to remove the petroleum ether by distillation for gravimetric quantification of FAME. Then, the FAME rich phase was analyzed by gas chromatography. Heptadecanoic acid methyl ester (C17:0) of chromatographic purity was used as internal standard for the quantification of FAME. FAME yield was calculated according to Hidalgo et al. (2014).

In the characterization of the methylated fraction (total fatty acid or TFA) formed by FFA, phosphatides and acyl-glycerides, FFA content was determined titrimetrically according to AOCS method (Cd 3d-63). Phosphatides content was evaluated using method Ca 12-55 (AOCS, 2012). Acyl-glyceride contents (tri-, di- and mono-glycerides) were determined by gas chromatography using tricaprine as internal standard.

Using the above procedure, the maximum FAME yield was obtained from the identification of suitable reaction parameters. Response surface methodology (RSM), which is an efficient statistical technique, was used in the optimization of FAME yield. Minitab (v. 16.1.0) was used to design and analyze experiments. Box-Behnken design of RSM was used to evaluate the effect of reaction parameters on FAME yield. Catalyst concentration (% wt of TFA), methanol: petroleum ether ratio (v/v) and moisture content (% wt) were the reaction parameters selected. The coded and un-coded levels of the independent variables are shown in Table 1. Two replications were carried out for all design points and the

experiments were performed in randomized order. Significance of regression coefficients was determined with a p-value of < 0.05.

Table 1. Independent variables and levels used for Box-Behnken design

Variables	Symbols	Levels		
		-1	0	1
Catalyst (% wt of TFA)	X_1	75	112.5	150
Moisture (% wt)	X_2	10(1.4*)	45(6.0*)	80(10.3*)
Methanol:co-solvent ratio (v/v)**	X_3	0.33:1	1:1	3:1
		(75)	(50)	(25)

    * Values in parentheses correspond to volume in solvent mixture (% v/v)
    **Total volume of solvent mixture: 7 ml. Values in parentheses correspond to the volume of co-solvent in solvent mixture (% v/v)

2.3 Determination of kinetics constants

In the kinetic study, the experimental analysis was developed from the optimum combination of reaction variables determined previously by the Box-Behnken design.

The kinetics parameters according to the proposed mechanisms has been fitted with experimental data obtained from the literature (Berrios et al., 2007; Farag et al., 2013; Nautiyal et al., 2014; Rani et al., 2013) to a non-linear regression model using XLSTAT (Version 2014.5.02) added to Microsoft Excel. The suitable selection of the model was based on the coefficient of determination (R^2) as well as visualization of fitted plots.

2.3.1 Kinetic model of esterification

For the first scenario, a kinetic model of esterification was evaluated due to high FFA and low acyl-glyceride content in microalgae (as shown in Table 2). The acid catalyzed

esterification reaction by which the conversion of FFA into FAME took place using methanol (MeOH) like acyl acceptor can be represented as:

$$FFA + MeOH \overset{K1}{\underset{K2}{\leftrightarrow}} FAME + H_2O \qquad \text{Eq. (1)}$$
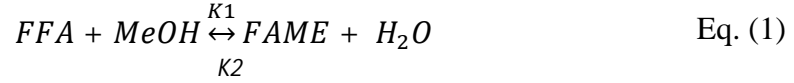
Table 2. Characterization of the methylated fraction of *B. braunii* lipids

Lipid composition	Content (%)
Triglycerides	2.1±0.4
Diglycerides	1.8±0.2
Monoglycerides	1.7±0.3
Free fatty acids	41.1±0.4
Phospholipids	18.4±0.7
Unidentified	34.9

The esterification kinetic model considered the following assumptions: the esterification reaction was a reversible homogeneous process; the rate of the non-catalyzed reaction was negligible compared to the catalyzed reaction; the esterification reaction occurred in the lipid phase; the methanol/fatty acids molar ratio used was high enough, thus the methanol concentration remained constant throughout the process (Berrios et al., 2007; Farag et al., 2013; Rani et al., 2013)

Under these conditions, the reaction was assumed to be pseudo-homogeneous, first-order in the forward direction and second-order in the reverse direction (Berrios et al., 2007). Hence the kinetic equation can be expressed as:

$$\frac{dX_{FAME}}{dt} = -\frac{dX_{FFA}}{dt} = k_1\,X_{FFA} - k_2\,X_{FAME}\,X_{Water} \qquad \text{Eq. (2)}$$

Where X_{FFA} denotes the molar fraction of FFA, X_{FAME} and X_{Water} are the molar fraction of FAME and waterformed during the reaction, respectively. K_1 and K_2 are the kinetic rate constants for the forward and reverse reactions, respectively.

It is assumed that at the reaction start (t = 0), X_{FAME} and X_{Water} are zero and $X_{FFA} = X_{FFA_O} - X_E$. X_E represents the molar fraction of X_{FFA} (molar fraction of FFA converted to FAME), and X_{FFA_O} is the initial molar fraction of FFA. Substituting in Eq (3) we obtain:

$$\frac{dX_E}{dt} = k_1\left(X_{FFA_0} - X_E\right) - k_2\,X_E{}^2 \qquad \text{Eq. (3)}$$

Integrating Eq. (3) according to Carberry (Carberry, 2001) we obtain:

$$2 \cdot k_2 \cdot \alpha \cdot t = Ln\frac{\left[X_{FFA0} + X_E \cdot \left(\beta - \frac{1}{2}\right)\right]}{\left[X_{FFA0} - X_E \cdot \left(\beta + \frac{1}{2}\right)\right]} \qquad \text{Eq. (4)}$$

Where

$$\alpha = \sqrt{\left(\frac{K^2}{4}\right) + K \cdot X_{FFA_0}} \qquad \text{Eq. (5)}$$

$$\beta = \frac{\alpha}{K} \qquad \text{Eq. (6)}$$

$$K = \frac{k_1}{k_2} \qquad \text{Eq. (7)}$$

The kinetic parameters (K_1 and K_2) of the proposed reaction mechanism, were obtained from the fit of experimental data to a non-linear regression model.

