

Review Paper

Microalgal biomass pretreatment for bioethanol production: a review

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HIGHLIGHTS

- > An overview on different aspect of 3rd generation bioethanol produced is presented.
- > Different pretreatment methods used for microalgal biomass are reviewed and discussed.
- > Different methods for starch and carbohydrates quantification are described.

GRAPHICAL ABSTRACT

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ABSTRACT

Biofuels derived from microalgae biomass have received a great deal of attention owing to their high potentials as sustainable alternatives to fossil fuels. Microalgae have a high capacity of CO₂ fixation and depending on their growth conditions, they can accumulate different quantities of lipids, proteins, and carbohydrates. Microalgal biomass can, therefore, represent a rich source of fermentable sugars for third generation bioethanol production. The utilization of microalgal carbohydrates for bioethanol production follows three main stages: i) pretreatment, ii) saccharification, and iii) fermentation. One of the most important stages is the pretreatment, which is carried out to increase the accessibility to intracellular sugars, and thus plays an important role in improving the overall efficiency of the bioethanol production process. Diverse types of pretreatments are currently used including chemical, thermal, mechanical, biological, and their combinations, which can promote cell disruption, facilitate extraction, and result in the modification the structure of carbohydrates as well as the production of fermentable sugars. In this review, the different pretreatments used on microalgae biomass for bioethanol production are presented and discussed. Moreover, the methods used for starch and total carbohydrates quantification in microalgae biomass are also briefly presented and compared.

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1. Introduction

In recent years, the use of petroleum has greatly increased resulting in an eminent depletion of these resources. In addition, fossil fuels produce the major fraction of CO₂ that causes the greenhouse effect, resulting in the need to develop new alternative energy sources to meet the global energy demands (Borines et al., 2013). Biofuels are alternatives to reduce the dependence on fossil fuels in a near future, being bioethanol one of the most important choices. According to the Renewable Fuels Association (RFA, 2015), the global production of bioethanol stood at 25,682 million gallons in the year 2015, with the USA (14,806 billion gallons) and Brazil (7,093 billion gallons) as the largest producers in the world. Nowadays, there are two types of gasoline-ethanol fuel blends for vehicles in the USA, i) E15 defined by the Environmental Protection Agency (USA) as the mixture of gasoline with 10.5 - 15% ethanol which can be used in conventional vehicles manufactured from 2001 onwards and ii) E85 (or flexible fuel) which is the mixture of ethanol-gasoline with high ethanol concentrations ranging between 51-83%. The latter is used in flexible fuel vehicles, leading to savings in fuel costs and reduced greenhouse gas (GHG) emissions (RFA, 2015; DOE, 2016).

Bioethanol can be produced through different biorefinery-based processes (Laurens et al., 2012). Nevertheless, as the first step, it is always necessary to find renewable raw materials with suitable compositions in terms of carbohydrates. According to the feedstock considered, there are three generations of bioethanol: i) first generation, where bioethanol is produced from human food/animal feed ingredients (e.g., soybean, wheat, rice, corn, sugarcane, etc.); ii) second generation bioethanol from lignocellulosic materials/agro-industrial residues (e.g., corn cob, wheat straw, sugar cane bagasse, agave bagasse, etc.), and iii) third generation bioethanol produced from aquatic biomass (such as cyanobacteria, macroalgae, and microalgae) (SAGARPA, 2011; Carvalho et al., 2013; de Vries et al., 2014; van Ejck et al., 2014; Cuellar-Bermudez et al., 2015). Among these, aquatic biomass especially microalgae biomass (and its application within the biorefinery framework) has been highlighted in recent years owing to its higher potential for bioethanol production. The ability to use solar energy and CO₂ on one hand and the capability of certain species to accumulate considerable quantities of starch on the other hand are among the most important advantages of such biomass. The high starch accumulation potential marks microalgae a potentially desirable feedstock for the transformation of starch into glucose and consecutively to ethanol by fermentation processes (Chen et al., 2014a; Kim et al., 2014).

Since starch is produced and retained intracellularly, it is necessary to increase its availability/accessibility to microbial fermentation. Accordingly, several pretreatment methods have been reportedly applied on aquatic biomass such as microalgae to extract the intracellular starch (John et al., 2011). The most common pretreatment methods used for aquatic biomass include, i) hydrothermal extraction, using microwave as unconventional type of heating, ii) ultrasound process, iii) enzymatic hydrolysis, and iv) electric pulses. It should be noted that these pretreatments use different kinds of reagents as catalysts, such as water, acids, bases, and supercritical fluids (Miranda et al., 2012; Cheng et al., 2013; Ho et al., 2013a; Chen et al., 2014a).

This review presents the different pretreatment methods that have been used or can be used in the future for microalgae biomass. In addition, the quantification methods for starch and total sugars as well as the other stages of bioethanol production (enzymatic saccharification) are also discussed.

2. Overview of microalgae biomass

Microalgae are photosynthetic microorganisms with a cell size between 2-200 µm and a high capacity for fixing CO₂. They can be autotrophic and heterotrophic and are capable of producing large amounts of biomass containing lipids, proteins, or carbohydrates (depending on the species and growth conditions) (Alaswad et al., 2015; Baroukh et al., 2015). From the composition point of view, microalgae are composed mainly of proteins, carbohydrates, lipids, ashes, and acids among other compounds. Lignin is not found in microalgae; and thus, its biomass has the advantage of facilitated pretreatment processing or in another word, facilitated enzymatic hydrolysis of the starch contained (Chen et al., 2014a).

