TÉCNICO LISBOA

Pilot scale production of metabolites from cyanobacteria

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Abstract

The great challenge of this work encompassed the direct production of ethanol at a pilot scale through a genetically modified (GMO) *Synechocystis sp*. PCC 6803 with an economically viable energy balance. In order to achieve that purpose, pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase genes (*adh*) from *Zymomonas mobilis* have been transformed into *Synechocystis* genome under the control of light-driven *psbA2* promoters, resulting in direct synthesis of ethanol.

This study was composed by two phases. The first involved the characterization and optimization of culture procedures by determining the specific growth rate of wild-type (WT) and GMO strains; determining ethanol productivity under controlled conditions to screen for stable transformants; studying their genetic stability and long term conservation through PCR based assays and cryopreservation, respectively. The validation of a disinfection method was developed to guarantee a safe GMO production. The second part focused in the scale-up of the GMO culture up to 1.1 m^3 photobioreactor (PBR).

During the scale-up procedure, it was observed a slow biomass accumulation due to ethanol production and accumulation, with average concentrations of 25 to 100 mg L^{-1}. In the outdoor PBR however, none ethanol was measured. This can be related to ethanol consumption by contaminants that proliferate in the system.

In face of the results, bioethanol production from microalgae still faces several challenges that need to be addressed in order for it to become a reality. Developing novel methods to enhance culture robustness and achieving higher ethanol production efficiencies are a priority for future studies.

Keywords: Bioethanol, GMO microalgae, *Synechocystis*, Pilot scale photobioreactor, Direct Ethanol From Microalgae.

Resumo

O grande desafio deste trabalho consistiu na produção de etanol, à escala piloto, através de um organismo geneticamente modificado (OGM), a cianobactéria *Synechocystis* sp. PCC 6803. Para tal, os genes piruvato descarboxilase (pdc) e álcool desidrogenase (adh) da bactéria *Zymomonas mobilis* foram inseridos no genoma de *Synechocystis* sob o controlo dos promotores *psbA2* resultando na síntese e excreção de etanol.

Este estudo foi dividido em duas fases. A primeira consistiu na caracterização e optimização dos procedimentos de cultivo através da determinação da taxa específica de crescimento das estirpes selvagem (WT) e OGM; da realização de testes de produção de etanol para determinar quais os OGMs mais estáveis; estudar a sua estabilidade genética e preservação através de ensaios baseados em PCR e criopreservação, respectivamente. Foi desenvolvido e validado um método de desinfecção para garantir uma produção de OGMs de acordo com as normas em vigor. A segunda parte centrou-se no *scale-up* da cultura OGM para fotobioreactor (FBR) de 1,1 m^3.

Durante o procedimento de *scale-up* foi observado uma diminuição de produção de biomassa devido à produção de etanol, obtendo-se concentrações médias em etanol de 25-100 mg L^{-1}. Contudo, foram medidas concentrações muito inferiores no FBR, o que poderá estar relacionado com o consumo de etanol pelos contaminantes presentes no cultivo.

A produção de biocombustíveis a partir de microalgas enfrenta ainda desafios que precisam ser ultrapassados para que esta se torne uma realidade. Melhorias na robustez da cultura e atingir eficiências de produção de etanol superiores são uma prioridade para estudos futuros.

Palavras-chave: Bioetanol, Microalgas OGM, *Synechocystis*, Fotobiorreactor escala piloto, *Direct Ethanol From Microalgae*

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Abbreviations and Acronyms List

3-PGA	3-phosphoglycerate
ADH	Alcohol dehydrogenase
ATP	Adenosine triphosphate
CH4	Metane
CO_2	Carbon dioxide
ctb_6f	Cytochrome b6f complex
DEMA	Direct Ethanol From MicroAlgae
DMSO	Dimethyl sulfoxide
DCW	Dry cell weight
EU	European Union
F6P	Fructose-6-phosphate
Fd	Ferredoxin
FNR	Ferredoxin-$NADP^+$
G3P	Glyceraldehyde 3-phosphate
G6P	Glucose-6-phosphate
GHG	Greenhouse gas
GMO	Genetically modified organism
HAVP	High-added-value products
NADPH	Nicotinamide adenine dinucleotide phosphate
NO_x	Nitrogen oxides
PBR	Photobioreactor
PC	Plastocyanin
PDC	Pyruvate decarboxylase
PGAL	1,3-biphosphoglycerate
PQ	Plastoquinone
PSI	Photosystems I
PSII	Photosystems II
PUFAs	Polyunsaturated fatty acids
RuBP	Ribulose bisphosphate
RuBisCo	Ribulose-1-5-bisphosphate carboxylase
SO_x	Sulphur oxides
TCA	Tricarboxylic acid
UVP	UV transilluminator
VCSS	Vapor compression steam stripping
WT	Wild type
wt%	Weight percent
Z. mobilis	*Zymomonas mobilis*

Chapter 1. Introduction

1. Introduction

1.1. Context and Motivation

Today's society has been struggling to meet the growing demand for alternative fuels, as a result not only of fossil fuels depletion but also of pronounced climate change due to its overuse. Although biofuels are currently more expensive than fossil fuels - biodiesel and bioethanol are produced through the fermentation of biomass from agriculture crops (1st generation biofuels) and residues (2nd generation biofuels) - their production is exponentially increasing worldwide.

The need to unveil more sustainable energy sources in order to reduce dependence on fossil fuels has led to the development of a 3rd generation of biofuels, which are produced from microalgae. Microalgae possess the advantage of having a high growth rate, carbon dioxide (CO_2) fixation ability and high production capacity of lipids or polysaccharides. Furthermore, they do not compete for land use with food or feed crops and can be produced on non-arable land. Therefore, microalgae can represent an effective alternative to the substitution of conventional fuel produced from edible crops and lignocellulosic biomass from dedicated non-edible crops like switchgrass and agricultural waste.

The EU has supported several R&D projects in order to fund biofuels technologies an alternative to fossil fuels. One of the main research activities of the European Union 7th Framework Program, Direct Ethanol from MicroAlgae (DEMA), is the production of bioethanol as a secretion from cyanobacteria.

Given this framework, the aim of this MSc dissertation, is the direct production of ethanol at a pilot scale through a genetically modified organism (GMO), *Synechocystis* sp. PCC 6803 at an economically viable energy balance. To do so, genetically engineered strains were cultivated in closed 1.1 m^3 PBR with optimized growth conditions for direct synthesis of bioethanol from sunlight.

1.2. Scope

Over the last years, microalgae have become an attractive candidate for developing clean and sustainable biofuel (either biodiesel or bioethanol) production because they combine the conversion of solar energy, carbon dioxide mitigation and biofuel production in a single organism (1). The first approach of ethanol production in a microorganism was made in 1999 by Deng and Coleman (2). They introduced heterologous genes encoding PDC (EC 4.1.1.1) and ADH (EC 1.1.1.1) from bacterium *Zymomonas mobilis (Z. mobilis)*, a natural ethanol producer, in a *Synechococcus* sp. PCC 7942, under the control of cyanobacterial *rbcLS* promoter.

To introduce the ethanol synthesis by GMO as a successful strategy, it is necessary to understand the cellular response of the microorganism to this new pathway and develop approaches to minimize the negative impact ethanol might have on the cells producing it. Also, combining the extraction of high-added-value products (HAVP) (such as pigments, antioxidants, polyunsaturated fatty acids (PUFAs) and vitamins) with the production of biofuels should be considered an option in order to enhance the overall cost-effectiveness of biofuel from microalgae approach (3) (4).

With this in mind, the aim of the current project is to develop a competitive technology that allows the production of bioethanol from microalgae with low-cost scalable PBR. In order to achieve this purpose, the photosynthetic capability of genetically modified *Synechocystis* sp. PCC 6803 is used to convert CO_2 into ethanol by assembling an ethanol-producing pathway as well as addressing other aspects, such as:

- Establishment of a cryopreservation method for *Synechocystis* sp. PCC 6803 strains;
- Ethanol tolerance test with wild-type species;
- Characterization of the genetic transformation by PCR;
- Development of a protocol for disinfection of biomass effluents at pilot scale;
- Development of strategies to cultivate *Synechocystis* sp. PCC 6803.

1.3. Dissertation Outline

This dissertation's structure is divided in five chapters. In this first chapter, the context and the motivation for this study are described, followed by this project scope presentation where the objectives to accomplish in this project were presented.

The second chapter provides a general review of biofuel production by photosynthetic microalgae and cyanobacteria, especially by *Synechocystis* sp. PCC 6803. Also the problems and limitations of the current biofuels generations are outlined, emphasizing the need to consider more sustainable options of biofuel production.

Chapter three describes the results of different experiments in order to characterize the microalgae and define the scale-up strategies.

The fourth chapter comprises the results of pilot scale cultivation of *Synechocystis* sp. PCC 6803 strains (WT and GMO), are compared and discussed.

In the fifth and final chapter, potential scenarios to be studied in the future are suggested based on the final conclusions achieved with this project.