2.3.2 Transesterification kinetic model

In the second scenario, transesterification reaction takes place. The reaction scheme for transesterification is presented according to following expression:

$$TG + MeOH \underset{K2}{\overset{K1}{\longleftrightarrow}} FAME + DG \qquad \text{Eq. (8)}$$

$$DG + MeOH \underset{K4}{\overset{K3}{\longleftrightarrow}} FAME + MG \qquad \text{Eq. (9)}$$

$$MG + MeOH \underset{K6}{\overset{K5}{\longleftrightarrow}} FAME + Gl \qquad \text{Eq. (10)}$$
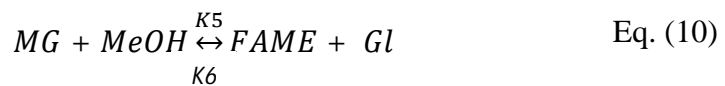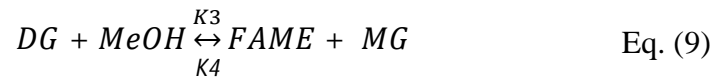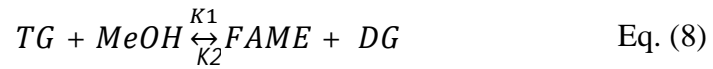
Transesterification reaction proceeds in 3 steps in which triglycerides (TG) react with methanol to produce diglycerides (DG), which further react with methanol to yield monoglycerides (MG) which finally react with methanol to produce FAME and glycerol (Jain et al., 2011). However, the overall transesterification reaction results in the formation of three moles of FAME (Shah et al., 2014).
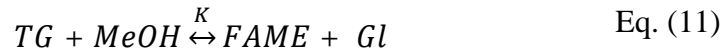
$$TG + MeOH \overset{K}{\leftrightarrow} FAME + Gl \qquad \text{Eq. (11)}$$

The overall transesterification reaction follows a first order kinetic model as a function of FAME concentration (Kusdiana & Saka, 2001; Shah et al., 2014). Thus, the reaction rate of the transesterification reaction can be expressed by Eq. (12).

$$r = \frac{dX_{FAME}}{dt} = k \cdot X_{FAME} \qquad \text{Eq. (12)}$$

Where X_{FAME} refers to the molar fraction of FAME obtained from acyl-glycerides (TG, DG and MG). Assuming that the initial molar FAME fraction at time t = 0 is X_{FAME_0} and that it increases to X_{FAME_t} at time t, the integration of equation (12) gives:

$$\int_{X_{FAME_0}}^{X_{FAME_t}} \frac{dX_{FAME}}{X_{FAME}} = \int_{o}^{t} k \cdot dt \qquad \text{Eq. (13)}$$

Solving equation (11) equation 13 is obtained:

$$\ln \frac{X_{FAME_t}}{X_{FAME_0}} = t \cdot k \qquad \text{Eq. (14)}$$

The kinetic contant *k* was determined from the fit of experimental data to a non-linear regression model by XLSTAT.

2.3.3 Simultaneous transesterification and esterification kinetic model

In the third scenario, transesterification reaction takes place simultaneously with the esterification reaction. Thus, a complete reaction mechanism (considering both reactions) was considered. The reaction scheme for the simultaneous transesterification and esterification reactions is presented in the following expressions:

$$FFA + MeOH \xrightarrow{k_1} FAME + H_2O \qquad \text{Eq. (15)}$$
$$TG + MeOH \xrightarrow{k_2} FAME + Gl \qquad \text{Eq. (16)}$$
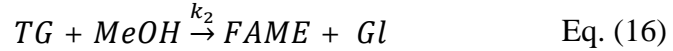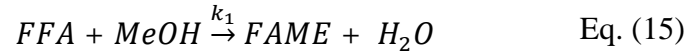
The reaction rate equation for simultaneous esterification and transesterification can be expressed as:

$$\frac{dX_{FAME}}{dt} = r_1 + r_2 = k_1(X_{FFA_O} - X_E) + k_2 X_{FAME*} \qquad \text{Eq. (17)}$$

Where:

$$r_1 = \frac{dX_{FAME*}}{dt} = k_1 X_{FFA}$$

$$r_2 = \frac{dX_{FAME*}}{dt} = k_2 \cdot X_{FAME**}$$

$$X_{FFA} = X_{FFA_O} - X_E$$

$$X_{FAME} = X_{FAME*} + X_{FAME**}$$

X_{FAME*} corresponds to FAME production from FFA and X_{FAME**} corresponds to FAME production from acyl glycerides (TG, DG and MG). X_E represents the molar fraction of X_{FFA} (or molar fraction of FFA converted to FAME). Reordering Eq. (17) Eq. 18 can be obtained:

$$\frac{dX_{FAME}}{dt} = k_1(X_{FFA_O} - X_E) + k_2(X_{FAME} - X_E) \qquad \text{Eq. (18)}$$

At the reaction start (t = 0), X_{FFA_O} and X_{FAME_0} are the initial molar fractions of FFA and

FAME respectively. X_{FAME_t} is the FAME molar fraction at time t. The integration of this

equation presented in Eq. (19) gives Eq. (20). Finally, through a fit of the experimental data

to a non-linear regression model the kinetic parameters were obtained.

$$\int_{X_{FAME_0}}^{X_{FAME_t}} \frac{dX_{FAME}}{k_1(X_{FFA_O}-X_E)+k_2(X_{FAME}-X_E)} = \int_o^t dt \qquad \text{Eq. (19)}$$

$$\ln \frac{\left[X_{FAME_t} +\left(\frac{k_1}{k_2}\cdot(X_{FFA_O}-X_E)-X_E\right)\right]}{\left[X_{FAME_0} +\left(\frac{k_1}{k_2}\cdot(X_{FFA_O}-X_E)-X_E\right)\right]} = t \cdot k_2 \qquad \text{Eq. (20)}$$

2.4 Chromatographic methods

2.4.1 FAME quantification

An Agilent Technologies 7890A GC system with FID and a polar capillary column (J&W

122-7031, 30 m x 250 um x 0.15 um) was used for FAME identification and quantification.

