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THIRD GENERATION BIOTEHANOL PRODUCTION

BY

ALEXANDRA CRISTINA BLAGA[1], LENUȚA KLOETZER[1], ALEXANDRA TUCALIUC[1], DAN CAȘCAVAL[1,*] and ANCA IRINA GALACTION[2]

[1]“Gheorghe Asachi” Technical University of Iași,
Faculty of Chemical Engineering and Environmental Protection
[2]“Grigore T. Popa” University of Medicine and Pharmacy, Iași,
Faculty of Medical Bioengineering

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Abstract. The algal biomass offers great potential as a sustainable feedstock which can be processed for the third generation bioethanol production, due to its many advantages: rapid growth rate, the ability to accumulate important amounts of carbohydrates, all the materials produced are nontoxic and biodegradable. However, in order to produce high concentrations of ethanol, it is necessary to convert all specific carbohydrate components of microalgae: glucan, sulphated polysaccharides, mannitol, alginate, agar, and carrageenan. This paper describes different types of algae and presents the main steps for the technology employed in the production of third generation bioethanol.

Keywords: bioethanol; continuous fermentation; microalgae; seaweed.

1. Introduction

The increase in fossil fuel prices and global energy consumption, cumulated with the environmental effects of greenhouse gas emissions have led to an overwhelming interest among researchers to develop economically viable processes for the production of alternative fuels (Farias Silva and Bertucco,

[*]Corresponding author; *e-mail*: dancasca@tuiasi.ro

2016; Kumar *et al.*, 2013). Among several biofuel candidates proposed to replace fossil fuels, in order to eliminate the vulnerability of energy sector, bioethanol has been accepted widely as a good renewable source of energy. According to F.O. Licht, the bioethanol market grew by 2.7% up to 115.1 million m^3 in 2015, 97.1 million m^3 being used as fuel (80%). The world leader in the production of bioethanol is the USA with 57.5 million m^3/year, followed by Brazil with 30.0 million m^3/year.

Due to an increasing requirement of energy, the evolution of bioethanol production in terms of converted feedstocks type was extremely rapid (Trivedi *et al.*, 2015) (Fig. 1). The first generation bioethanol seemed to be the most feasible short-term alternative to fossil fuels, but it was based on edible crops: sugarcane, corn, wheat and soybeans. However, limited arable lands and water supply, the excessive utilization of harmful pesticide and fertilizer in production of first generation crop plants, which strongly affect the environment, led to an increased interest on other feedstock. The second generation bioethanol was mainly obtained from agricultural crops or agricultural waste, like rice straw and switchgrass, but this process was limited especially by the high costs involved in converting lignocellulosic materials into ethanol: the high lignin content in the lignocellulosic biomass increases significantly the difficulty of the saccharification process. All these mentioned drawbacks indicated that algal biomass, a diverse group of autotrophic organisms, can represent a more sustainable feedstock for the bioethanol production. The main advantage of this source is the less resistance to its conversion into simple sugars compared to that of agricultural crops (Yanagisawa *et al.*, 2013).

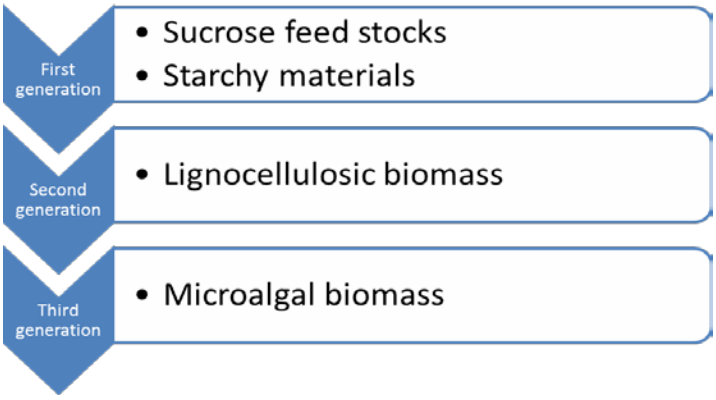


Fig. 1 – Schematic evolution of converted feedstocks for bioethanol production.