Chapter 2.
Literature Review

2. Literature review

2.1 Microalgae: definition & industrial applications overview

Microalgae are prokaryotic and eukaryotic microorganisms with high photosynthetic efficiency that can be found in a wide range of marine and freshwater environments. Although the photosynthetic mechanism is similar to the one found in higher plants, microalgae are able to convert more efficiently sunlight energy into chemical energy (biomass) due to their simple cellular structure (5).

Cyanobacteria are a common example of prokaryotic microalgae, being also referred to as blue-green algae, which are closely related to Gram-negative bacteria. On the other hand, eukaryotic microalgae are classified into three major categories according to their pigmentation, life cycle and basic cellular structure: green microalgae (*Chlorophyta*), red microalgae (*Rhodophyta*) and diatoms (*Bacillariophyta*) (5).

Microalgae can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure, being able to complete a cell cycle in a few hours (5). This fact, allied to their genetic tractability, turn microalgae into an ideal target organism for biotechnological utilization. Indeed, recent years witnessed the bloom of industrial scale applications of microalgae cultivation for different purposes: food, feed, wastewater treatment, cosmetic and pharmaceutical industry, agriculture and biofuel production (3) (4).

Nowadays, a new frontier is being developed where the overall strategy can be enhanced by metabolic engineering or genetic methods in order to optimize the biosynthetic pathways of microalgae to produce specific metabolites. Therefore, coupled with sequestration of CO_2 emissions from power plants, its usage in wastewater treatment, production of high-value compounds and biofuel production, microalgae industrial cultivation may prove to be environmentally sustainable, cost effective and profitable.

2.2 Microalgae products: commercial applications & market

The potential applications of microalgae ranges from direct use of its biomass (which is in general rich in carbohydrates, proteins or lipids/natural oils) in human nutrition, aquaculture feed or bio-fertilization; to indirect use via the extraction of HAVP (such as pigments, antioxidants, β-carotenes, polysaccharides, triglycerides, PUFAs and vitamins) with economic impact in food, cosmetics and pharmaceutical industries –Table 2.1 (4) (5).

Microalgae HAVP can derive from the primary metabolism, which furnishes intermediates for the synthesis of essential macromolecules; or the secondary metabolism which is of restricted distribution. According to Cardozo et al. (3), “although chemical research on the algae products is very active, biosynthetic studies have been few and mainly concerned with secondary metabolism, which present a high structural diversity, due to modifications and combinations of reactions from the primary metabolic pathways. However, with the emergence of molecular biology tools, metabolic pathways

have been clarified, paving the way for generating novel metabolites in quantity by genetic engineering" (3).

Table 2.1. Biotechnological applications of products from microalgae.
[Adapted from(3),(4)].

Product	Applications	Microalgae producers
Polyunsaturated fatty acids (PUFA)		
Eicosapentaenoic acid (EPA)	Nutritional supplements, aquaculture feed	*Pavlova*, *Nannochloropsis*, *Monodus* & *Phaeodactylum*
Docosahexaenoic acid (DHA)	Infant formula, nutritional supplements, aquaculture feed	*Crypthecodiuimu* & *Schizochytrium*
γ-linoleic acid (GLA)	Nutritional supplements	*Spirulina*
Arachidonic acid (ARA)	Nutritional supplements	*Phorpyridium*
Phycobiliproteins		
Phycocyanin	Natural dye for health food and cosmetics antioxidant	*Spirulina platensis*
Phycoerythrin	Fluorescent agent, tool for biomedical research, diagnostic tool	Red algae (*e.g. Porphyridium cruentrum*)
Carotenoids		
β-Carotene	Food colourant, antioxidant cancer-preventive properties	*Dunaliella salina*
Astaxanthin	Pigmenter for salmon, antixodiant	*Haematococcus pluvialis*
Zeaxanthin	Eye health, antixodiant	*Synechocystis* sp.
Mycosporine-like amino acids (MAA)	UV-screening agent, sunscreen	Aphanizomenon *flos-aquae*
Polysaccharides	Viscosifiers, lubricants and flocculants for industrial applications; antiviral agent	*Porphyridium cruentum*
Phycotoxins		
Okadaic acid, gonyautoxins & yessotoxins	Experimental tools for investigators on neurodegenerative diseases	Dinoflagellates (*e.g. Amphidinium, Prorocentrum* & *Dinophysis*)
Lipids		
Triglycerides	Biofuels	*Chlorella protothecoides* *Botryococcus braunii* *Nannochloropsis* sp.

The added-value of these metabolites (Table 2.2) promotes a continuous investment in microalgae business leading to new technologies and market development – which exists long before the interest for biofuel production. However, the interest in microalgae for biofuels production has brought huge attention on microalgae; investment which allows the current dissemination of research and development carried out in worldwide.

Table 2.2. Market prices and producers for the main microalgae HAVP.
[Adapted from (6)].

Product	Microalgae	Price (USD)	Producer
β-carotene	*Dunaliella*	300–3,000/kg	AquaCarotene (USA) Cognis Nutrition & Health (Australia) Cyanotech (USA) Nikken Sohonsha Corporation (Japan) Tianjin Lantai Biotechnology (China) Parry Pharmaceuticals (India)
Astaxanthin	*Haematococcus*	10,000/kg	AlgaTechnologies (Israel) Bioreal (Hawaii, USA) Cyanotech (Hawaii, USA) Mera Pharmaceuticals (Hawaii, USA) Parry Pharmaceuticals (India)
Whole-cell dietary supplements	*Spirulina* *Chlorella*	50/kg	BlueBiotech International GmbH (Germany) Cyanotech (USA) Earthrise Nutritionals (USA) Phycotransgenics (USA)
Whole-cell aquaculture feed	*Tetraselsmis* *Nannochloropsis* *Isochrysis* *Nitzshia*	70/L	Aquatic Eco-Systems (USA) BlueBiotech International GmbH (Germany) Coastal BioMarine (USA) Reed Mariculture (USA)
PUFA	*Crypthecodinium* *Schizochytrium*	60/g	BlueBiotech International GmbH (Germany) Spectra Stable Isotopes (USA) Martek Biosciences (USA)
Heavy isotope labeled metabolites	N/A	1000–20,000/g	Spectra Stable Isotopes (USA)
Phycoerythtin (fluorescente label)	Red algae Cyanobacteria	15/mg	BlueBiotech International GmbH (Germany) Cyanotech (USA)
Anticancer drugs	N/A	N/A	PharmaMar (Spain)
Pharmaceutical proteins	*Chlamydomonas*	N/A	Rincon Pharmaceuticals (USA)
Biofuels	*Botryococcus* *Chlamydomonas* *Chlorella* *Dunaliella* *Neochloris*	N/A	Cellana (USA) GreenGuel Technologies (USA) LiveFuels, Inc. (USA) PetroAlgae (USA) Sapphire Energy (USA) Solazyme, Inc. (USA) Solix Biofuels (USA)

2.3 Biofuels: background

Worldwide interests in renewable energy sources, such as biofuels, emerges from the depletion of fossil fuels (oil, natural gas and coal) – which contribute to 80 % of worldwide energy supply – and the need to address climate change due to increasing greenhouse gas (GHG) emissions – mainly CO_2 and methane (CH_4). Over past years, it has been an increase in the number of countries which have developed strategies to deliver biofuels as a means to reduce reliance on fossil fuels and GHG lowering emissions (7).

By definition, biofuel is a mixture of volatile, flammable hydrocarbons produced from renewable resources such as plant biomass, vegetable oils and treated municipal and industrial wastes (7) . Bioethanol, biodiesel and biomethane comprise the main used biofuels as alternatives to fossil fuels, which are non-renewable and raise serious environmental and social concerns. Biofuels may have different biological sources and are currently classified in generations: the criterion is based on the type of feedstock, the processing technology or their level of development (8).

2.3.1 First generation biofuels

The 1st generation biofuels, also known as conventional biofuels, are based on biofuels that are made from feedstock such as starch, sugar, animal fats and vegetable oil, using well-known processes or technologies. Some of the most popular first generation biofuels are sugarcane ethanol in Brazil, corn ethanol in Unit States, oilseed rape biodiesel in Germany and palm oil biodiesel in Malaysia (7).

Bioethanol results from the conversion of sugar and starch from sugarcane, of corn and of agriculture residues through a fermentation process. Bioethanol can be used directly in internal combustion engines or as a gasoline additive (Figure 2.1). Biodiesel is produced from vegetable oils or liquid animal fats, through transesterification and esterification reactions. It can be used with no or few modifications in diesel engines or blended with petroleum diesel (Figure 2.2) (9). On the other hand, biogas is a mixture of methane (50-75%) and carbon dioxide (25-50%) produced by anaerobic fermentation of organic matter such as animal manure or municipal waste. This gaseous biofuel can be purified to obtain natural gas, a high quality methane-rich fuel, or it can be compressed and used in vehicle engines (Figure 2.3) (10) (11).