Helium was used as carrier gas (0.7 ml min^{-1}) and the sample was injected (1 μL) with split

injection (ratio 100:1). The following temperature program was used: 60°C for 2 min, then

there was an increase of temperature up to 200°C at a rate of 10°C min^{-1}, finally a rise up to

240°C was performed at rate of 5 °C min^{-1}.

2.4.2 Acyl-glycerol quantification

A Hewlett Packard 6890 series GC system with FID and a polar capillary column (J&W

123-5711, 15 m x 320 um x 0.10 um) was used for acyl-glycerol analysis. Helium was used

as carrier gas (1 ml min^{-1}). A temperature program of 15 °C min^{-1} from 50 to 180 °C, 7 °C

min^{-1} from 180 to 230°C and finally an increase at a rate of 10 °C min^{-1} up to 370°C was applied.

3 Results

3.1 Process optimization

A Box-Behnken design of 17 experiments for studying the interactive effects of three critical variables of the *in-situ* transesterification process such as catalyst concentration (X_1), moisture content (X_2) and methanol:petroleum ether molar ratio (X_3) on FAME yield is presented in Table 3. Different combinations of variables resulted in FAME yields varying from 60.7 to 79.7 (% wt). The predicted values calculated using the model ranged between 61.4 and 80.3 (% wt).

Table 4 shows the statistical analysis of variance (ANOVA) to study the significance, the effects of significant individual terms and their interactions on the responses as well as multiple regression coefficients. The prediction model derived from the regression coefficients in terms of coded factors is:

$$FAMEyield(\%\,wt) = 72.78 + 1.69X_1 - 2.19X_2 + 6.77X_3 + 0.078X_1X_2 + 2.02X_1X_3 \quad \text{Eq.(21)}$$
$$1.09X_2X_3 - 1.75A^2 - 0.02B^2 - 2.51C^2$$

Table 3 Experimental Box–Behnken matrix design

Run	Catalyst (% wt)	Moisture (% wt)	Methanol: petroleum ether (v/v)	FAME yield experimental (% wt)	FAME yield predicted (% wt)
1	112.5	45	1:1	74.1	72.8
2	75	80	1:1	66.2	67.1
3	150	45	3:1	79.7	79.0
4	150	45	0.33:1	60.7	61.4
5	150	80	1:1	69.7	70.6
6	150	10	1:1	75.7	74.8
7	75	10	1:1	72.4	71.6
8	112.5	45	1:1	73.1	72.8
9	112.5	80	3:1	73.9	73.7
10	112.5	45	1:1	72.3	72.8
11	75	45	3:1	72.3	71.6
12	112.5	10	0.33:1	64.4	64.6
13	75	45	0.33:1	61.4	62.1
14	112.5	45	1:1	74.2	72.8
15	112.5	45	1:1	70.2	72.8
16	112.5	10	3:1	78.7	80.3
17	112.5	80	0.33:1	64.0	62.4

Table 4 ANOVA of the polynomial model

Source	Coefficient estimate[a]	Sum of squares	df	Mean square	F-Value	P-value
Model		490.09	9	54.45	18.13	0.0005*
Intercept	72.78					
A-Catalyst	1.69	22.84	1	22.84	7.60	0.0282**
B-Moisture	-2.19	38.20	1	38.20	12.72	0.0091**
C-Methanol:petroleum ether ratio	6.77	366.18	1	366.18	121.89	< 0.0001*
AB	0.08	0.02	1	0.02	0.01	0.9312
AC	2.02	16.27	1	16.27	5.42	0.0528
BC	-1.09	4.78	1	4.78	1.59	0.2476
A^2	-1.75	12.87	1	12.87	4.28	0.0772
B^2	-0.02	0.00	1	0.00	0.00	0.9832
C^2	-2.51	26.59	1	26.59	8.85	0.0207
Residual		21.03	7	3.00		
Lack of Fit		10.11	3	3.37	1.23	0.4071
R-Squared	0.9589					
Adeq Precision	14.192					

[a]Coefficients refer to the model given. *Significant at level $p<0.001$. ** Significant at level $p< 0.05$

According to ANOVA results, the model prediction was significant with a p-value of less than 0.0001 to predict FAME yields. The R-squared value of model prediction was 0.9589. It implies that 95.89% of the total variation in FAME yield can be attributed to the studied experimental variables. Lack of fit value, which is the weighed sum of squared deviations between the mean response at each factor level and the corresponding fitted value (Zabeti et al., 2010) was not significant for the response with a p-value of 0.4071. Thereby the model prediction is fitted to all data. Moreover, the adequate precision (14.192) is much higher than 4 for the model prediction, indicating adequate model discrimination.

Linear terms of catalyst concentration, moisture content and methanol:petroleum ether ratio, and quadratic term of methanol:petroleum ether ratio were significant for the model with p-values of less than 0.05. A positive value of the linear term catalyst (X_1) and methanol:petroleum ether ratio (X_3) coefficients indicates a favorable effect on FAME yield. The increase of acid catalyst concentration improved FAME yield. On the opposite, the decrease of catalyst concentration during the reaction because side reactions such as cell wall hydrolysis, produced a low FAME yield (Velasquez-Orta et al., 2013b).