Microalgae are complex organisms and are classified into very diverse group. For instance, based on their structural characteristics, they can be rhizoids or cocoides, may or may not have flagella, and live in colonies or aggregates (Tomasselli, 1997). Their cell wall is mainly composed of cellulose, but it can also include pectin and sulfated polysaccharides. Intracellular starch is found in the plastids, ranging from 20 to 50%. The majority of lipids are found intracellularly with concentrations ranging from 20 to 60% (Ho et al., 2013b; Chen et al., 2014a). Other components such as proteins can also be found in concentrations between 20 and 50%. The concentration of these biochemical compounds depends on the growth conditions and culture medium in which the microorganisms are cultivated, **Table 1** presents a comparison of the composition of different microalgae species.

Microalgae biomass is considered important for producing assorted products such as biofuels, bioactive compounds vitamins (e.g., amino acids and vitamins) for human and/or animal consumption, etc. within the biorefinery concept (Ross et al., 2008; John et al., 2011; Kee and Teong, 2015). For instance, Cyanotech Corp. (USA) is the main producer of *Spirulina* and owns about 9 ha of production in Hawaii, commercializing *Spirulina* tablets with prices ranging from USD 87.47-142.82/Kg. The Japanese company Yaeyama Shokusan Co. Ltd. also distributes different products of *Chlorella* in form of tablets, powder, fine powder, and liquid. They have 10 ha of microalgae production making them important producers of microalgae biomass, with selling prices around \$100.00/Kg of microalgae powder. There are also other industrial companies in the world producing microalgae including Cyano Biofuels GmbH. (Germany), AlgaFuel, S.A. (Portugal), Oil Fox (Argentina), as well as Algae Food and Fuel (Netherlands). Currently there are over fifty companies that produce some types of aquatic biomass either for use as supplement, additives, colorants, food or bioenergy (Klöck, 2010).

Table 1.
Composition of different species of microalgae (all results are presented in % dry matter basis).

Biomass	Carbohydrates	Proteins	Lipids	References
<i>Chlorella vulgaris</i>	20.99	15.67	41.51	Wang et al. (2013)
<i>Spirulina platensis</i>	30.21	13.30	48.36	Jena et al. (2011)
<i>Chlorella sorokiniana</i>	35.67	9.90	18.81	Chen et al. (2014b)
<i>Nannochloropsis oceanica</i>	22.70	24.80	19.10	Cheng et al. (2014)
<i>Scenedesmus obliquus</i>	13.41	4.66	30.38	Chen et al. (2014c)
<i>Dunaliella tertiolecta</i>	21.69	2.87	61.32	Shuping et al. (2010)
<i>Dunaliella salina</i>	32.00	57.00	9.00	Castrillón et al. (2013)
<i>Scenedesmus dimorphus</i>	21 - 52	8 - 18	16 - 40	Castrillón et al. (2013)
<i>Chlorococum humicola</i>	32.50	-	-	Harun and Danquah (2011a)
<i>Chlamydomonas reinhardtii</i>	22.60	64.70	12.60	Mahdy et al. (2014)
<i>Spirogyra</i> sp.	33 - 64	6 - 20	11 - 21	Milano et al. (2016)
<i>Porphyridium cruentum</i>	40 - 57	28 - 39	9 - 14	Milano et al. (2016)
<i>Dunaliella salina</i>	85.58	8.46	11.47	Pirwitz et al. (2016)

3. Microalgae biomass pretreatment

In the production of bioenergy from aquatic biomass, as in any types of biorefineries, a very important step is the pretreatment of the raw materials, where the objective is to make available the intracellular compounds such as carbohydrates, proteins, lipids, and added-value compounds such as short chain oligosaccharides, antioxidants, pigments, etc. (Harun et al., 2014; Demuez et al., 2015). The two main objectives for which pretreatment of microalgae biomass is necessary are the breakdown of the cell wall and the modification of the structure of intracellular carbohydrates. The carbohydrate from microalgae can be found in the cell wall in the form of cellulose and in the plastids in the form of starch as the major sugar reserve (Fig. 1). Such information is relevant to select the biomass with the highest sugars content for bioethanol production (Chen et al., 2013). Cell wall is composed of cellulose, pectin (polygalacturonic acid), and sulfated polysaccharides, that can be impregnated with inorganic substances such as calcium carbonate, silica, and magnesium (Castrillón et al., 2013; Chen et al., 2013). It is also composed of two layers, i.e., external and internal cell walls (Fig. 2). The former is formed principally by a matrix of polysaccharides like pectin, agar, alginate, and algaenan polymers; while the latter is composed of pectin, fucans, hemicellulose, and glycoproteins in a microfibrils matrix of cellulose, and traces of fucose, xylose, rhamnose, arabinose, and galactose (Scholz et al., 2014). Depending on the composition, the cell wall can result in more or less rigid structure and therefore, it may be more difficult to break, such is in the case of *Chlorella* sp. that has a harder wall contrary to those of *Scenedesmus* sp. and cyanobacteria *Arthrospira* sp.