The use of algal biomass for bioethanol production offers many other advantages (Guo *et al.*, 2013; Posada *et al.*, 2016; Lam and Lee, 2012; Jiang *et al.*, 2016):

– microalgae grow faster (100 times faster than the terrestrial plants), fix CO_2 with higher rate than the terrestrial plants, and can produce large amount of lipids and carbohydrates;

– microalgae do not require soil and agricultural input such as fertilizer or pesticides, they can be grown all year depending on the climate, and are highly biodegradable;

– microalgae-based carbohydrates are mainly in the form of starch and cellulose (rather zero content of lignin), therefore being much easier converted to monosaccharides compared to the lignocellulosic materials;

According to literature, there are three possible ways to convert algal biomass into bioethanol (Farias Silva and Bertucco, 2016; Hallenbeck *et al.*, 2016) (Fig. 2): the traditional one involving hydrolysis and fermentation, the dark fermentation route, and the use of engineered cyanobacteria in "photofermentation".

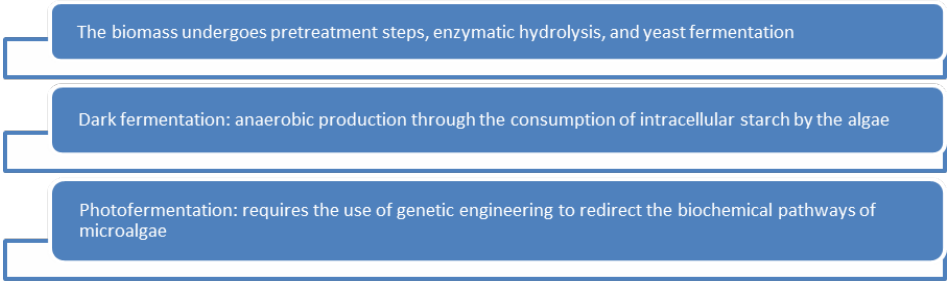


Fig. 2 – Schematic routes for bioethanol production from algae.

Many studies are focused on exploiting microalga's ability to directly use their enzymatic or anaerobic digestion systems to produce bioethanol, either by screening high starch accumulating microalgae or by generating efficient mutants (by genetic engineering), in order to obtain an algae strain that produce enzymes as amylases and cellulases necessary for the hydrolysis step (Alaswad *et al.* 2015; Lam and Lee, 2012; Vassilev and Vassileva, 2016).

2. Microalgae as a Feed Stock

Algae, from small, single-celled organisms (phytoplanktons-microalgae) to multi-cellular organisms (seaweeds – macro-algae), represent a vast variety of photosynthetic species that require sunlight, CO_2 and water to produce biomass (Hallenbeck *et al.*, 2016; Dragone *et al.*, 2010). The autotrophic algae use photosynthesis and are able to fix the inorganic carbon from atmospheric CO_2 and convert it into reserve food materials such as carbohydrate, while the heterotrophic algae can grow in the absence of light, using organic carbon sources dissolved in the culture media. The following

microalgae are capable to biosynthesize ethanol in the absence of light: *Chlamydomonas reinhardtii, Chlamydomonas moewusii, Chlorella vulgaris, Oscillatoria limosa, Chlorococcum littorale, and Spirulina sp.* (Farias Silva and Bertucco, 2016).

The macro-algae are multicellular organisms with differentiated cell structure and function, with simple reproductive structures (fast-growing: can reach sizes of up to 60 m in length), that can grow in fresh or salt water. The macro-algae can be classified, in relation with the type of pigments, into: brown seaweed or *Phaeophyceae*, red seaweed or *Rhodophyceae* and green seaweed or *Chlorophyceae* (Jiang *et al*., 2016).