The increase of methanol:petroleum ether ratio has a positive effect on FAME yield due to a raise of methanol content in the reaction. However, a polar pure solvent like methanol limited the interaction with freestanding neutral lipid globules. The incorporation of a non-polar co-solvent in the extractive mixture has been used to ensure a complete lipid extraction from cells (Halim et al., 2012). Thereby, free lipids and lipids associated to membrane such as polar lipids (phospholipid and glycolipids) were extracted with a polar/non-polar solvent mixture (Halim et al., 2012; Hidalgo et al., 2014a).

Moreover, a negative value of the linear term moisture content (X_2) coefficient indicates an unfavorable effect on FAME yield. The increase of moisture content in the reaction favored side reactions such as hydrolysis of fatty acids (Kildiran et al., 1996a; Yucel & Terzioglu, 2013). Nevertheless, FAME conversion from microalgae samples with moisture content lower than 30% was not inhibited, suggesting that water volumes in this range are diluted by using high solvent mixture volumes. In fact, at this level of microalgae samples moisture, the water content in the solvent mixture was close to 5% v/v.

In the interactions shown in Figure 1 (a and b), it is observed that a higher volume of petroleum ether produced a decrease in FAME formation. This could be related to a decrease in both selectivity and affinity to polar lipids (Lee et al., 2010; Shahidi, 2005).

Besides, the use of monophasic solvent mixtures in lipids extraction have shown a positive effect, as higher lipids extraction yields are obtained (Bligh & Dyer, 1959a). Methanol:petroleum ether in ratios of 1:1 to 3:1 (v/v) are in a stable monophasic state when the water content in the solvent mixture is lower than 10 % v/v (Wang & Gustafson, 1994). Thereby, the raise of methanol:petroleum ether ratio produced a stable monophasic state, which could have favored lipids recovery and FAME conversion yield. Moreover, once the reaction was stopped by the addition of petroleum ether and water, a biphasic system was formed, where the upper layer contained the produced FAME.

On the opposite, when methanol:petroleum ether ratio is lower than 1:1 (v/v), two systems can be observed: a biphasic mixture formed when water content in the solvent mixture is higher than 10 % v/v; and an unstable monophasic mixture when water content is lower than 10% (Wang & Gustafson, 1994). Even though during the reaction the miscibility of the mixture is enhanced by a temperature and agitation increment, when these variables values decrease, the monophasic state disappears. Therefore, water and petroleum ether

presence produced the phase separation. However, FAME distribution in each phase can be uneven, diminishing FAME yield.

The diminishment of FAME presence in the supernatant could be related to the formation of a hydrophilic FAME micellar complex. Wang and Gustafson et al (1994) reported an uneven distribution of lipids in triphasic systems (polar- non-polar solvent - water) due to the formation of a micellar complex. The accumulation of FAME in the bottom water phase could be related to the formation of a micellar complex of FAME, thereby causing its accumulation in the water-rich phase. The formation of the micellar complex could be promoted by the presence of phospholipids in the biomass.

A theoretical maximum FAME yield of 80.2% (77.3 ±1.5 %, experimental maximum) derived from the polynomial model was obtained. This maximum was reached using a 28.5% moisture content in microalgae, a catalyst dosage of 148.7 %, and adding a methanol:petroleum ether ratio of 2.9:1 v/v (24.5% v/v of co-solvent) into the reaction mixture.

According to the obtained results, the reaction occurred even using a biomass with a high moisture content. At this moisture level, the reaction was not inhibited due to the high solvent volume in the reaction medium. Besides, according to the literature, the result of FAME yields obtained in this study were higher than the maximum conversion of 60% reached by Velasquez et al (2013) when using wet biomass with 10% moisture content (Velasquez-Orta et al., 2013b) or the 66% of FAME yield obtained by Johnson and Wen (2009) with dry biomass (Johnson & Wen, 2009).