As for the starch, it is in the form of semi crystalline granulose particles composed of high molecular weight amylose polymers and highly branched amylopectin (Silva and Bertucco, 2016; Huang et al., 2017). The starch granules in their crystalline form contain smaller amounts of water leading to their greater stability and are therefore, more difficult to hydrolyze by enzymes. Hence, it is necessary to change their structure by gelatinization through chemical, physicochemical, or biological pretreatment methods, or combinations of these (Kuakpetoon and Wang, 2007; Cheng et al., 2013; Ho et al., 2013a; Chen et al., 2014a). Among these are acid, alkaline, or biological pretreatments, as well as alternative methods such as ultrasound, microwave, electric pulses, etc. (Miranda et al., 2012; Zhao et al., 2013). Table 2 compares the different studies performed on pretreatment of microalgae biomass for the extraction and hydrolysis of different compounds (i.e., carbohydrates, proteins, lipids, pigments, etc.). Figure 3 shows a general scheme of the third generation bioethanol production process based on the biorefinery concept.

3.1. Enzymatic pretreatment

Compared with various pretreatment methods, enzymatic hydrolysis has been shown promising (Liang et al., 2012; Demuez et al., 2015). The main advantages of the enzymatic hydrolysis are its high specificity, no severe conditions requirements, and that it is easy to carry out at industrial scale. The

Fig.1. Diagram of starch production in microalgae.

Fig.2. Microalgae cell wall composition.

main disadvantage is the high cost of enzymes used (Günerken et al., 2015). Enzymatic hydrolysis is performed using different enzymes among which cellulases, amylases, and amyloglucosidases are meant to hydrolyze the cell wall polysaccharides. The use of proteases has also been reported to hydrolyze the glycoproteins present in the cell walls of some microalgae

Table 2.
Different pretreatments of microalgae biomass for extraction of different compounds.

Biomass	Pretreatment method	Conditions	Extracted compounds	References
<i>Scenedesmus</i> sp.	Hydrothermal	Water 1:13 (w/v) 147 °C 40 min	Glucose	Yuan et al. (2016)
Mix of microalgae (<i>Scenedesmus</i> , <i>Chlorella</i> , <i>Ankistrodesmus</i> , <i>Micromonas</i> , <i>Chlamydomonas</i>)	Acid hydrolysis	H ₂ SO ₄ 1 M 80 – 90 °C 120 min	Carbohydrates	Castro et al. (2015)
<i>Scenedesmus obliquus</i>	Enzymatic	Endogalactouronase 800 U/g Esterase 3600 U/g Protease 90 U/g pH 6 50 °C 24 h	Carbohydrates	Ometto et al. (2014)
<i>Scenedesmus obliquus</i> , <i>Scenedesmus quadricauda</i> , <i>Nitzschia</i> sp. <i>Aphanothece</i> sp. <i>Desmodesmus spinosus</i> <i>Nitzschia palea</i>	Alkaline-peroxide	H ₂ O ₂ 1 – 7.5 % (w/w) 50 °C 1 h	Carbohydrates and byproducts	Juárez et al. (2016)
<i>Scenedesmus obliquus</i>	Acid hydrolysis	H ₂ SO ₄ 2 N 120 °C 30 min	Carbohydrates	Miranda et al. (2012)
<i>Chlorococcum</i> sp.	Ultrasound	65 – 130 W 40 kHz 25 min	Carbohydrates	Halim et al. (2012)
	High pressure homogenization	500 – 850 bar 15 min		
	Bead milling	Glass beads 1 mm 1:2 or 1:3 (v/v) 4 min		
	Acid hydrolysis	H ₂ SO ₄ 3 – 8 % (v/v) 120 – 160 °C 15 – 45 min		
<i>Synechocystis</i> sp.	Ultrasound	300 W 20 – 25 kHz 30 min	Proteins	Zhou et al. (2014)
	Bead milling	200 µL glass beads 10 min cycles 30 s vortexing/30 s cooling on ice		
	Freezing / thawing	3 cycles 10 min freezing - 80 °C 5 min thawing 37 °C		
<i>Synechocystis</i> sp.	Pulsed electric field	17.9 – 71.7 kWh/m ³ 36 – 54 °C	Cell disruption	Sheng et al. (2011)
<i>Nannochloropsis oculata</i> <i>Porphyridium cruentum</i>	High pressure homogenization	10 mL compression chamber 50 – 270 MPa 3 °C	Cell disruption	Montalescot et al. (2015)
	Bead milling	Glass beads (0.375, 0.625, 1.30, and 2.15 mm) Zirconia beads (0.200, 0.600, 1.25 mm) Rotation speed (8, 10, 14 m/s) 20 °C		

Table 2.
Continued.