The classification of microalgae is related to the type of pigments, chemical nature of storage products and cell wall constituents. The microalgae include dinoflagellates, the green algae or *Orchlorophyceae*, the golden algae or *Chryosophyceae*, and diatoms or *Bacillariophyceae* (Lam and Lee, 2012; John *et al*., 2011).

Algae can produce carbohydrates (and lipids and proteins), over a short period of time, that can be used as carbon source or substrate for fermentation (Table 1).

Table 1
The Highest Concentrations of Carbohydrates in Different Algae

Algae	Carbohydrates	Ref.
Macroalgae		
Alaria	39.8	Alaswad *et al*., 2015
Enteromorpha	64.9	
Gracilaria	61.75	
Laminaria	39.3	
Monostroma	63.9	
Porphyra	45.1	
Ulva	42	
Microalgae		
Anabaena cylindrica	25-30	Sydney, 2010
Dunaliella salina	32	
Porphyridium cruentum	40-57	
Spirogyra sp.	33-64	
Chlamydomonas reinhardtii UTEX 90	60	Chow *et al.*, 2015

The algae's carbohydrates composition (Table 1) is different between strains and varies between 2-65% d.w. (Dragone *et al.*, 2010) including monosaccharides (glucose, mannose, ribose/xylose, rhamnose, and fucose) and the following polysaccharides (glucan and non-glucan):

– cellulose, starch, sulphated polysaccharides as ulvan in green seaweeds;

– cellulose, laminarin, mannitol and alginate, in brown seaweeds;
– cellulose, floridean starch, agar or carrageenan, in red seaweeds.

Macroalgae usually contains more carbohydrates compared to microalgae, but microalgae have higher contents of starch compared to macroalgae (except green algae). The decreasing order of their bulk contents in carbohydrates is: red algae > brown algae > green algae (Alaswad *et al*., 2015).

The ethanol production has been reported from different seaweeds or microalgae (Sudhakar *et al*., 2016; Bharathiraja *et al.*, 2015):

– green: *Ulva lactuca, Ulva pertusa, Ulva fasciata, Chaetomorpha linum*

– red: *Kappaphycus alvarezii, Gelidium amansii, Gelidium elegans, Gracilaria salicornia, corda, tenuistipitata* and *varrucosa, Eucheuma cottoni*

– brown: *Alaria crassifolia, Laminaria japonica, Laminaria hyperborea, Saccharina latissima, Sargassum fulvellum, Undaria pinnatifida.*

Production pathways for bioethanol are referred to the fermentable sugars (*i.e*. glucose, sucrose, etc.) converted to ethanol by fermentation. In order to obtain a high productivity in ethanol, as many as possible of these carbon sources need to be converted in compounds that could be used during fermentation.

3. Technological Process for Algal Biomass Use for Bioethanol Production

The production of bioethanol from biomass involves the following steps: the biomass containing carbohydrates, from different cultivation systems, can be harvested for their extraction by mechanical means or by using enzymes (Fig. 3). The resulting sugar is then used for fermentation to yield bioethanol.

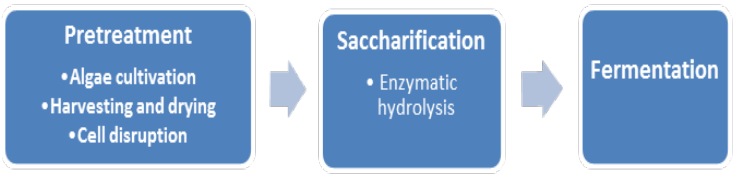


Fig. 3 – Main steps in the production of bioethanol from algal biomass.