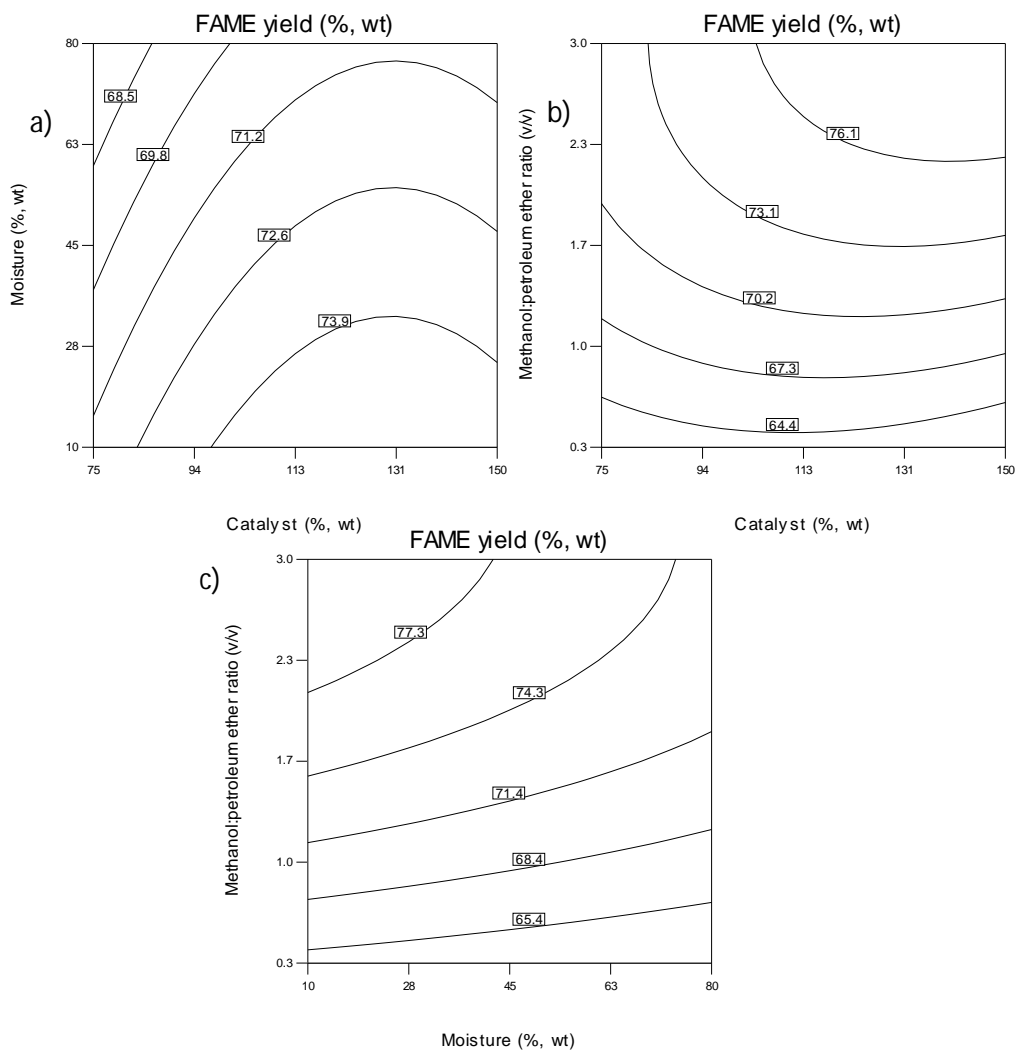Figure 1. Contour plots of FAME yields with respect to variable interactions a) Moisture content-catalyst b) Methanol:petroleum ether-catalyst c) Methanol:petroleum ether-moisture content.

3.2 Reaction kinetics

3.2.1 Esterification kinetic model

An esterification kinetic model for the transformation of FFA into FAME was evaluated, assuming a pseudo-homogeneous reaction of first-order in the forward direction (products

formation) and second-order in the reverse direction. The kinetic constants were obtained from Eq. (3) and Eq.(4) after fitting the experimental data to a non-linear regression model. In Figure 2 the fit of the experimental data to the predictive model is shown. It can be seen that the model predicts quite satisfactorily the experimental results as a high correlation between the experimental data and the predictive model is observed. The coefficient of determination (R- squared: 0.97) found implies that the accuracy of the predictive model is adequate. Thus, the esterification reaction follows a of first order kinetic model in the forward direction and second-order in the reverse direction.

The reaction rate constants found were 0.031 min^{-1} and 0.00011 min^{-1} for K_1 and K_2 respectively (see Table 5). It can be concluded that K_2 value is negligible compared to K_1 value, which indicates that hydrolysis is the main reaction taking place.

These reaction rate constants are in a similar range of already reported values in the literature. The reaction rate constants obtained for FAME production from lipids with high FFA content has been reported in the range of 0.01–0.08 min^{-1} for K_1 and lower than 0.001 for K_2 (Berrios et al., 2007; Farag et al., 2013; Nautiyal et al., 2014; Rani et al., 2013).

Farag *et al.* (2013) reported higher reaction rate constants (K_1: 0.015 to 0.025 min^{-1} and K_2: 0.00016 to 0.0001 min^{-1}) for FAME production from waste cooking oil assuming pseudo-homogeneous first-order kinetic in the forward direction and second-order in the reverse direction. On the opposite, Rani et al (2013) found values of 0.003 and 0.001 L mol^{-1} min^{-1}) for K_1 and K_2 respectively in FAME production from jatropha oil, assuming a second order kinetic model for both the forward and backward reaction.

Figure 2. FAME formation from FFA. Reaction condition: 28.5% moisture content, catalyst dosage of 148.7%, methanol:petroleum ether ratio of 2.9:1 v/v and 60°C. Filled circle: experimental data; continuous line: model. Filled square: FFA content.

3.2.2 Transesterification kinetic model

A transesterification kinetic model for the transformation of acyl-glycerides into FAME was evaluated from Eq. 12 and Eq. 14. In Figure 3 experimental data of FAME conversion and the fit using a predictive model is observed. The predictive model appropriately adjusted to the experimental data with a coefficient of determination of 0.94, implying the accuracy of the predictive model. The reaction rate constant found for transesterification of acyl-glycerides was 0.0037 min^{-1} (see Table 5). According to the results already reported in the literature, Nautiyal *et al.* (2014) reported a reaction rate constant of 0.001 min^{-1} for FAME production from *Spirulina platensis* by *in-situ* transesterification, assuming a first order kinetic model as a function of the products formation. Jain and Sharman (2011) and Jain *et al.* (2010) obtained a reaction rate constant of 0.0031 min^{-1} for the acid catalyzed

transesterification of *Jatropha curcas* oil and waste cooking oil (Jain & Sharma, 2010; Jain et al., 2011).