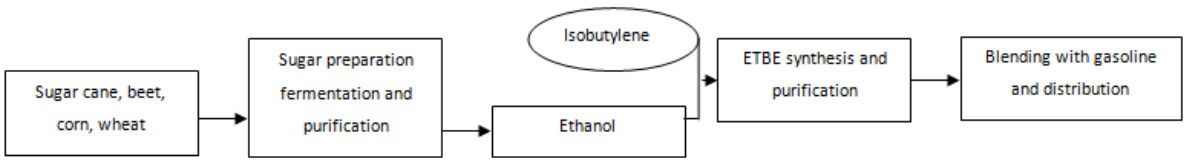


Figure 2.1. Bioethanol manufacturing process and transformation to ETBE. Abbreviation: Ethyl tert-butyl ether (ETBE).
[Adapted from (9)].

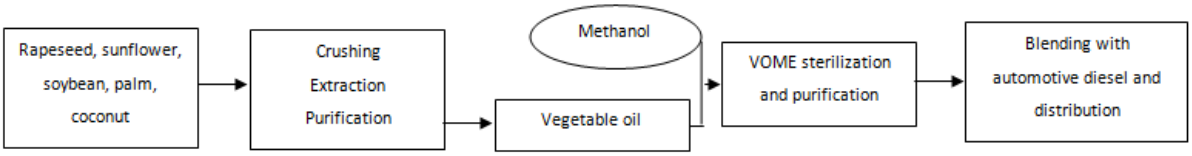


Figure 2.2. Biodiesel manufacturing process Abbreviation: Vegetable oil methyl ester (VOME).
[Adapted from (9)].

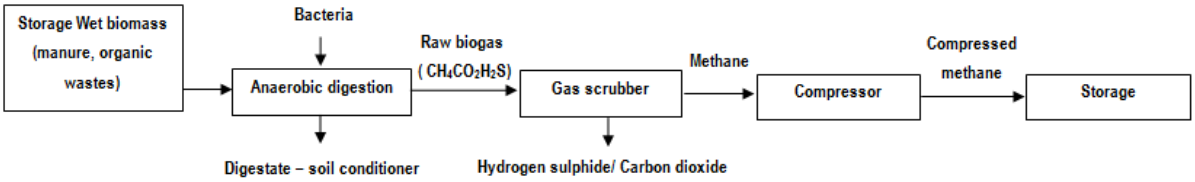


Figure 2.3. Biomethane manufacturing process.
[Adapted from (11)].

During recent years, the demand for food and biofuels has increased so much that the sustainability of the first generation biofuel production has been questioned (Figure 2.4). The major concerns related to first generation biofuels are (11):

- Competition over arable land and water resources used in agriculture, contributing to the raise of food prices and animal feeds;
- Deforestation;
- Negative impact on biodiversity;
- Expensive alternative, without government grants or subsidies;
- Limited effectiveness to reduce global carbon emissions;
- Life-cycle assessment frequently approaching the traditional fossil fuels.

All these factors have contributed to draw attention to the so-called second-generation biofuels, which are based on non-edible biomass and on a promise to avoid the sustainability concerns related to first-generation biofuels production (8) (12).

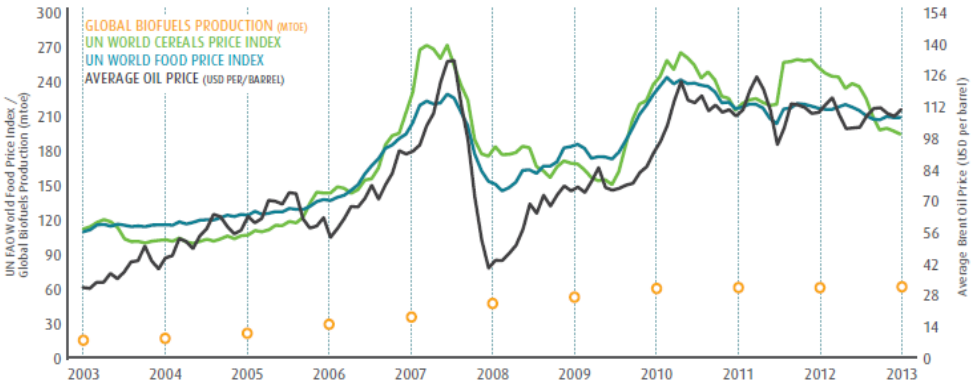


Figure 2.4. Biofuel production and food and oil prices from 2004 to 2013.
[Adapted from (11)]

2.3.2 Second generation biofuels

The 2nd generation consists on biofuels produced from non-food feedstocks *i.e.* from waste, agricultural and forestry residues. The carbon source for this type of biofuels is mainly based in lignocellulosic biomass such as straw, wood and grass, which do not compete with food production.

An example of second generation biofuels is cellulosic ethanol, which is produced from agricultural residues rich in cellulose such as leaves, stems and stalks of plants and trees. Cellulosic ethanol has the same chemical characteristics as any other type of ethanol.

The main drawback of these biofuels production is related to the conversion of the lignocellulosic biomass into fermentable sugars. Lignocellulose is a complex molecule consisting of cellulose, hemicellulose and lignin, thereby requiring an additional step (of thermo-chemical treatment using enzymes) to convert cellulose into simple sugars to be fermented to ethanol. This costly technology requires a conservative approach when considering large scale deployment of such technologies (8) (12).

2.3.3 Third generation biofuels & future trends

Currently, bioethanol is produced mainly from 1st and 2nd generation biofuels through the fermentation of biomass from agriculture crops and residues, respectively. However, the costs associated to the conventional processes (collecting, harvesting, storing and processing the biomass) as well as concerns over agriculture lands, water and cereals used for fuel, along with the high energy input associated to fermentation, led to the development of novel biological approaches where microalgae are used not only as biomass that is converted in biofuel but also as producers – cell factory concept – through the introduction of genes encoding a metabolic pathway – 3rd generation biofuel (11).

Production of biodiesel and bioethanol from microalgal biomass includes the following steps: cultivation of microalgae under optimized growth conditions, followed by harvesting and then lipid extraction for biodiesel production through transesterification or, for bioethanol, conversion of starch in fermentable sugars by adding amylolytic enzymes. These sugars are fermented and distilled into bioethanol using conventional ethanol distillation technology (Figure 2.5).

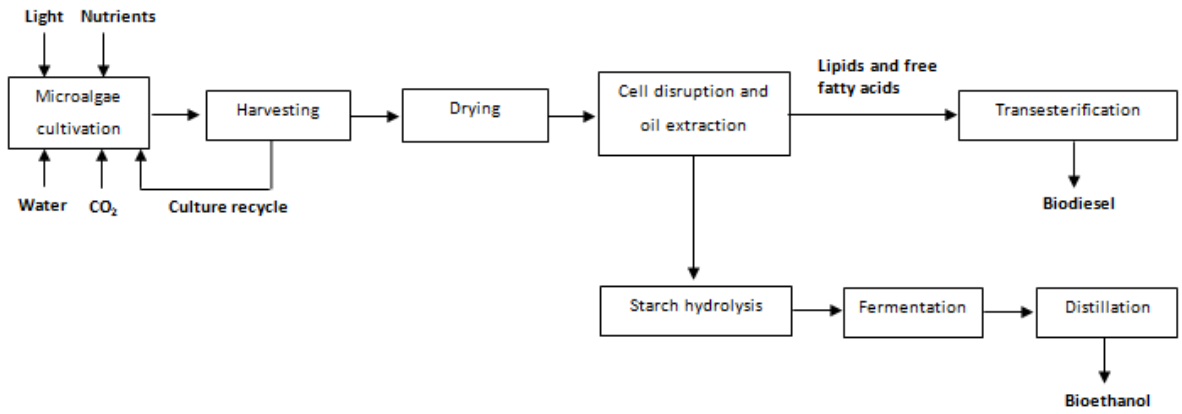


Figure 2.5. Integrated process for biodiesel and bioethanol production from microalgae.
[Adapted from (8)].

Microalgae characteristics favor its industrial cultivation, with the conception of simple and easy processes. These characteristics are (14):

- Higher growth rates and photosynthetic efficiencies in comparison with higher plants (biomass productivity could be ca. 50 times more than that of switchgrass, which is the fastest growing terrestrial plant);
- Reduced nutritional demands;
- Freshwater is not required; wastewater could provide some nutrients (particularly nitrogen and phosphorus) that are essential for microalgae growth and, at the same time, treat the organic effluents;
- Microalgae could be cultivated in brackish water on non-arable land, not competing with agriculture resources;
- High-efficiency CO_2 mitigation (1 kg of dry biomass corresponds to 1.83 kg of CO_2) which improves the air quality;
- Possible incorporation of gas streams with high CO_2 content (6–15 % (v/v) of CO_2);
- Synthesis and accumulation of large quantities of neutral lipids (20-50 % of dry weight (DW) of biomass)
- Extraction of valuable co-products with application in several industrial sectors;
- Herbicides or pesticides are not necessary.

Therefore, biofuel production from microalgal biomass will not compromise production of food, fodder and/or other products derived from crops (15). Some microalgae with potential feedstock for bioethanol production are *Chlorella vulgaris* and marine green algae *Chlorococcum littorale.* The first one is able to accumulate high levels of starch and the second one can produce bioethanol via self-fermentation (450 µmol ethanol g^{-1} at 30 ºC, in dark fermentation) (8).