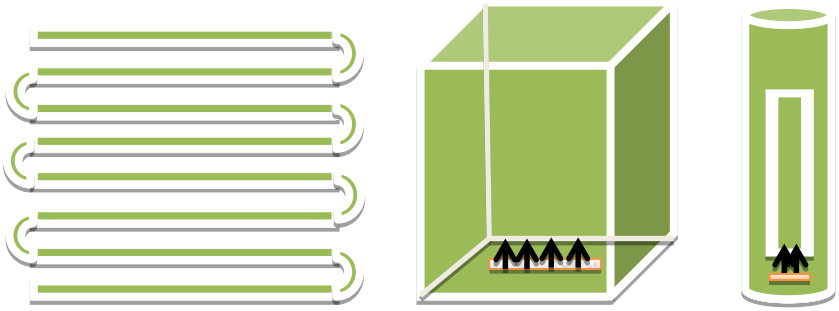
Cultivation of algal biomass uses two main systems (Posada *et al.*, 2016; Lam and Lee, 2016):

– open pond, that refers to a simple open tank or natural ponds. They are relatively cheap, easy to clean, require low energy inputs and easy maintenance, but offers poor productivity, require large area of land and high harvesting cost, provide only poor mixing, light and CO_2 utilization and are easily contaminated.

– enclosed photobioreactor (PBR), where algae are cultivated in suspension in a closed system, offers advantages in terms of yield and control: high productivity, low contamination, continuous operation and controlled growth conditions, but with some limitations regarding low light penetration and sophisticated construction (Caşcaval *et al.*, 2007).

From the many different types of photobioreactors the following three designs, showed schematically in Fig. 4, were used for algae: tubular (*a*), flat plate (*b*), and column (*c*).

Tubular photobioreactors can be helical, manifold, serpentine, and α-shaped, with different positions: horizontal, near horizontal, vertical, inclined and conical-shaped, have a large illumination surface area and have fairly good biomass productivities. The flat photobioreactors can be alveolar panels or glass plates and offer large surface area exposed to illumination and high densities of photoautotrophic cells. The column may be bubble columns and airlift, and are usualy placed vertically, aerated from the bottom, and illuminated through transparent walls (Olivieri *et al.*, 2014).



a *b* *c*

Fig. 4 – Schematic designs of main types of photobioreactors for algae.

Since 1950s photo-bioreactor technology has evolved, different designs being developed and investigated to improve control strategies for long-term stability and reliability of operations. In order to choose the most appropriate, one must consider not only the yield, the increase in photosynthetic efficiency and enhancement of gas exchange rate, but also the capital investment and operating costs, to prove that it can be commercially feasible. Many challenges are still to be overcome in developing models for radiative transfer mechanism, hydrodynamics, but also for photosynthetic and growth kinetics (Lee and Lam, 2012; Olivieri *et al.*, 2014; Hallenbeck *et al.*, 2016). Photo-bioreactors can be illuminated by artificial light, solar light or both, the open ponds are usually naturally illuminated while laboratory-scale photo-bioreactors are artificially illuminated using fluorescent lamps or other light distributors (photosynthesic pigment-sensitized solar cells).

The algal biomass production worldwide is about 12 million tonnes for macroalgae and 9200 tonnes for microalgae (d.w.), mostly grown in open ponds, with a potential bioethanol production of 23400 kg/year from macroalgae (Vassilev and Vassileva, 2016).

The accumulated biomass needs to be harvested and the methods depend on the type of algae (Singh *et al*., 2011):

– macro-algae: use of nets, saving energy;

– micro-algae: sedimentation, flotation, centrifugation, filtration with higher capital and operation costs.

The grown biomass is subjected to a disintegration process of the cell's walls by mechanical means or by dissolution with enzymes. The next important step of the global process is saccharification, since it provides the glucose that can be metabolized by yeast to produce bioethanol. The enzymatic hydrolysis process requires freeing the monosaccharide components from the biomass, in order to enhance the ethanol fermentation productivity.

Enzymatic hydrolysis is carried out under mild conditions (pH and temperature) trying to obtain high sugar yields (no inhibitory byproducts) with low maintenance costs, but the selection of enzyme is extremely important for an efficient hydrolysis of algal biomass, due to their specificity. Also, the difficulty in recovering the enzyme from the products and the long hydrolysis time are factors that need to be considered when choosing the appropriate hydrolysis method (Trivedi *et al.*, 2015).