Biomass	Pretreatment method	Conditions	Extracted compounds	References
<i>Dunaliella tertiolecta</i>	Microwave	Acetone 50 W 56 °C 5 min	Pigments	Pasquet et al. (2011)
<i>Ankistrodesmus falcatus</i>	Pulsed electric field and solvents	1 cm electrode distance 45 kV/cm Ethyl acetate / methanol / water	Lipids	Zbinden et al. (2013)
<i>Chlorella vulgaris</i> <i>Neochloris oleoabundans</i> <i>Tetraselmis suecica</i>	Bead milling	Bead size (0.3, 0.4, 0.65, 1 mm) 65 % (v/v) 25 °C	Carbohydrates and proteins	Postman et al. (2016)
<i>Chlorella vulgaris</i>	Ionic liquid and solvent	Ionic liquid 1 h ambient temperature Adding hexane mixture 30 s 15 min	Cell disruption and lipids	Orr et al. (2016)
<i>Chlorella pyrenoidosa</i>	Enzymatic	Cellulase 140 mg/m ² pH 4.6 50 °C 24 h	Carbohydrates and lipids	Fu et al. (2010)
<i>Oocystis</i> sp.	Hydrothermal	110 and 130 °C 1.2 and 1.7 bar 15 and 30 min	Cell disruption	Passos and Ferrer (2015)
<i>Nannochloropsis salina</i>	Hydrodynamic cavitation	Orifice plate with 13 holes 0.5 mm diameter Upstream / downstream pressures 4 and 0.4 bar 26.21 m/s velocity	Cell disruption and lipids	Lee and Han (2015)
<i>Arthrospira platensis</i>	Pulsed electric field	15 – 25 kV/cm 60 – 150 µs 10 – 40 °C	Pigments	Martinez et al. (2016)
<i>Chlorella vulgaris</i>	Hydrothermal	Water 140, 160, 80 °C 3, 6, and 10 bar 10 – 20 min	Compounds solubilisation	Mendez et al. (2014)

species (Günerken et al., 2015; Pirwitz et al., 2016), resulting in more efficient cell lysis and extraction of target compounds. Liang et al. (2012) used a mixture of neutral and alkaline proteases to promote cell degradation. For the conversion of the polymers present in the cell wall of microalgae, endo-β-(1,4)-D-glucanase is needed to break the cellulose matrix and the cellulosic linkages, exo-β-(1,4)-D-glucanase to hydrolyze cellulose into smaller oligosaccharides, and β-glucosidase to degrade glycosidic bonds into glucose and maltose. Zheng et al. (2016) reported the use of a mixture of cellulases along with different kinds of polymers to enhance the hydrolytic effect of the enzymes by protecting their structure leading to their longer lifetime without alterations. It is important to mention that depending on the type of microalgae, its cell wall may or may not be permeable to other compounds, whereby specific enzymes are not often required to break the cell wall since these (the enzymes) can reach up intracellular carbohydrates. Table 3 tabulates different enzymes used in the degradation of microalgae cell wall.

3.2. Hydrothermal pretreatment

Hydrothermal processing is an alternative method to break down microalgae cell walls and to gelatinize the intracellular starch. This technology is also used for pretreatment of both agro-industrial residues (wheat straw, sugarcane bagasse, softwoods, etc.) and aquatic biomass (seaweed). Temperatures ranging from 60 – 180 °C and short reaction times below 60 min are used in this method. Moreover, in this pretreatment, acids,

Fig.3. General scheme for 3rd generation pretreatments of bioethanol production.

Table 3.
Enzymes used in microalgae cell wall degradation.

Microalgae species	Enzymes	Conditions	Saccharification rate	References
<i>Chlorella vulgaris</i>	Cellulase Pectinase Xylanase β -glucosidase Amylase Chitinase Lysozyme Sulfatase	Cellulase (0.122 FPU/mg); Pectinase (240 IU/mg protein); Amylase (16 FAU/mL); β -glucosidase (10 U/mL); Xylanase (100 U/mL); Chitinase (0.2 U/mL); Lysozyme (4000 U/mL); Sulfatase (50 U/mL) 50 °C and pH 4.8 for 72 h	79 %	Kim et al. (2014)
<i>Chlorella vulgaris</i> <i>Chlamydomonas reinhardtii</i>	Glucanase Protease	Glucanase (0.3 mL/g biomass); Protease (0.2 mL/g biomass); 50 °C and pH 4.5 for 5 h	86 - 96 %	Mahdy et al. (2014)
<i>Chlorella sorokiniana</i>	Cellulases Amylases	Celluclast 1.5 L (60 μ L/3 g biomass); Novozyme 188 (30 μ L / 3 g biomass); 55 °C and pH 4.5 for 72 h	100 %	Hernández et al. (2015)
<i>Dunaliella tertiolecta</i>	Amyloglucosidase	0.4 mL enzyme/g biomass; 55 °C pH 5.5	80.9 %	Lee et al. (2013)
<i>Chlamydomonas reinhardtii</i>	α -amylase Amyloglucosidase	120 KNU/g (300 AGU/mL); 50 - 65 °C and pH 4.5 - 5.5 for 10 - 60 min	56 %	Choi et al. (2010)

alkalis or only water can be used as reaction catalyst (Chen et al., 2013; Ruiz et al., 2013a and 2015). Using only water as catalyst for biomass pretreatment presents great benefits and has raised great interest in recent years due to the inexistence of toxic waste, no need to neutralize the treated samples, being thus considered as an environmentally friendly process (Hu et al., 2011). However, it is necessary to perform the process at high temperature and pressure values and as a result, hydrothermal pretreatment requires the use of specific equipment that can lead to increased production costs. Some new technologies (ultrasound, microwave, electric pulse, etc.) could also be used in combination to further enhance cell wall disruption resulting in a more efficient pretreatment with lower energy costs and higher recovery of target compounds. Concerning the structural modification of starch, Rubens and Heremans (2000) reported that as temperature and pressure were increased, a better gelatinization was induced, but increasing these parameters to much higher levels could lead to unfavorable outcomes, i.e., the degradation of starch granules.