The transesterification reaction rate constant was lower compared to that obtained by esterification. During the start of the reaction under acid-catalyzed conditions, both FFA and acyl-glycerides initially require the activation of their carboxylic/carbonyl functions by protonation. The alkyl chain of an acyl-glyceride molecule however can interfere with the activation of its carbonyl group (Shu et al., 2011). Thus, acyl-glycerides are more difficult to activate compared to FFA. Moreover, the presence of intermediate reactions of acyl-glycerides interferes in the transformation into FAME, making acid catalyzed transesterification reaction rate slower (Freedman et al., 1986). Indeed, Figure 3 shows that transformation of acyl-glycerides into FAME was lower and incomplete compared to esterification of FFA.
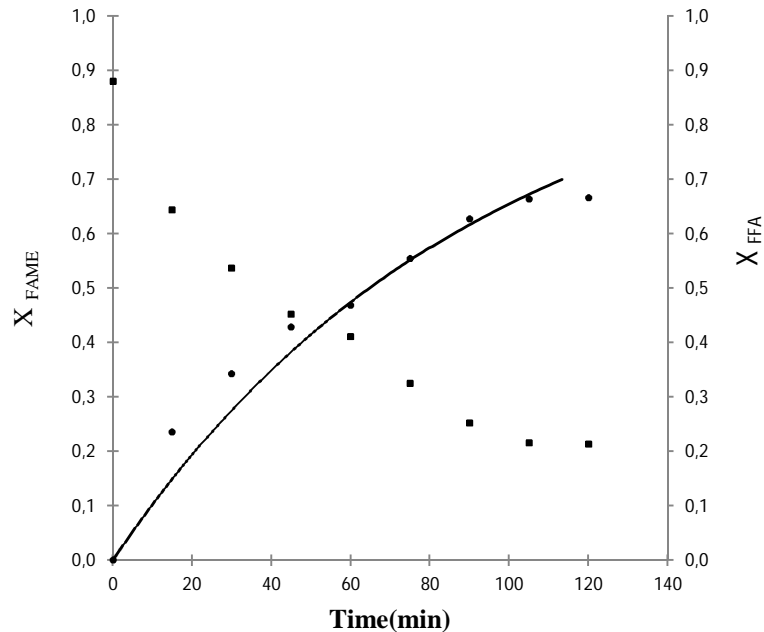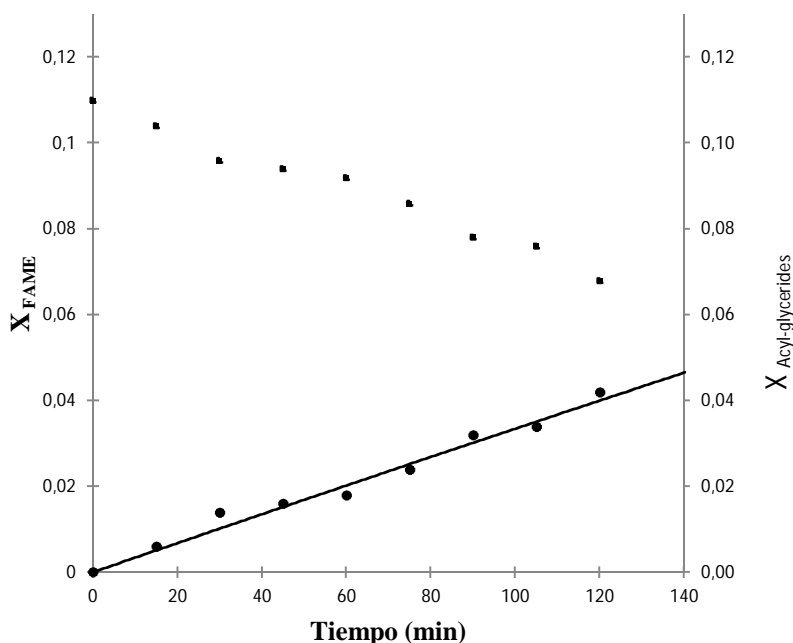

Figure 3. FAME formation from acyl-glycerides. Reaction condition: 28.5% moisture content, catalyst dosage of 148.7%, methanol:petroleum ether ratio of 2.9:1 v/v and 60°C. Filled circle: experimental data; continuous line: model. Filled square: FFA content.

3.2.2 Simultaneous esterification and transesterification kinetic model

Although the reaction rate constant of acyl-glycerides transesterification into FAME is low,, the reaction took place. Therefore this reaction must be considered in the evaluation of the kinetic mechanism involved in FAME production.

The kinetic model of simultaneous esterification and transesterification for the transformation of lipids into FAME was evaluated from Eq. (18) and Eq. (20). Figure 4(a) shows that the fit of experimental data to a predictive model is suitable presenting a coefficient of determination of 0.94.

As already mention, the transesterification reaction rate constant was lower compared to that obtained by esterification. For the simultaneous model, values of 0.034 min^{-1} and 0.003 min^{-1} for K_1 (esterification reaction) and K_2 (transesterification reaction) were obtained, respectively.