Cellulose, the main algae's carbohydrate, is hydrolysed by a suite of enzymes, including cellulase and β-glucosidase and the process is influenced by numerous factors such as cellulose crystallinity, substrate surface area, cell wall thickness, porosity, mass transfer. Cellulase includes endocellulase and exocellulase, the first are acting on internal bonds of cellulose while exocellulase are breaking terminal bonds from the free ends of chains produced by endocellulase to form cellobiose witch is hydrolysed by cellobiase (β-glucosidase) releasing glucose monomers. In addition, most of cellulase mixtures contain hemicellulase that facilitates hemicellulose hydrolysis to assist with the overall effectiveness the enzymatic hydrolysis. Amylases break the algal starch and release mainly glucose. Mechanical or chemical (acid or alkaline) pre-treatment, but also ultra-sonication or autoclaving may be necessary to increase the reaction area and give more accessibility to the polysaccharides for hydrolytic enzymes (Harun and Danquaha, 2011).

Hernandez *et al.* (2015) tested different techniques for the saccharification: acid, alkaline, microwave and enzymatic hydrolysis, obtaining the higher value in glucose (128 mg/g dry weight) for the enzymatic hydrolysis with amylases for *Chlorella sorokiniana*, pretreated with 10% sulphuric acid; for *Nannochloropsis gaditana* the combination of acid hydrolysis followed by enzymatic hydrolysis produced 129 mg/g.

Different types and combinations of enzymes have been tried for the hydrolysis step (Table 2), the higher result (55.74 mg glucose/g d.w.) being obtained by using an acid pre-treatment method and a mixture of enzymes for the hydrolysis.

Table 2

Enzymes Used for Hydrolysis of Algal Biomass

Substrate	Enzymes	Glucose	Ref.
Scenedesmus abundans	H_2SO_4+celullase	5.730 g/L	Guo *et al*., 2013
	H_2SO_4+amylase	4.016 g/L	
Mychonaster afer	H_2SO_4+celullase	6.223 g/L	
	H_2SO_4+amylase	6.057 g/L	
Chlorella vulgaris FSP-E	β-glucosidase+amylase	12.00 g/L	Ho *et al*., 2013
	Amylase+Glucanase+Xylanase	8.6 g/L	Marsalkova *et al*., 2010
	Cellulase+Xylanases+Amylases	23.3 g/L	Rodrigues and Bon, 2011
Kluyveromyces marxianus KCTC7150.	Viscozyme L	8.5 g/L	Ra *et al*., 2016
	Celluclast 1.5 L (β-glucanases and β-glucosidases)	9.7 g/L	
	Viscozyme L + Celluclast 1.5 L	13.5 g/L	
Macrocystis pyrifera	2 vol.% H_2SO_4 + cellulases + β-glucosidase + alginate lyase + oligoalginate lyase	55.74 mg glucose/g dry algae	Ravanal *et al*., 2016
Ulva prolifera	Depolymerase isolated from *Catenovulum sp. LP*	ND	Li *et al.*, 2016

Hou *et al.* (2015) obtained a high conversion rate of 84.1% glucose recovery by enzymatic hydrolysis using *Laminaria digitata*, (56.7% glucose content) using only milling as a pretreatment. This study showed that even in the absence of any exo-enzyme, 13.4% of glucose was gradually released from *L. digitata* biomass during 48 h incubation, indicating the possible existence of inherent enzymes for glucan hydrolysis in this seaweed biomass.