In spite of the number of advantages, bioethanol production derived from microalgae faces some constraints in order to become an alternative energy source. The major challenges associated are:

- Low biomass concentration reached in the microalgae cultivation due to limitation of light penetration;
- Harvesting and drying biomass is costly due to the small size of microalgae cells and the high water content, respectively;
- Higher capital costs of microalgae cultivation in relation to a conventional agricultural farm;
- Incorporating flue gases containing poisonous compounds such as nitrogen oxides (NO_x) and sulphur oxides (SO_x);
- Lack of data regarding large scale plants because there are only a few commercial plants in operation.

Indeed, investment and production costs – despite the continuous improvements – still remain the greatest challenge towards industrial large scale deployment of biofuel production from microalgae.

Nonetheless, its competitiveness will continue to increase as fossil fuels – crude oil, natural gas… - prices raise and reach the critical point.

In Europe, the current biofuel R&D and production is heavily dependent on legislative framework and subsidies (16), however, it is expected that biofuels market opportunities expand accompanying a large socio-economic impact. Indeed, there is the consensus that biofuels technologies and potential is worth developing as it is considered an alternative to fossil fuels. In the European Commission there is currently a strategy for biofuels with the following vision (17):

> *"*By 2030, the European Union (EU) covers as much as one quarter of its road transport fuel needs by clean and CO_2-efficient biofuels. A substantial part is provided by a competitive European industry. This significantly decreases the EU fossil fuel import dependence. Biofuels are produced using sustainable and innovative technologies; these create opportunities for biomass providers, biofuel producers and the automotive industry*."*

In support of this strategy, the EU enacted Framework Programmes 6 and 7 which included dedicated financing to biofuel research, supporting European competitiveness and fuel sources diversification while reducing GHG. Biofuels production is expected to increase in the next years (18). Also, emerging technologies to sustain the demand and downsizing investment and operational costs as well as carbon footprint are expected to have a major contribution.

2.4 Bioethanol: overview & perspectives

Bioethanol, ethanol obtained from fermentation processes, is the most common biofuel and accounts for nearly 90 % of the biofuel usage worldwide (7). A variety of starchy biomass can be used, such as corn, maize, sugarcane, as well as organic wastes, while yeasts of the species *Saccharomyces cerevisiae* are the main catalyst. The conventional bioethanol production process is based on enzymatic conversion of starchy biomass into sugars and fermentation of 6-carbon sugars with final distillation of ethanol to fuel grade.

The production of ethanol by converting cereals into fuel through fermentation processes is relatively simple at either small or large scales and is well understood. Since the 1970s, the capacity for bioethanol production has continued to increase in several countries and world regions, in particular in Brazil and the USA – which have a long history of government support for their domestic industries. Since 2005, the USA has been the world's largest producer of ethanol, overtaking Brazil. Recent news showed that in 2013 the USA bioethanol industry, whose feedstock is mainly corn and maize, produced and consumed around 50 billion liters, followed by Brazilian sugarcane bioethanol with approximately 23 billion. Europe is the world's third largest producer of bioethanol, equaling 6.7 billion liter in 2014 (Figure 2.6.a). Currently, ethanol production can be found in 20 countries, with France being the Europe´s leading bioethanol producer (Figure 2.6b). According to EU Cereal Balance, the bioethanol produced in Europe, which is mainly from maize and wheat, uses only about 0.7 % of total EU agriculture land and 2 % of Europe's cereals supply (Figure 2.7). Crop diversity allows the producers to have some flexibility and possibility to adjust to market circumstances and changes.

(a) (b)

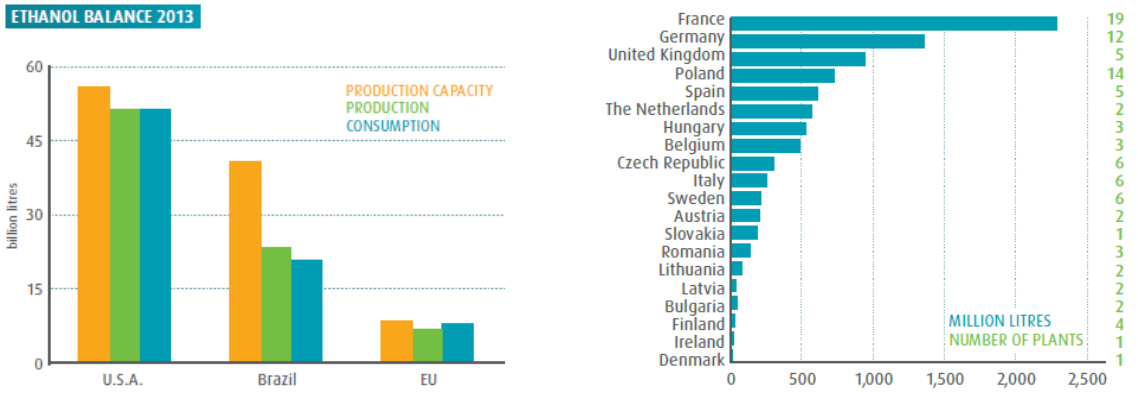


Figure 2.6. (a) Global ethanol balance in the major producing countries in 2013 (billion liters); (b) Production capacity of bioethanol in EU countries in 2014.
[Adapted from (19)].

(a) (b)

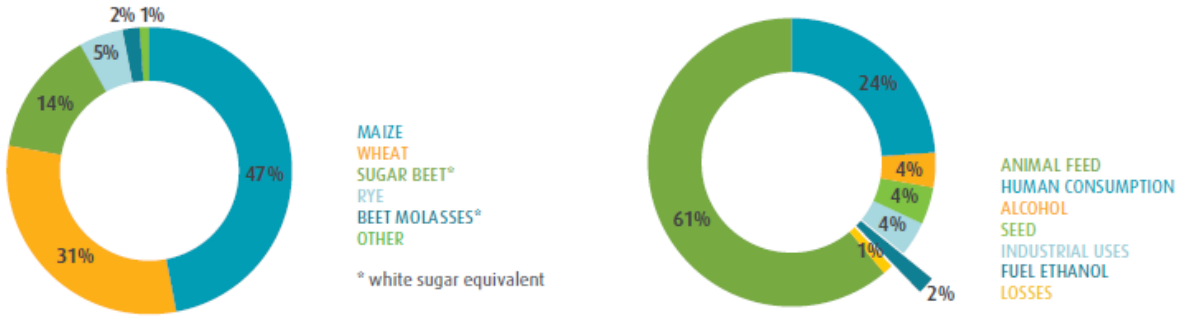


Figure 2.7. (a) Estimated feedstock used in EU bioethanol production; (b) End use of EU cereals supply From EU Cereals Balance, DG AGRI, European Commission.
[Adapted from (19)].

For all the described above, current bioethanol demand will continue to increase; therefore, research carried out seeking new technologies/strategies aiming for cost reduction will be a major development area. The 3rd generation biofuels will aim to introduce a new approach and succeed such challenge.

2.5 Bioethanol as metabolite from microalgae

Despite the interest in producing bioethanol using microalgae as feedstock for fermentation, the commercialization of algae-derived bioethanol is still in its infancy stage. Therefore, several studies have been made in order to convert microalgae into a cell factory, *i.e.* production of biofuel (bioethanol) that is not biomass based but by introduction of genes encoding the proteins required to synthesize the desired products *in vivo* (20).

The genetic and metabolic engineering approach will allow enhancing the production of microalgae biofuel. Some examples of molecular engineering that are currently studied are:

- Increase of photosynthetic efficiency to enable increased biomass yield on light;
- Enhance biomass growth rate;
- Increase oil content in biomass;
- Improve temperature tolerance to reduce the expense of cooling;
- Eliminate the light saturation phenomenon so that growth continues to increase in response to increasing light level;
- Reduce photo-bleaching that actually reduces growth rate at midday light intensities that occur in temperate and tropical zones;
- Reduce susceptibility to photo-oxidation that damages cells (20).

The advantages of metabolically engineered microorganisms in the production of fuels, chemicals, biomaterials, etc. is obtaining the desired product directly without the need to harvest or adding further downstream procedures.

2.5.1 *Synechocystis* sp. PCC 6803 – Cell Factory

Cyanobacteria, also known as blue-green algae, are one of the most promising groups of organisms from which novel and biochemically active natural products can be extracted or produced (Figure 2.8). Among cyanobacteria species, *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis* 6803), a fresh-water, non-filamentous, non-nitrogen fixing microorganism capable of heterotrophic growth, was one of the first strains to be fully characterized in terms of physiology, biochemistry and genetics. The entire genome sequence, which includes four endogenous plasmids (pSYSX, pSYSM, pSYSA, pSYSG), was completely sequenced and over 3000 genes have been annotated (1) (13)(21).

Synechocystis 6803, being a prokaryote, is highly amenable to be genetically engineered, as opposed to eukaryotic microalgae. Furthermore, this species is naturally competent, *i.e.* it is able to uptake foreign DNA and integrate it into the chromosome by homologous recombination.