Different studies have reported on the use of hydrothermal treatments for preparation and solubilization of organic matters and cellular rupture of aquatic biomass. For instance, Passos et al. (2015) reported the use of a hydrothermal treatment at temperatures between 110 - 130 °C. Mendez et al. (2014) evaluated more severe conditions with temperatures ranging between 140 - 180 °C. In a different investigation, Pirwitz et al. (2016) used temperatures up to 200 °C for more than 60 min under hydrothermal liquefaction treatment. It is important to note that the treatment used would result in a broad spectrum of other compounds in addition to simple sugars and therefore, it is essential to select the best pretreatment conditions to most efficiently break the cell wall, modify the structure of carbohydrates, and obtain added-value compounds. In better words, since the use of very severe conditions could possibly degrade certain types of compounds like proteins or carbohydrates, thus generating degradation products like acids or furfurals, the choice of pretreatment conditions is critical (Ruiz et al., 2013b).

3.2.1. Acid and alkaline hydrothermal pretreatment

Concentrated or diluted acid and alkaline solutions could also be used under hydrothermal conditions resulting in shorter reaction times, lower costs, and higher capacity to hydrolyze polymers and oligosaccharides to monosaccharides (Ometto et al., 2014). Higher concentrations of these chemicals decrease reaction times, avoid the use of enzymes, while the use of low concentrations makes necessary higher temperature and pressure values to achieve favorable hydrolysis efficiencies (Gíro et al., 2010; Lenihan et al., 2010; Talebina et al., 2010). Nevertheless, the use of these chemicals at high temperature involves the formation of high concentrations of degradation compounds. Moreover, these compounds are undesirable for the subsequent

fermentation process since they are inhibitory to the microorganisms involved in the process (Duarte et al., 2009; Ruiz et al., 2013c). In addition to these, they can also cause equipment corrosion, contaminate residues, and damage the environment. The need to neutralize the samples once treated and the consequent increases in the overall cost of the operation is also among the disadvantages associated with the application of concentrated reagents (Duarte et al., 2009; Zu et al., 2014; Günerken et al., 2015).

The acid-based method commonly employs sulfuric acid and hydrochloric acid in concentrations ranging from 1 to 10% at temperatures between 60 - 180 °C. It promotes the degradation of cellulose matrix contained in the cell wall, depolymerization of hemicellulose, and hydrolysis of starch into simple molecules in order to avoid the enzymatic hydrolysis step (Harun and Danquah, 2011b; Miranda et al., 2012). The alkaline-based method mainly uses sodium hydroxide. This method is characterized by creating solvation and saponification reactions, forming pores in the cell wall thereby allowing the intracellular compounds to get out of the cell, decreasing the size of the starch polymers as well as the crystallinity of cellulose and starch (Brienzo et al., 2010; Harun et al., 2011c; Sui et al., 2012).

3.3. Ultrasound pretreatment

Ultrasound technology has been widely used in the field of organic chemistry for the acceleration of chemical reactions and for the extraction of bioactive compounds from various plant species. The ultrasound process involves the use of sound waves that travel through a liquid medium and creates areas of compression and rarefaction, where pressure changes occurs creating the cavitation phenomenon inducing the formation of bubbles in the elastic medium (Cervantes-Cisneros et al., 2015). Meanwhile, gas that cannot be retained in the bubbles is condensate, releasing a large amount of energy causing a violent collapse of the bubbles creating shock waves and regions of very high temperature and pressure. In fact, the cavitation process is effective on heat and mass transfer, creating hot spots that can cause the acceleration of chemical reactivity in the medium (Picó, 2013). This type of technology can help break the cell wall of microalgae because when bubbles collapse on the surface of a solid, the pressure and elevated temperature create microjets that allow the solvent to penetrate into the raw material and a rupture of the cell wall occurs (Luo et al., 2014). In a previous work reported by Jeon et al. (2013), *Scenedesmus obliquus* biomass was submitted to ultrasound treatment between 10 - 60 min to facilitate the accessibility of bacteria to ferment the sugars present intracellularly in the biomass, with best pretreatment found within a time

period of 15 min. This pretreatment method is an alternative for cell disruption where water, acid, or alkalis could be used as catalysts for cell wall disruption of microalgal biomass. Ferreira et al. (2016) successfully extracted some compounds (carbohydrates, lipids, proteins, and pigments) within the biorefinery concept from diverse types of microalgae such as *C. vulgaris*, *Nannochloropsis oculata*, and *S. obliquus*, employing low frequency ultrasound and different solvents.

The use of ultrasound also offers the opportunity to modify and improve some important features of bioactive compounds without removing their biological properties (Jambrak et al., 2010). For instance, in third generation bioethanol production, the starch present in microalgae can be modified leading to improvements in the subsequent enzymatic hydrolysis. The effect of ultrasound pretreatment is shown through the distortion of the crystalline regions and improvements in the water uptake of the starch granules, which might aid in the accessibility of the enzymes which could consequently lead to a more efficient hydrolysis process of the carbohydrates (Zheng et al., 2013).