Pancha *et al.* (2016) analyzed the chemo-enzymatic hydrolysis of mixotrophically grown *Scenedesmus sp. CCNM 1077* de-oiled biomass, using amylase, cellulase and Viscozyme-L (mixture of arabanase, cellulase, betaglucanase, hemicellulase and xylanase) with a saccharification yield of 6.13, 16.20 and 33.46 w/w of de-oiled biomass. These results underlined the fact that, in this case, a mixture of enzymes that is capable of solubilizing all microalgal carbohydrates is more efficient than the single enzyme. Wu *et al.* (2014) combined the acid hydrolysis (0.1 N H_2SO_4, 121°C, 1 h) with enzymatic hydrolysis (pH 4.5, 50°C, 100 rpm, 6 h) on red macroalgae *Gracilaria sp.* producing 26.8 g/L galactose and 6.1 g/L glucose. Comparative results were obtain by Tan and Lee (2016) using Novozyme 188 (β-glucosidase) and Celluclast 1.5 L combining solid acid hydrolysis (6% w/v Dowex (TM) Dr-G8,

120°C, 1 h) with enzymatic hydrolysis (50°C, pH 4.8, 48 h) for red macroalgae *Eucheuma cottonii*, with a total sugars released in the hydrolysate of 82.1 g/L.

Duan *et al.* (2016) developed a high-efficiency method to hydrolyze carrageenan from *Eucheuma cottonii* (dried seaweed) using cellulase (pH 5.0 and 50°C for 2 h) and recombinant κ-carrageenase, produced by *Escherichia coli BL21-HTa-cgkZ* (35.7°C, pH 5.9 and 7.9 U carrageenase per gram of dry mass) recovering oligosaccharides with a 38% yield.

Viral infection was used by Cheng *et al.* (2013), for inducing microalgal cell lysis coupled with presaccharification steps in a *Chlorella variabilis NC64A* (30°C, 150 rpm, 5 days) using *Paramecium bursaria chlorella virus* (*PBCV-1*) and amylases. A total carbohydrate hydrolysis of 30.5% was obtained. Matsumoto *et al.* (2003) used an amylase-producing marine bacterium (*γ-Proteobacteria-Alteromonadales*, *Pseudoalteromonas undina*) for an efficient saccharification step of green microalga NKG 120701 in saline conditions.

The hydrolysis on *Chlamydomonas reinhardtii*, using a mixture of amylase (produced by *Bacillus licheniformis* at 90°C for 30 min) and glucoamylase (produced by *Aspergillus niger* at 55°C for 30 min) obtained 94% conversion of the carbohydrates (Choi *et al.*, 2010).

Table 3
Comparison of Ethanol Yield from Various Algal Feedstocks

Algae	Microorganism for fermentation	Ethanol (g/L)	Ethanol yield	Ref.
Gracilaria verrucosa	*Saccharomyces cerevisiae*	14.89	0.43 (g/g sugar)	Kumar *et al.*, 2013
C. vulgaris FSP-E	*Z. mobilis ATCC 29191.*	11.66	0.233 (g/g sugar)	Ho *et al.*, 2013
Eucheuma cottoni	*Saccharomyces cerevisiae (YSC2)*	11.6	75%	Jiang *et al.*, 2016
Ulva pertusa	*Saccharomyces cerevisiae (ATCC24858)*	12.4	–	Jiang *et al.*, 2016
Ulva pertusa	*Saccharomyces cerevisiae (IAM4178)*	27.5	80.6%	Yanagisawa *et al.*, 2011
Kappaphycus alvarezii	*Saccharomyces cerevisiae (CBS1782)*	14.3	105 L /ton d.w.seawead	Hargreaves *et al.*, 2013
U. fasciata	*Saccharomyces cerevisiae MTCC No. 180*	–	0.44 (g/g sugar)	Trivedi *et al.*, 2015
Gelidium amansii	*S. cerevisiae KCTC 7906*	3.78	84.9%	Kim *et al.*, 2015
Ulva lactuca	*Saccharomyces cerevisiae*	13.3	52.15%	El-Sayed *et al.*, 2016
L. japonica	*E. coli KO11*	29	–	Kim *et al.*, 2011

After the enzymatic hydrolysis, sugar monomers can then be fermented to ethanol by yeasts of the genus *Saccharomyces,* by bacteria of the genus *Zymomonas,* or ethanologenic *Sphingomonas sp. A1A, E. coli BAL1611 or KO11.* A comparison of ethanol yield from various algal feedstocks is presented in Table 3.