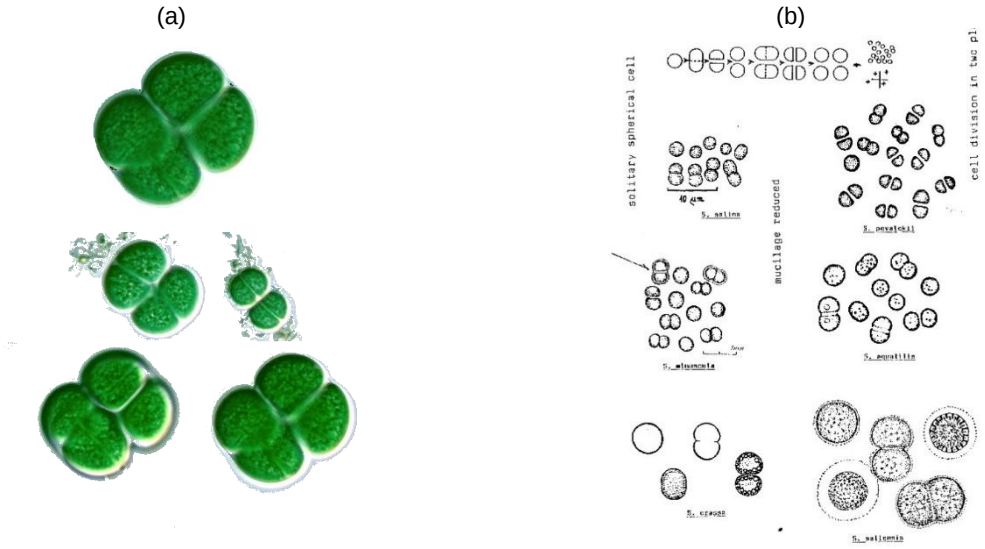


Figure 2.8. (a) *Synechocystis* cell photographs [Adapted from (22)].
(b) Cell division schemes of *Synechocystis* [Adapted from (23)].

Considering their unique characteristics, cyanobacteria have been used as a model organism to produce a wide range of fuels. For instance, ethanol and hydrogen production attempts in three cyanobacteria strains (*Synechocystis* 6803, *Synechococcus* 7002 and *Synechococcus* 7942 - a parental strain) have already been reported as shown in Table 2.3.

Table 2.3. Application of cyanobacterial species as a cell factory. Abbreviations: Dry cell weight (DCW). [Adapted from (21)].

Strains	Chemicals	Productivity	Growth conditions	References
Synechocystis 6803	Ethanol	5.50 g L^{-1}	Photoautotropic; Sparging with 5% CO_2 - air	(24)
	Fatty acids	197 mg L^{-1}	Photoautotropic; Bubbled with 1% CO_2	(25)
	Isoprene	50 µg gDCW $^{-1}$ day^{-1}	Photoautotropic;	(26)
	Alk(a/e)nes	2.3 mg L^{-1} OD_{730}^{-1}	Photoautotropic;	(27)
	Fatty alcohols	761 µg g DCW $^{-1}$	Photoautotropic;	(28)
	Sucrose	35 mg L^{-1} OD_{730}^{-1}	Photoautotropic with 600 mM NaCl	(29)
	Hydrogen	186 mmol mg chl-a^{-1} h^{-1}	Nitrogen-limiting in the dark	(30)
Synechococcus 7002	Hydrogen	14.1 mol day^{-1} 10^{17} $cells^{-1}$	Anaerobic in the dark	(31)
	Sucrose	71 mol 10^{17} $cells^{-1}$	Under hypersaline condition	(32)
Synechococcus 7942	Ethanol	0.23 g L^{-1}	Photoautotrophic	(2)
	Isobutyraldehyde	1.1 g L^{-1}	Photoautotrophic with $NaHCO_3$	(33)
	Isobutanol	0.45 g L^{-1}	Photoautotrophic with $NaHCO_3$	(33)
	Fatty acids	80 mg DCW^{-1}	Photoautotrophic; Bubled with CO_2	(34)
	Hydrogen	2.8 µmol h^{-1}mg Chl-a^{-1}	Anaerobic in the dark	(35)

2.5.1.1 Photosynthesis

Being a photosynthetic prokaryote, *Synechocystis* 6803 is able to convert light energy, water and CO_2 to obtain its own energy source. The production of carbohydrate reserves is also followed by the release of oxygen as a waste product (1). Photosynthesis can be described by Equation 2.1.

$$2H_2O + CO_2 + Light \rightarrow (CH_2O) + H_2O + O_2 \qquad \text{Equation 2.1}$$

The photosynthetic process comprises two different stages: the first stage is light-dependent and the second one is light-independent. The first stage reactions, also called light reactions, take place in specific pigment-protein complexes called phycobilisomes of the thylakoids membranes. In contrast to the chloroplasts of eukaryotic cells, thylakoids of cyanobacteria are invaginations of the cytoplasmic membrane and mostly localized at the periphery of the cells, forming concentric circles parallel to the

cytoplasmic membrane. Inside the thylakoid membrane space, also called lumen, light energy is converted into energy storage molecules, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), and oxygen. On the other hand, the dark stage takes place in the cytoplasmic region, where CO_2 and energy molecules from the light phase are converted into carbon storage compounds (36).

2.5.1.1.1 Light Phase

During the light phase, a linear electron-transport system is used for the conversion of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to its reduced form (NADPH). During this phase, photosystems I and II (PSI and PSII) use the light energy to excite the magnetic element of chlorophyll (magnesium), creating a lack of electrons. To replace them, molecular water is oxidized through several enzymatic reactions leading to the formation of two atoms of hydrogen positively charged, molecular oxygen, which is released to the atmosphere, and two electrons (Equation 2.2) (36).

$$2H_2O + Light \rightarrow 2H^+ + 2e^- + \frac{1}{2} O_2 \qquad \text{Equation 2.2}$$

Through several intermediates such as coenzymes and cofactors, the additional electrons are transferred to plastoquinone (PQ) forming the charged form PQH_2. PQH_2 transfers its electrons to PSI via a cytochrome b6f complex (ctb_6f) and the resulting protons are released into the lumen along with the protons generated by the oxidized water, creating a proton gradient across the thylakoid membrane. This proton gradient is then used for ATP synthesis. In ctb_6f, electrons pass through several intermediates to plastocyanin (PC), the electron donor to PSI (36).

Regarding PSI, electrons from the pigment are excited by light energy. In order to compensate the lack of electrons, PC donates electrons from PSII and complex reactions take place leading to the formation of Fd (Ferredoxin). Then, ferredoxin-$NADP^+$ oxidoreductase (FNR) transfers the electrons to $NADP^+$ leading to the final production of NADPH. In Figure 2.9 a simplistic scheme of the light phase reaction is presented (36).

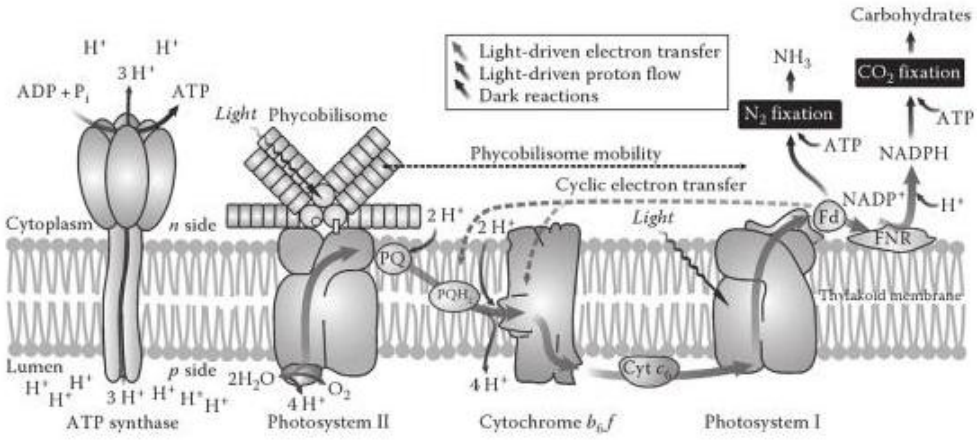


Figure 2.9. Diagram of the complexes involved in the light phase reactions.
[Adapted from (36)].

2.5.1.1.2 Dark Phase

Although the dark phase does not require light, it is most likely to occur during the day, when ATP and NAPH are highly produced. This pathway is also known as Calvin cycle (or Calvin-Benson cycle) and comprises three main steps: CO_2 fixation, CO_2 reduction and regeneration of ribulose bisphosphate (RuBP). The first step consists on CO_2 fixation by ribulose-1-5-bisphosphate carboxylase (RuBisCo) – which is localized in specific sub-cellular structures called carboxysomes - leading to the formation of 3-phosphoglycerate (3-PGA) (3C). In presence of enzyme triose phosphate dehydrogenase, 3-PGA is reduced into 1,3-biphosphoglycerate (PGAL) through the conversion of ATP into ADP. PGAL is then reduced to glyceraldehyde 3-phosphate (G3P) (3C) in presence of NADH derived from the light-dependent stage - CO_2 reduction (37).