Moreover, higher FAME conversion yields (see Table 5) were reached when the simultaneous esterification and transesterification reaction mechanism was taken into account. This trend was also observed in Figure 4(b), where is showed the differences of FAME obtained from the models evaluated.
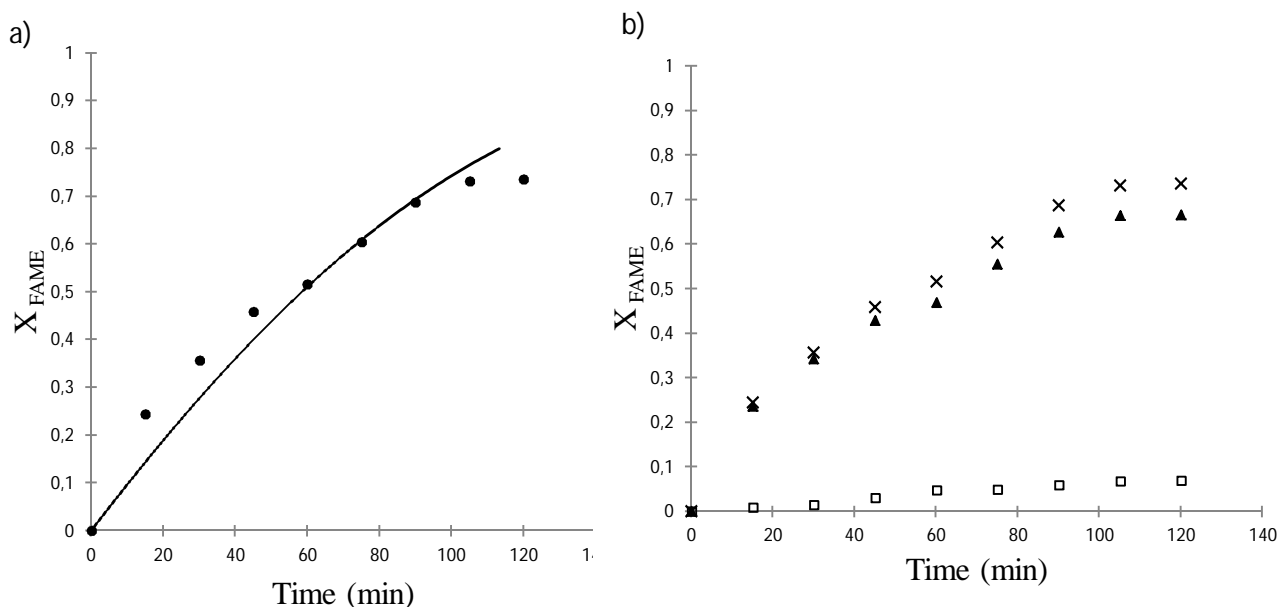
Figure 4. FAME formation from simultaneous esterification and transesterification.(a) FAME formation from TFA (methylated fraction) and model fitted (Filled circle: experimental data; continuous line: model) (b) FAME formation from FFA (filled triangle), acyl-glycerides (empty square) and TFA (cross). Reaction condition: 28.5% moisture content, catalyst dosage of 148.7%, methanol:petroleum ether ratio of 2.9:1 v/v and 60°C.

Table 5. Kinetic parameters for esterification and transesterification reaction.

	Esterification of FFA	**Transesterification of acyl-glycerides**	**Esterification and Transesterification**
Kinetic constants	K_1: 0.031 min^{-1} (forward direction)	K: 0.0037 min^{-1}	K_1 : 0.034 min^{-1} (Esterification reaction)
	K_2: 0.0011 min^{-1} (reverse direction)		K_2 : 0.0030 min^{-1} (transesterification reaction)
FAME yield (%)	64.1 ±1.1 %	7.4±1.1 %	71.5 ±1.1. %

4. Conclusion

In the study of FAME production from wet microalgae using a single *in-situ* transesterification, the experimental parameters evaluated were significant for the process. A theoretical maximum FAME yield of 80.1% was reached according to the experimental

model, using microalgal biomass with a 28.5% moisture content, a catalyst dosage of 148.7%, and using a methanol:petroleum ether ratio 2.9:1 v/v in the reaction mixture.

The obtained results allow us to conclude that the use of wet biomass is suitable for FAME production, diminishing the intensity of the required drying step of microalgae, without affecting FAME conversion due to inhibition by water in the system. The reaction medium was tolerant to a biomass moisture content of up to 30% wt, mainly because of the high solvent volume used in the reaction. Regarding catalyst concentration, a high dosage is necessary to reach high FAME yields due to catalyst consumption by side reactions such as hydrolysis of cell wall and other cell constituents. Although the incorporation of a co-solvent into reaction medium increased FAME yield, a high co-solvent volume provoked a negative effect on lipids extraction and FAME production, due to a polarity decrease in the extractive mixture.

Regarding reaction kinetics, a model of esterification, transesterification and simultaneous esterification and transesterification reactions were evaluated. The esterification model satisfactorily represented the overall reaction due to high FFA content in lipids. However, when analyzing the transesterification reaction model, it was observed that the reaction took place but with a low FAME yield. Thereby, transesterification effect cannot be dismissed in the overall FAME production rate.

As esterification and transesterification took place, the third studied reaction mechanism of simultaneous esterification and transesterification was evaluated. The results show a higher FAME yield with respect to the other kinetic models evaluated, as expected.

Chapter 7

General discussion, Concluding remarks and Future directions

7.1 General discussion

In the present study we aimed to develop a technology for the production of biodiesel by *in-situ* transesterification from the biomass of the microalga *Botryococcus brauni*. This technique is of great value for biodiesel production from microalgae, because it is able to extract and convert the lipids into alkyl esters of fatty acid in a single stage. In this process it is possible bypassing the use of large quantities of organic solvents used in the lipid extraction stage.

In order to achieve the above, we approach the subject in the following parts: Evaluation of different operational strategies for biodiesel production by direct transesterification of microalgal biomass (in Chapter 3), where two reaction systems were evaluated: conventional batch reactor (CBR) and Stirred extraction reactor (SER). The highest biodiesel production yields (80.6%) after 5 h with a solvent: fatty acids ratio of 840:1, was obtained in the reflux extraction reactor. On the opposite, 64.5% FAME yield in the conventional batch reactor but used methanol/fatty acids molar ratio of 293:1. In both systems the yield is limited by the extracted lipids which are transformed into FAME by the action of acyl acceptors present in excess. However, in CBR the solvent acts as acyl acceptor and simultaneously helps to improve the mixture homogenization. In RER, the solvent is always in excess to move the reaction equilibrium of esterification to the products side. However, in this system is very difficult to calculate the real solvent ratio, as it will depend on the design and configuration. Instead CRB, the control of solvent volume used is easy.