3.4. Microwave pretreatment

This type of pretreatment is also used in a large part in the organic chemistry for the acceleration of reactions and extraction of compounds, and it has already been used in the pretreatment of lignocellulosic residues to produce second generation bioethanol and high value compounds in a study carried out by Velazquez-Lucio et al. (2015). The action of this alternative energy is through the interaction with the polar molecules of the solvent, generally water, which form hot nuclei allowing very efficient and rapid heating. Consequently, the reactions could be carried out more quickly, with better yields and greater selectivity (Wang and Lu, 2013; Aguilar-Reynosa et al., 2017). This heating is performed by two mechanisms: 1) by the rotation of the dipoles where the polar molecules try to align in the electromagnetic field that changes rapidly by the microwaves and 2) by the ionic conduction consisting of the instant superheating of the ionic substance due to the friction of the ionic molecules generated by the movement that produces the electric field (Sarker and Nahar, 2012). With the application of microwave irradiation in starch, the native crystallinity of the starch granules is lost and a completely amorphous material is created, i.e., the granule is practically destroyed (Rozi and Singh, 2000; Sjöqvist and Gatenholm, 2005). Microwave pretreatment promotes starch digestibility which can enhance, depending on the conditions of the pretreatment, the accessibility of enzymes to the pretreated substrate (Emami et al., 2012). Ma et al. (2014) reported the use of microwave pretreatment on *Chlorella* sp. biomass using 2,450 MHz; 530 W; during 45 and 75 s as pretreatment conditions and obtained up to 82% cellular rupture. In a different research work, Ali and Watson (2016) applied the microwave pre-treatment at 943 W for 5 min in *N. oculata* and claimed 70% cell wall destruction. Overall, since microalgae are grown in water and given the ionic nature of water, microwave radiation is well absorbed by the medium and consequently, it is an efficient and rapid way to carry out the pretreatment.

3.5. Pulse electric field pretreatment (PEF)

The PEF pretreatment method is a simple technique based on electricity; it is non-thermal and is performed within a short time duration ranging from nanoseconds to milliseconds but at great amplitude from 100 – 300 V cm⁻¹ to 300 kV cm⁻¹. This pretreatment works well both at low and high cell concentrations in liquid and therefore, could be used directly with the microalgal culture. During this treatment, an effect called electroporation or electropermeabilization occurs. In fact, the electric field created induces a power differential through the cellular membrane and electroporation occurs when a certain threshold value is exceeded (between 0.5 – 1.5 V) (Goettel et al., 2013; Vorobiev and Lebovka, 2015). When electroporation occurs, the permeability of cell membrane increases. Cell membranes are mostly negatively charged and are formed by ions and different proteins. In this site, a transmembrane potential exists due to internal and external ionic gradients, and when an external electric field above the said potential is applied, a remarkable cellular polarity will be induced, creating a charge separation and producing a dipole moment that is parallel to the external field (Sheng et al., 2011; Zbinden et al., 2013).

When a critical electric field is applied, the electric forces cause a dielectric break that increases the permeability together with the formation of pores that are usually irreversible. Parniakov et al. (2015) reported the successful use of

PEF and different pH values for extracting different compounds such as carbohydrates, proteins, and pigments from the microalgae *Nannochloropsis*. Recent reports have claimed that the use of hot liquid at temperatures between 20 – 80 °C during the electroporation process could increase the effect of the pretreatment on the cellular membrane (Vorobiev and Lebovka, 2015; Postma et al., 2016). Han et al. (2009) studied the effect of the electric pulses on corn starch and observed that by increasing the strength of the pulses they managed to decrease the temperature and enthalpy of gelatinization. Moreover, after the pretreatment with PEF, the starch lost its granule form and its crystallinity level was decreased. Such findings could be indicative of the effectiveness of this technique using microalgae biomass as well. Nonetheless, to the best of our knowledge, the extraction of compounds using this technology has not been thoroughly studied, and this represents a great opportunity for performing further studies with a focus on PEF-based pretreatment of not only microalgae biomass, but also the other types of renewable biomass in order to extract sugars and high added value compounds without damaging or degrading the raw materials used.

3.6. Mechanical methods

Conventional mechanical methods are the most used techniques in the industry, offering favorable outcomes at large scale as well as high recovery of the targeted products. Among them, high-pressure homogenization, hydrodynamic cavitation, bead milling and ball milling are mostly in use. The homogenization by high pressure works based on a simple principle and could lead to considerable cellular disruption of the biomass. More specifically, cells are placed in a suspension and are displaced and directed by a pump to an orifice with especially designed valves to resist pressure. Subsequently, the flow rate is rapidly increased and the pressure of the fluid decreases over a short distance as it exits the system. Overall, cell disruption by high pressure homogenization is attributed to different causes or mechanisms such as fluid shear, turbulence, shock velocity, and cavitation (Spiden et al., 2013; Yap et al., 2015; Xie et al., 2016).

An alternative form of this technique has also emerged namely hydrodynamic cavitation, generated by passing a liquid or slurry into a large transverse cavity directed to a very small cavity called a throttle valve which causes the constriction of the suspension. This process produces a pressure drop when it falls below the vapor pressure forming microbubbles that collapse when the pressure returns above the normal vapor pressure values. This collapse produces shock waves and increases the pressure and temperature which ultimately results in cell disruption (Lee and Han, 2015).