G3P is the main product of Calvin Cycle so it can be converted into other intermediates or connected to other metabolic pathways. For instance, fructose-6-phosphate (F6P), which came from G3P, is converted in glucose-6-phosphate (G6P) and glucose-phosphate, which is the substrate for the pentose phosphate pathway, the synthesis of cellulose (a major component of cell wall) and glycogen (the major form of carbohydrate reserve). On the other hand, pyruvate is directed to the partial tricarboxylic acid (TCA) cycle for the synthesis of amino acids or it can be used as a substrate for ethanol synthesis, through the glycolytic pathway (Figure 2.10) (37).

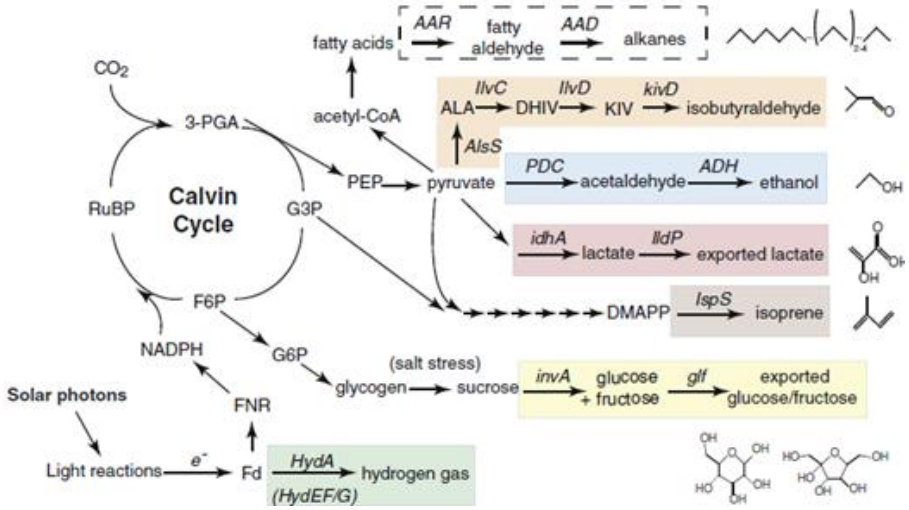


Figure 2.10. Diagram of the dark phase reactions.
[Adapted from (38)]

To complete the Calvin cycle, RuBP must be regenerated. Therefore, RuBP is converted into RuBisCo in the presence of ATP and phosphopentose kinase. Overall, 5 out of 6 carbons from the 2 G3P molecules are used for this purpose, leaving only 1 net carbon produced to play with for each turn. Thus, it takes three turns of the Cycle to have a net gain of one PGAL which can be used to form glucose ($C_6H_{12}O_6$), with a net cost of nine molecules of ATP and six of NADPH (37).

2.5.2 From R&D to industrial scale cultivation

Complementing the several works described in Table 2.3, extensive R&D work is being carried out through public financed projects, such as the European Commission Seventh Framework Programme (FP7) projects – DEMA (39) or DirectFuel (40). Also, large scale deployments of the concept have been attempted, in particular, for the case of Algenol (see section 2.5.3.1). However, despite the innumerous patents submitted by Algenol, the economic viability of well-established ethanol production via *Synechocystis* 6803 ethanol producing strain still remains a challenge.

2.5.2.1 DEMA

The DEMA project aims for the production of ethanol at pilot scale from a genetically modified organism (GMO) *Synechocystis* 6803 containing bacterial genes in order to create a novel pathway for ethanol production through cyanobacteria. The idea consists in developing synthetic cell factories through the genetic modification of *Synechocystis* 6803. The produced GMO should then be able to assemble an ethanol-producing pathway, where organic metabolites can be metabolically converted into ethanol by-products using photosynthesis (39) .

This project comprises two important stages. The first consists in the genetically modification of blue-green algae. *Synechocystis* 6803 cell factory carries the ethanol genes from an obligate anaerobe *Z. mobilis* under *psbA2* promoter encoding PDC (EC 4.1.1.1) and ADH (EC 1.1.1.1). Ethanol production is directly coupled with the pyruvate metabolism (Figure 2.11) and the ATP synthase, as well as with some reactions from central carbon metabolism (glycolysis and pentose phosphate pathway). In addition, it is partially coupled to the Calvin cycle under autotrophic conditions competing for carbon source.

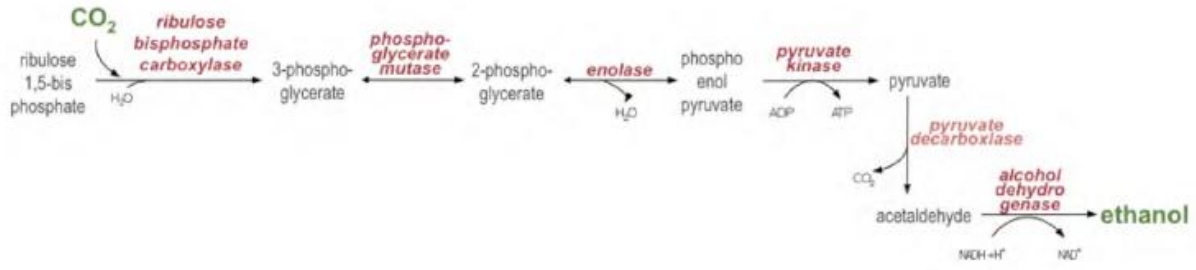


Figure 2.11. Metabolic pathway of ethanol production.

[Adapted from (41)].

The second stage aims for the scale-up of the engineered ethanol producing cyanobacteria, in order to obtain the production of ethanol at a pilot scale at concentration levels greater than 1-2 % (v/v). *Synechocystis* 6803 would be cultivated in 1.1 m^3 conventional tubular PBRs with optimized sun exposure, in liquid medium supplemented with CO_2, nitrogen, phosphate and micronutrients for direct synthesis of bioethanol from sunlight. The bioethanol produced would be continuously extracted from the culture media using a membrane technology process (39).

The DEMA project, which began in December 2012 and is set to conclusion in May 2017, comprises nine partners, from six European countries, spread from academia to industrial areas (Table 2.4). The project, with a total budget of 5 M€ from the EU under the energy strand of the FP7 platform, is coordinated by the University of Limerick (Ireland) (39).

Table 2.4. Partners of FP7 project DEMA.
[Adapted from (39)].

Participant	Participant legal name	Country	Organisation type
1 UL	University of Limerick	Ireland	University
2 A4F	A4F - AlgaFuel, S.A.	Portugal	*SME tech. provider*
3 UVA	University of Amsterdam. Swammerdam Institute	Netherlands	University
4 UCAM	The Chancellor, Masters and Scholars of the University of Cambridge	United Kingdom	University
5 UTU	Imperial College of London	United Kingdom	University
6 PTN	Photanol BV	Netherlands	*SME tech. provider*
7 LNEG	LNEG National Institute for Energy	Portugal	State Lab
8 TERE	Tereos	France	Multi-national IND
9 PTECH	Pervatech BV	Netherlands	*SME tech. provider*

2.5.3 Industrial Ethanol Production from GMO *Synechocystis* 6803

Currently there are three commercial PBRs reported to grow *Synechocystis* 6803: Algenol's flexible plastic film 1st generation PBR (42); Joule's flat plate reactor and Photanol's air-lift design (Figure 2.12) (43) (44).

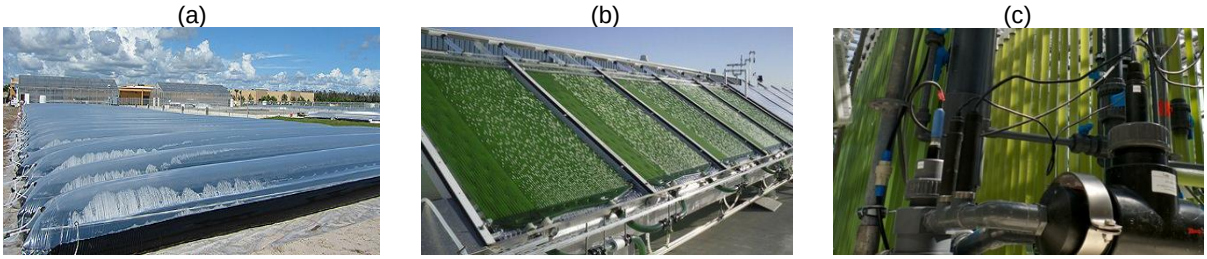


Figure 2.12. (a) Algenol, (b) Joule and (c) Photanol photobioreactors design.
[Adapted from (42), (43), (44)].

2.5.3.1 Algenol – case study

Algenol is an advanced industrial biotechnology company founded in 2006 with headquarters in Naples, Florida. Algenol is commercializing patented algae technology for production of ethanol (DIRECT TO ETHANOL®) and other biofuels such as gasoline, diesel or jet fuel. The concept of DIRECT TO ETHANOL® is the autotrophic culture of a blue-green strain capable of producing ethanol directly through the over expression of genes encoding for fermentation pathway enzymes, ADH and PDC. Strains of engineered microalgae are cultivated in seawater in 4.5 m^3, 15 m x1.5 m wide flexible film PBRs made of polyethylene film with special additives and coatings to optimize performance (Figure 2.13). The ethanol-freshwater condensate is collected and concentrated into fuel grade

ethanol through downstream separation technologies - vapor compression steam stripping (VCSS) and conventional distillation system (45) (46).