Even though the methanol/fatty acid molar ratio used were different in both systems,   in RER were higher than SBR. In SBR a solvent volume very high could produce the dilution of catalyst, thus affect its role in the disruption of cell wall.

 Then, in Chapter 4 we analyzed the effect of particle size reduction on FAME yield obtained by in-situ transesterification using a conventional batch reactor. From the results obtained we found that the decrease in the particle size improved the access of solvent into biomass due to increasing in surface area. Thus, this resulted in an increase on lipid extraction and its subsequent conversion to FAME. In the evaluation of the effect of methanol:fatty acids molar ratio on the different particle size fractions, a maximum FAME yield with particle size fraction <150 µm (58.12%   wt using a 280:1 methanol: total fatty acids molar ratio) was found, while for a particle size between 150 µm and 500 µm was 53.10% using a 281:1 methanol: total fatty acids molar ratio. An increment in the methanol: total fatty acids molar ratio over this value provoked a diminishment in FAME yield. On the other hand of the optimization of process, evaluating the synergistic effect of particle size, co-solvent dosage, temperature and catalyst dosage a maximum theoretical FAME yield of 80.2% (78.3 ±1.2 %, experimental maximum) was found. This maximum was reached using a particle size <150 µm, a catalyst dosage of 125%, reaction temperature of 67°C and co-solvent dose of 27% in the reaction mixture. The evaluation of time to reach maximum FAME yield, was not presented in this chapter. The maximum FAME yield was reached in 2 h low the tested conditions.

In Chapter 5 in order to optimize the *in-situ* transesterification in a RER, due to higher FAME yield obtained with this configuration in Chapter 3. In this configuration there a physical separation of the zone of extraction and reaction that not limit the diffusion of lipid

outside of the microalgae. In this configuration there a physical separation of the zone of extraction and reaction that not limit the diffusion of lipid.

In the evaluation of the methanol:fatty acids molar on FAME yield was observed that the increase of FAME yield was directly proportional to the increase of methanol:fatty acid molar ratio until 151:1. At higher molar ratios, FAME yield was maintained constant. According to this result is possible decrease the solvent volume used in RER, however require of large reaction time to reach the FAME extraction complete (5 h). Moreover, of the optimization of process, evaluating the synergistic effect of catalyst and co-solvent dosage in this system a maximum theorical of FAME yield of 95% (93 ±1.5 %, experimental maximum) was found, using a catalyst dosage of 104% and 47% of co-solvent, respectively. The incorporation of cosolvent increased, the lipid extraction of low polarity,with an increase close to 15% of the FAME yield.

In general, the studies of in-situ transesterification from microalgal biomass has been developed mainly using dry biomass, due to negative effect of water in the reaction. In Chapter 6, we show the feasibility of FAME production from wet microalgal biomass via a single extraction–transesterification stage in a conventional batch reactor. A theoretical maximum FAME yield of 80.2% (77.3 ±1.5 %, experimental maximum) using a biomass with a 28.5% moisture content, a catalyst dosage of 148.7%, and adding a methanol:petroleum ether molar ratio of 2.9:1 v/v was found (24.5% v/v% of co-solvent).

While the reaction was tolerant to a moisture content of the biomass low to 30% wt., require of high levels of methanol and catalyst dosage. From kinetic model, as esterification and transesterification took place, the mechanism of simultaneous esterification and transesterification represent the reaction.

In thesis, we hypothesized also that a continuous extraction system of microalgae biomass using a mixture of methanol/co-solvent could promote the oil extraction, esterification and transesterification reactions simultaneously to reach higher productivity compared to a conventional biodiesel production process. While, the incorporation of a co-solvent into reaction increased the nonpolar lipid extraction that have a low affinity to methanol, the selectivity to extract the polar lipid constituents of membranes was decreased. Moreover, the selectivity toward neutral non-saponifiable lipid as sterols, was increased, thereby affecting the quality of sample.

7.2 Concluding remarks

Taking into account the main results, it can be concluded that:

- The highest FAME yields were obtained in the reflux extraction reactor, where the extraction zone and reaction zone is coupled. In this systems is easy the separation of biomass of the product (FAME mixture) and acid catalyst. This could favor the recycling of the acid alcohol (methanol + acid catalyst) in subsequent process of transesterification. However, in this system is very difficult to calculate the real solvent ratio, as it will depend on the design and configuration. Besides, require of large reaction time to reach the FAME extraction complete. However, the time de reaction could be resolved by pretreatment of biomass.

- In a conventional batch reactor, where the solvent is in direct contact with the biomass, even if lower FAME yield was reached, the control of solvent volume is

simple. Besides, the maximum FAME yield was reached to lower time reaction than in reflux extraction reactor. However, is difficult the recycling of the acid alcohol in subsequent reactions due to high content of impurities (pigments, hydrolyzed protein, waste microalgae, among others).

- Finally, the incorporation of unpolar co-solvent into reaction, increased both the lipid contents (mainly of unpolar lipids) and the content of saturated and monounsaturated FAME. Added to the effect of reducing the requirements of methanol in the system.

7.3 Future directions

Results of this thesis support the hypothesis that a continuous extraction system of microalgae biomass using a mixture of methanol/co-solvent could promote the oil extraction, esterification and transesterification reactions simultaneously to reach higher productivity.

However, we believe that is absolutely necessary further research in the laboratory to test this hypothesis using other solvents that improve the extraction of saponifiable lipids during the *in-situ* reaction. Therefore, more studies are necessary to evaluate the effect of other solvents less toxic and cheaper in the reaction. Besides, it is necessary to evaluate to a temperature range higher, the conventional batch reactor, for compare them with this study.

Lastly is necessary assess the possibility of use it again the residual solvent (acid alcohol) in the reaction.

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