The two mechanical pretreatment processes most used in the industry in batch or continuous mode for grinding of minerals, ceramics, powders, among other compounds, are the bead mill and the ball mill. In the field of biotechnology, these technologies have already been used for cellular disruption of some microorganisms and are affected by different parameters such as feed rate of the cell suspension, agitation speed, agitator design, diameter and size of the balls, as well as design of the grinding chamber. Besides biomass concentration, density of the suspension, and microorganism morphology, it is also necessary to take into account the interactions of the equipment with the biomass derived from different microorganisms (Montalescot et al., 2015; Postma et al., 2015). Overall, in spite of the effectiveness of these pretreatments for cellular disruption of microalgae biomass, their main drawback is that they do not directly affect the structure of the intracellular carbohydrates, and therefore, a further step would be needed to modify the starch structure. Moreover, these methods are also very energy-intensive. Hence, future studies are still needed and the results obtained through the combination of these methods with others could possibly promote or limit their application.

3.7. Freezing / thawing pretreatment

The mechanism of freezing and thawing pretreatment method is simple, i.e., a slow freezing is carried out at temperatures as low as 10 °C below the freezing temperatures of water. This promotes the creation of ice crystals, mechanically breaking the cell walls (Yang et al., 2015). Intracellular compounds such as carbohydrates, lipids, proteins, and pigments are released into the medium during thawing, while cell membrane compounds and water soluble organic compounds can also be extracted (Carbonell et

al., 2006; Ando et al., 2016). This pretreatment can be handled in cycles, i.e., the freeze-thaw process is repeated to increase cell disruption thereby, improving the extraction of carbohydrates or other compounds of interest, which are deeply rooted in cellular organelles. Although this pretreatment has been used only for cell disruption, but a recent study on certain lignocellulosic biomasses (which are more difficult to pretreat compared with microalgae biomass) performed by Smichi et al. (2016) proved it to be an efficient alternative and a promising pretreatment for breaking biomass cells leading to a better accessibility of the contained polysaccharides for enzymatic attack. It should also be noted that this pretreatment does not produce any degradation compounds. Nevertheless, the application of this technology is controversial since it can increase energy consumption and the time required to complete the process. In better words, the main disadvantage of the freezing-hawing pretreatment is the need to perform it for at least 24 h cycles, which would considerably increase the overall time of the production process.

4. Enzymatic hydrolysis of carbohydrates in microalgae

As mentioned earlier, the production of bioethanol from microalgae biomass includes three stages: pretreatment, saccharification, and fermentation. One of the advantages of the use of microalgae biomass is that in some pretreatment

methods, saccharification can be carried out followed by the pretreatment step without having to implement any different equipment or excessive treatment of the sample. Enzymatic hydrolysis (saccharification) is one of the most important steps to obtain essential sugars such as glucose and mannose for the subsequent fermentation and bioethanol production (Harun and Danquah, 2011a; Milano et al., 2016). More specifically, one of the enzymes used is an endo-amylase attacking internal α -1-4 glycosidic bonds of starch producing dextrans. Then, the enzyme amyloglucosidase hydrolyzes alpha α -1-6 glycosidic bonds, leading to the production of glucose and other sugars such as maltose (Chen et al., 2013; Ometto et al., 2014; Hernández et al., 2015). Other enzymes such as cellulases and hemicellulases can also be used to obtain simple sugars from cell wall and intracellular polysaccharides (cellulose, hemicellulose, etc.), as reported by Mahdy et al. (2015) who used enzymatic cocktails (Celluclast 1.5 L, Viscozyme L, and Pectinex-Ultra SP- L) on *C. vulgaris* and *Scenedesmus* sp. obtaining conversion efficiencies as high as 84% of total sugars.

The enzymatic hydrolysis of the sugars present in microalgae biomass offers many advantages over chemical hydrolysis with acids or alkalis. Those include no requirement for expensive equipment (because it is carried out under mild conditions), no generation of degradation products or toxic compounds (that can potentially affect the subsequent fermentation

Table 4. Comparison of methods used for starch and total carbohydrates determination.

Method	Reagents / Equipment	Determination method	Advantage	Disadvantage	References
Starch					
Perchloric acid	5 - 10 mg sample Ethanol 80% (v/v) HClO ₄ 35% (v/v)	Anthrone	- Little sample requirement - Low cost - Good reproducibility	- Corrosive reagents - Low specificity	Rose et al. (1991) Brányiková et al. (2011)
Hydrochloric acid	5 - 10 mg sample Acetone Ethanol 80% (v/v) HCl 1.1 % (v/v)	Anthrone	- Low reagent control and precautions - Little sample requirement	- Probability of human error - High interference	Meyer et al. (1988) Fernandes et al. (2012)
Enzymatic	5 mg sample Ethanol 80% (v/v) α -amylase Amyloglucosidase Glucose oxidase/peroxidase	Absorbance	- High specificity - Established method - Low interference - High reproducibility	- High cost - High reagent control and precautions requirement	Megazyme (2009)
Total Carbohydrates					
Chemical	10 mg sample Diluted HClO ₄ / HCl	Anthrone Phenol-Sulfuric	- Little sample requirement - Low cost - Good reproducibility - Low reagent control and precautions - Little sample requirement	- Corrosive and toxic reagents - High interference - Low specificity	Fernandes et al. (2012) Margarites and Costa (2014)
Physical	10 mg sample H ₂ O 10 mL Mortar / Ultrasound 10 min or Freezing/thawing 12 h	Anthrone Phenol-Sulfuric	- Little sample requirement - Moderate cost - Good reproducibility - Low reagent control and precautions requirement	- Corrosive and toxic reagents - High interference - Low specificity - Probability of human error	Laboratory of fermentations and Biorefinery Group (2017)*
HPLC	300 mg sample H ₂ SO ₄ 72% (v/v) Column 87 H RI detector H ₂ SO ₄ 0.025 M/0.005 M 0.6 mL/min flow; 50 °C	HPLC	- Little sample requirement - High specificity - High reproducibility - Environmental friendly residues	- High cost - Specific equipment requirement - High precaution with corrosive reagent	NREL (2008)

and bioethanol production), and finally higher yields of simple carbohydrates.