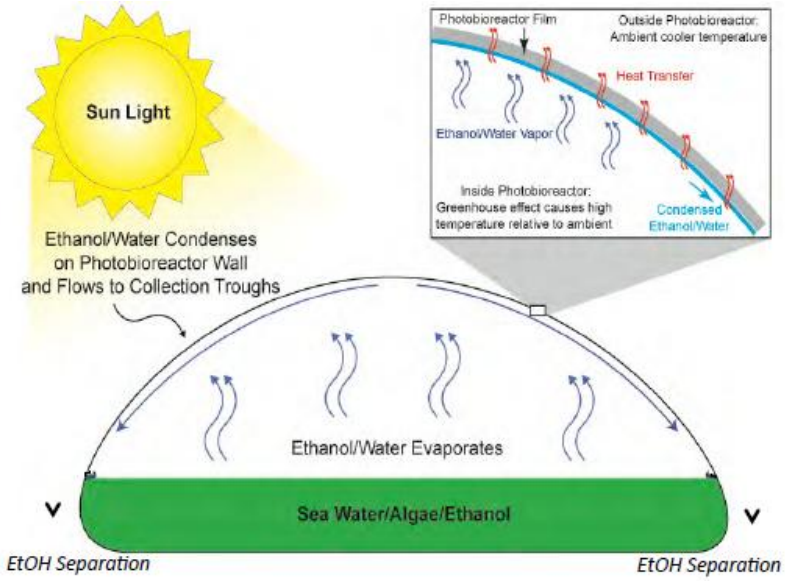


Figure 2.13. Algenol 1st generation photobioreactors.
[Adapted from (46)].

The horizontally oriented 20 cm deep 1st generation PBR required low capital cost and had magnetically coupled mixing systems. However, these reactors had the following limitations: (i) low gas exchange efficiency; (ii) poor light distribution; (iii) difficult culture temperature management; (iv) low productivity. Therefore, and concluding one year of R&D, it was unveiled Algenol's 2nd generation reactor (see Figure 2.14). Opposed to the 1st generation reactors, the new design is vertically oriented which result in better light use, better gas exchange dynamics, less temperature stress and ultimately in higher productivities (46).

However, it is believed that the major drawback of Algenol's reactor design is the concept of scale-up, which consists in replicating the PBR and not in increasing PBR volume.

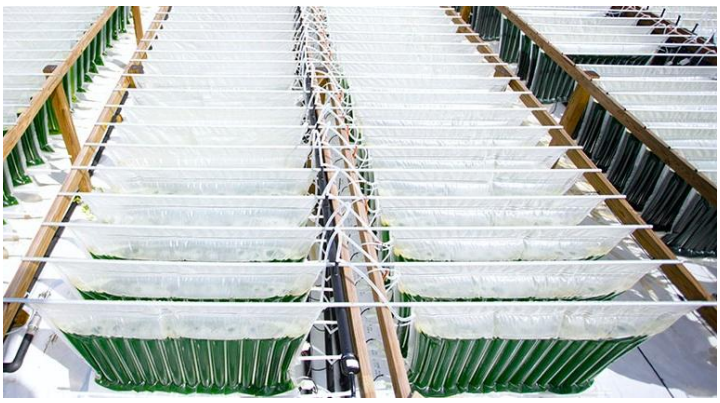


Figure 2.14. Algenol's 2nd generation PBR design.
[Adapted from (46)].

Algenol's technology allows the production of ethanol, gasoline, jet and diesel fuel for around $1.30 per gallon at production levels of 8000 total gallons of liquid fuel per acre per year.

2.5.4 Summary of *Synechocystis 6803* patents

Table 2.5 shows a compilation of the most relevant patens regarding the cyanobacterium *Synechocystis* 6803 and the production of ethanol. Most patents applicants are: (i) Algenol Biofuels with approximately 13 patents about their Direct to Ethanol® product technology; (ii) Joule Unltd Technologies with more than 40 patents on production of ethanol and other organic compounds from genetically engineered cyanobacteria.

The patents are divided in 4 main scientific fields: (i) genetic alterations to cyanobacteria; (ii) PBRs design; (iii) ethanol recovery and recycling methods and (iv) biomass valorization.

Table 2.5. Compilation of most relevant patents about *Synechocystis* 6803.

Field	Applicant	Patent	Title
Genetically modified *Synechocystis* sp. PCC 6803	ALGENOL BIOFUELS	US2010297736	Genetically modified photoautotrophic ethanol producing host cells, Method for producing the host cells, constructs for the transformation of the host cells, Method for testing a photoautotrophic strain for a desired growth property and Method of producing ethanol using the host cells.
	UNIV HAWAII	US2009155871	Methods and compositions for ethanol producing cyanobacteria.
	PHOTANOL	US2013071895	L-lactate production in cyanobacteria.
	UNIV AMSTERDAM	WO2009078712	Light.driven CO_2 reduction to organic compounds to serve as fuels or as industrial half products by an autotroph containing a fermentative gene cassette.
	JOULE UNLTD TECHN.	US2013252300	Ethanol production in microorganisms.
PBR Design	ALGENOL BIOFUELS	US2013109085	Closed photobioreactor system for continued daily *in situ* production of ethanol from genetically enhanced photosynthetic organisms with means for separation and removal of ethanol.
	ALGENOL BIOFUELS	WO2012/116335	Magnetically coupled system for mixing.
	JOULE UNLTD TECHN.	US2013244320	Photobioreactors, solar energy gathering systems, and thermal control methods.
	JOULE BIOTECHN. INC	WO2010068288	Solar biofactory, photobioreactors, passive thermal regulation systems and methods for producing products.
	JOULE UNLTD TECHN.	WO2013006681	Bioreactors circulation apparatus, system and method.
Recovery and purification: ethanol, CO_2	ALGENOL BIOFUELS	WO2011/103277	Vapor compressions steam stripping.
	ALGENOL BIOFUELS	US2012137727	Membrane-augmented distillation with compression and condensation to separate solvents from water.
	ALGENOL BIOFUELS	US2012171752	Water/Carbonate stripping for CO_2 capture adsorbed regeneration and CO_2 delivery to photoautotrophs.
	JOULE UNLTD TECHN.	US2012298498	Fractional condensation processes, apparatuses and systems.
Biomass valorisation	COGNIS IP MAN GMBH	US2007190595	Process for obtaining zeaxanthin from algae.

2.6 Conclusion

Currently, bioethanol is produced mainly from 1st and 2nd generation biofuels through the fermentation of biomass from agriculture crops and residues, respectively. However, the costs associated to the conventional processes (collecting, harvesting, storing and processing of the biomass) as well as concerns over agriculture lands, water and cereals used for fuel, along with the high energy input associated to fermentation, led to the development of novel biological approaches where microalgae are used not only as biomass that is converted in biofuel but also as producers – cell factory concept – through the introduction of genes encoding a metabolic pathway – 3rd generation biofuel.

This novel approach involves a metabolically engineered microorganism for the production of biofuel which would combine the conversion of sunlight, mitigation of greenhouse gas CO2 and biofuel production in a single organism. The first approach of ethanol production was made in 1999, by Deng and Coleman. They integrate *pdc* e *adh* genes from ethanol producing *Z. mobilis* into the *Synechococcus* sp. PCC 7942 chromossome, under the control of cyanobacterial *rbcLS* promoter.

The EU has supported several R&D projects in order to fund biofuels technologies as an alternative to fossil fuels. One of the main research activities of the FP7 is the DEMA project which concept is to culture, autotrophically, a cyanobacterial strain that is capable of directly producing ethanol at an economically viable energy balance. The cyanobacterium selected is one of the first microorganisms well-characterized – *Synechocystis* 6803. The entire genome is completely sequenced and annotated which has allowed for the establishment of techniques for precise genome manipulation.

Despite the interest of developing a renewable energy, the ethanol production titers and rates from *Synechocystis* 6803 cannot compete with that from the biomass fermentation or agricultural crops and residues. Therefore, more research is needed in order for it to become a reality.

Chapter 3. Microalgae Characterization & Scale-up Strategies

3. Microalgae Characterization & Scale-up Strategies

This research project was carried out within the R&D group of A4F – Algafuel, S.A. (A4F), located at the Lisbon Laboratory which is a GMO Class II certified laboratory. The main objective of the project was the production of ethanol at a pilot scale through a microalgae organism and comprised two important stages. The first stage consisted in the characterization and preservation of a modified strain of ethanol producing cyanobacteria. The second stage focused on the scale-up of the engineered ethanol producing cyanobacteria, in order to obtain the production of ethanol at pilot scale.

3.1 Strains, nutritive media, pre-culturing & preservation

Strains

The *Synechocystis* 6803 WT and GMO strains used in this study were provided by the University of Limerick, Ireland, a partner in the FP7 funded DEMA project. The photosynthetic cyanobacteria were genetically altered to produce ethanol by insertion of DNA constructs encoding for *adh* and *pdc* enzymes in the photosystem II (PsbA) gene. The genetic material encoding the *adh* and *pdc* enzyme was isolated from *Z. mobilis.* Also, a kanamycin resistance gene was inserted to act as selective pressure on transformed cells. The strains were maintained isolated in the microalgae culture collection of A4F, in both liquid and solid media and also cryopreserved, which will be addressed below.