Saccharification is used to degrade polymeric chains into monomeric sugars that becomes substrates for subsequent fermentation and can be a separate step from the fermentation (SHF - separate hydrolysis and fermentation) or can be realized simultaneously with fermentation (SSF - Simultaneous saccharification and fermentation). SSF has been widely used in studies recently, because most enzymes and ethanol producing microorganisms have similar temperature and pH values for their optimum conditions, this making the two steps compatible. The main advance of SSF is that monomeric sugars released from the enzymes action can be utilized directly by the microorganisms instead of being accumulated, which could led to product inhibition for the enzymes, or substrate inhibition for the microorganisms. A comparative study between SSF and SHF showed that SSF produces lower amounts of toxins (levels that allow fermentation) having some in situ detoxification effect. Tan and Lee (2016) obtained 0.559 g/g ethanol for SHF and 0.909 g/g of bioethanol for SSF, proving that SSF is more effective for *Saccharomyces cerevisiae*.

The pretreatment and hydrolysis methods can generate inhibitors, with an important impact on microbial fermentation. Immobilized cells were tested with very good results to reduce the negative effects of inhibitors. The results proved that this method can offer other advantages: increase cell density, shorten fermentation times, increase product tolerances, the possibility of using continuous fermentation (higher bioethanol production efficiency). Lee *et al.* used *Saccharomyces cerevisiae* immobilized in sodium alginate and the particle could be reused for 5 consecutive batch runs. *S. cerevisiae VS3* immobilized on rice straw was reused for 8 consecutive batch runs.

The production cost for different algae and cultivation system proved that a more distant offshore location for the seaweed production increases the cost substantially: for *Gracillaria sp.*, in a tidal flat farm the production cost is 21-33 $/ton d.w. while for rope farm (offshore) it can reach up to 409 $/ton d.w. (Fasahati *et al*., 2015). Despite the economic challenges that constrain the third generation bioethanol commercialization, the current technologies could be efficiently improved in the future. A detailed study of optimization in enzymatic hydrolysis and fermentation is required for the development of an efficient, advanced and significant bioethanol production process from third generation feedstock.

4. Conclusions

The use of algal biomass as an alternative feedstock can be a sustainable and eco-friendly approach to generate bioethanol. Based on the literature, the process should focus not only the increase of the carbohydrate content of the biomass but also on its higher productivity. The percentage of carbohydrates is different with the strain and the conditions provided during its growth, so a judicious choice of the strain and the growth conditions needs to be made for the desirable product. An important cost reductions may be achieved if the water can be obtained at low cost: there are possibilities to grow these easily cultivable microorganisms even in marine or other waste waters, or in coupled processes with biodiesel production.

Some drawbacks of the algal production of ethanol process are recognised due to an extensive energy input compared to terrestrial energy crops, but they could be overcome by developing cost effective cultivation and harvesting strategies. In order to obtain high concentration of ethanol all the carbohydrates from the algae biomass need to be converted to simpler sugar, the development of an efficient hydrolysis method is of great interest to produce high concentrations of total sugars and the fermentation of every sugar to ethanol.

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OBȚINEREA BIOETANOLULUI DE GENERAȚIA A TREIA PRIN PROCESE BIOTEHNOLOGICE

(Rezumat)

Biomasa obținută din alge oferă un potențial important ca materie primă durabilă, care poate fi prelucrată în vederea obținerii etanolului de generația a treia, datorită multiplelor avantaje: viteză de creștere rapidă, capacitatea de a acumula cantități importante de carbohidrați, materialele produse lipsite de toxicitate și biodegradabilitate. Cu toate acestea, în scopul de a produce concentrații mari de etanol, este necesar ca toate componentele glucidice specifice algelor: glucan, manitol, alginat, agar și caragenan să poată fi convertite cu ușurință la glucide simple. Această lucrare descrie diferite tipuri de alge, și prezintă principalele etape ale tehnologiei folosite în producția de bioetanol de generația a treia.