5. Starch and total carbohydrates determination methods

The main polysaccharide found in microalgae biomass which is used for third generation bioethanol production is starch, and therefore, it is fundamental to know the different techniques used for the quantification of this compound. This section describes some techniques used for the determination of starch as well as the methods used for the quantification of total carbohydrates.

5.1. Starch determination methods

As reported by Fernandes et al. (2012), there are different approaches for the determination of starch in microalgae: i) perchloric acid method - in this method, first, interfering compounds (pigments, soluble sugars, and lipids) are extracted with acetone or ethanol, then microalgae starch is extracted and solubilized with perchloric acid, and finally, total sugars are quantified by colorimetric Anthrone method (Rose et al., 1991; Brányiková et al., 2011); ii) hydrochloric acid method - in this method, interfering compounds are first extracted with acetone followed by an ethanolic extraction to ensure complete removal of these substances. After obtaining the starch extract, it will be hydrolyzed by hydrochloric acid followed by total sugars determination by the Anthrone method (Meyer et al., 1988). Finally, iii) enzymatic method - this method is based on enzymatic degradation of starch to glucose with α -amylase and amyloglucosidase and it is performed by total starch assay described by Megazyme (2009), endorsed by Association of Analytical Communities (Official Method 996.11) and American Association of Cereal Chemists (Method 76.13). As in the previous methods, in order to extract interfering compounds, the previously ground biomass is incubated in a water bath, followed by the addition of a thermostable α -amylase with pH 7.0. Subsequently, amyloglucosidase with pH 4.5 is added and finally glucose oxidase, peroxidase, and 4-aminoantipyrine (GOPOD reagent) are added to the sample and the mixture is incubated. The absorbance of the sample is measured at 510 nm by a spectrophotometer using a blank solution (water), GOPOD reagent, and a control sample (D-glucose). Table 4 shows a comparison between starch and total carbohydrate determination methods, including their advantages and disadvantages.

5.2. Determination methods for total carbohydrates

Total carbohydrates in microalgae biomass have been determined by diverse colorimetric methods. For all these methods, it is first necessary to break microalgae cells either by using chemical or physical methods. i) chemical methods: these can be used as in the cases of starch quantification methods previously mentioned (see Section 5.1) (Fernandes et al., 2012; Margarites and Costa, 2014). Physical methods: these include grinding with mortar or ultrasound probe (where the sample is subjected to these treatments for around 10 min) and freezing/thawing in 12 h cycles in the presence of water as solvent. The resulting extract obtained through chemical or physical methods is then used for the quantification of total carbohydrates by the phenol-sulfuric method (Dubois et al., 1956) or Anthrone method (Dreywood, 1946). iii) High-performance liquid chromatography (HPLC)-based total carbohydrate determination: this method is based on the analytical procedures of the National Laboratory of Renewable Energy - USA (NREL, 2008). Briefly, microalgae biomass sample should first be prepared by concentrated acid hydrolysis. More specifically, H_2SO_4 (72% w/w) is added into dry biomass in a test tube and the mixture is left for 1 h at 30 °C to react. The sample is then diluted to 4% with distilled water and autoclaved at 121 °C for 1 h. Subsequently, the solid and liquid fractions are separated and the liquid fraction is used for the HPLC determination in which an ion-exclusion column such as Bio-Rad HPX-87H or Agilent MetaCarb 87H is required in conjunction with a refractive index detector. The following HPLC conditions can be used to perform the analysis; H_2SO_4 0.025 M or 0.005 M as mobile phase with a flow ratio between 0.5 to 0.6 mL/min and 50 °C column temperature. Among the carbohydrates that can be quantified with this method are glucose, xylose, cellobiose, arabinose, as well as different byproducts including oxalic, formic, acetic, butyric, succinic and levulinic acids (Juárez et al., 2016).

6. Conclusions and future prospects

The extraction of carbohydrates from microalgae biomass is essential to produce third generation bioethanol and the efficiency of this process is dependent on distinct factors, such as morphology (cell wall composition) and algae species. Depending on these characteristics, it is possible to choose the most appropriate pretreatment for a certain type of algal to be used. In the production of bioethanol from microalgae and to make this process viable, it is important to take into account other aspects, such as improving the culture conditions to enhance the accumulation of different compounds of interest (e.g., carbohydrates, lipids, proteins, pigments, etc.), application of the culture water in the pretreatment, and the use of solvents or catalysts that are environmentally friendly. The use of algal biomass in the concept of integrated biorefinery should also be considered, taking advantage of all the compounds present in the biomass and in order to avoid the generation of waste streams.

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