Nutritive media

Based on years of experience A4F has developed a nutritive media for laboratory cultivation of several microalgae strains. This nutritive media is composed by the macro-nutrients – nitrogen, phosphorous and iron; micro-nutrients – *e.g.* magnesium, zinc, etc., and is supplemented with vitamins and further sterilized by filtration (Ø = 0.2 µm, Whatman, USA).

Pre-culturing

For scale-up purposes, the *Synechocystis* 6803 cultures were platted under sterile conditions (class II GMO biosafety cabinet) on nutritive medium solidified with 1.5 wt% Agar (HIMEDIA) and incubated at 25 ºC under cold white fluorescent light (170 µmol $m^{-2}s^{-1}$). The working cultures were transferred from the isolated microalgae agar plates into liquid mineral media. The strains were cultivated autotrophically at pH=8.0 in 250 mL Erlenmeyer flasks containing a total culture volume of 100 mL; with 20 mg L^{-1} of kanamycin – for selective pressure. The Erlenmeyer flasks were closed with cotton plugs and aluminum foil and placed in an orbital shaker (Orbital incubator Agitor 200, Aralab) at 170 rpm and 25 ºC, under continuous light intensity of 30 µmol $m^{-2}s^{-1}$.

Preservation

For long term conservation and genetic stability, cryopreservation was successfully tested in the studied cyanobacteria. The culture cells, which grown in the agar plates, were transferred in aliquots into lab medium with 8 % (v/v) dimethyl sulfoxide (DMSO) and stored under -70 º C.

Procedure

Cyanobacteria were cultured in Erlenmeyer flasks (250 mL) with agitation, at 25 °C and under continuous illumination of 30 µmol $m^{-2}s^{-1}$. After two days, pre-cultures were re-inoculated onto 100 mL of fresh medium supplemented with kanamycin 20 mg L^{-1} and sodium bicarbonate 2 mM with an $OD_{730\ nm}$ of ≈0.02. In a biosafety cabinet, cultures were collected and centrifuged at 3500 rpm for 30 minutes. The supernatant was discarded and fresh medium was added. Then, the dense culture was dispensed into cryo-vials containing cold sterile filtered DMSO at a final concentration of 8 % (v/v). To ensure adequate homogenization, cryo-vials were rapidly homogenized and transferred to a storage box with ice. Finally, the filled cryo-vials were placed in a freezer for at least one week at -70 ºC. During this procedure and in order to avoid cryo-protectant cell damage, the vials were protected from the light.

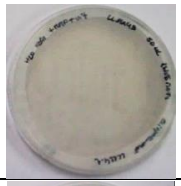
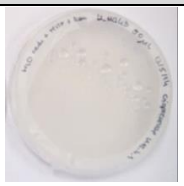
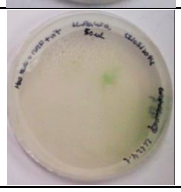
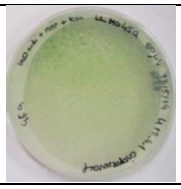
Viability assay

A quick thawing was performed by immersing and agitating the cryo-tubes in a pre-heated water bath (40 ºC) until every ice crystal has melted. The cyanobacteria cultures were immediately centrifuged for a few seconds and the supernatant discarded. Then, new culture medium has added and cultivation could be carried out.

A viability assay was done by spreading 50 µL of 7 days cryo-preserved cyanobacteria cultures on agar plates with nutritive media using glass beads (Ø = 2 mm). The cultures were also tested in liquid medium by transferring 100 µL into a sterilized tube containing 10 mL of fresh medium with 10 mM of sodium bicarbonate. The liquid cultures were first maintained under low light conditions and then under normal growth conditions – to account for the physiological stress of cryopreservation. Two sets of samples were prepared: (1) using only nutritive media; (2) nutritive media in presence of 20 mg L^{-1} kanamycin antibiotic.

After seven days in cultivation, samples were analyzed, in both liquid and solid cultures, with and without the antibiotic (Table 3.1). The existence of growth and the microscopic examination (data not shown) proved the cells viability. As expected, when cultivated with kanamycin – a broad spectrum antibiotic - only the genetically modified strain was able to sustain growth. This results from the insertion of the kanamycin resistance gene.

Table 3.1. Plating and liquid culture results after 7 days of incubation using two culture media. WT and GMO *Synechocystis* 6803 were cryopreserved during 7 days at -70 ºC using DMSO as cryo-protectant.

Strain	Cultivation media			
	Solid		Liquid	
	without kanamycin	with kanamycin	without kanamycin	with kanamycin
WT				
GMO				

3.2 Tolerance to ethanol

It is described that *Synechocystis* 6803 cell membrane permeability to ethanol is high and therefore continuous secretion of ethanol to the media is expected; however the overall process technology requires a concentration of 10 g L^{-1} of ethanol in the media before proceeding to extraction. Thus, the first important factor to address is the cyanobacteria tolerance to ethanol and also, the effect of the ethanol presence on the contaminants proliferation.

3.2.1 Ethanol tolerance test with WT strain

This experiment aimed to test different operating conditions, in terms of photoperiod and daily aeration period, in WT cultures growing in 1 L bubble column reactors in the presence of increasing ethanol concentrations. This ethanol tolerance test allows the evaluation of contaminant proliferation and the impact of ethanol toxicity in the cyanobacteria (loss of productivity, pigment leaching or agglomeration). With this experiment, it was also possible to reproduce conditions of outdoor production (photoperiod), while analyzing the effects of stopping nocturnal aeration.

Experimental conditions

The test was carried out at 25 ºC in four 1000 mL glass bubble columns (designated by AL7, AL8, AL9 and AL10) containing 700 mL of culture, during 7 days. Turbulence was provided by bubbling air enriched with CO_2 through the gas distributor. Table 3.2 presents the growing conditions using during the test.

Table 3.2. Experimental conditions of the test.

System	[EtOH]	Volume	Daily renewal	CO_2 source	Air flow	Light intensity	Temp. / Pressure	Photoperiod
AL7 (control)	0 g L^{-1}	700 mL	15 %	Air + 0.5 % CO_2	Day and night: 5 mL/min	170 µmol/m^2/s	25 ºC / Ambient pressure	10:14 h
AL8	~ 25 g L^{-1}							
AL9 (control)	0 g L^{-1}				Day: 5 mL/min Night: no air flow			
AL10	~25 g L^{-1}							

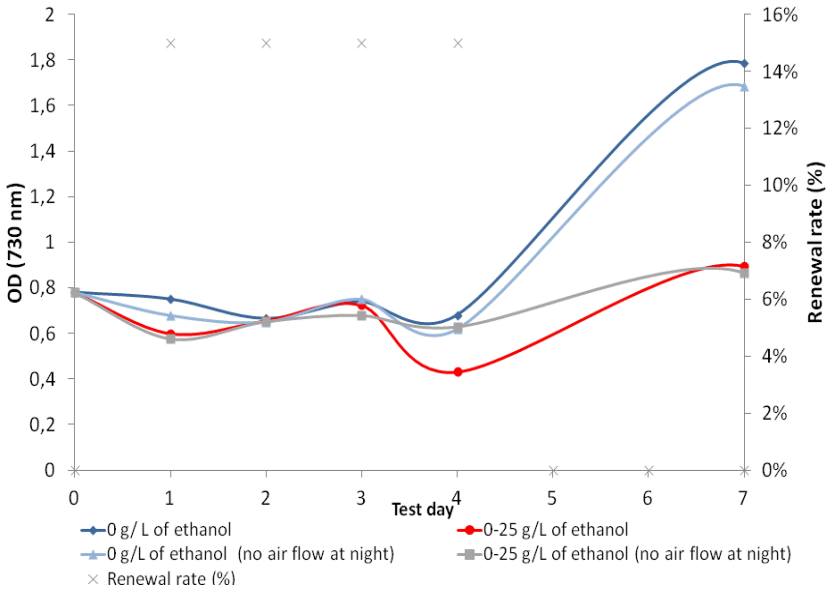
The ethanol concentration was progressively added at a rate of 2 g $L^{-1}day^{-1}$ up until 25 g L^{-1}, being then measured twice a day. The photoperiod was set up manually by covering the bubble columns with aluminum foil at 6 pm and removing it on the following day at 10 am. During the same period, the aeration was switched off for the respective ALs. On weekends, all reactors remained uncovered and aerated.

The objective of the test was to cultivate WT *Synechocystis* 6803 with photoperiod and daily renewals, containing 25 g L^{-1} of ethanol in the medium. Ethanol concentration was measured daily and fresh ethanol was added to compensate for the quantities entrained due to aeration.

Results

Regarding cell growth and, consequently, productivity (Figure 3.1), there were no significant differences between bubble columns with and without aeration during the first 4 days. The abrupt increase of OD from day 4 is related to the stop of daily renewals, strong aeration and changes in light availability (24h).

(a)



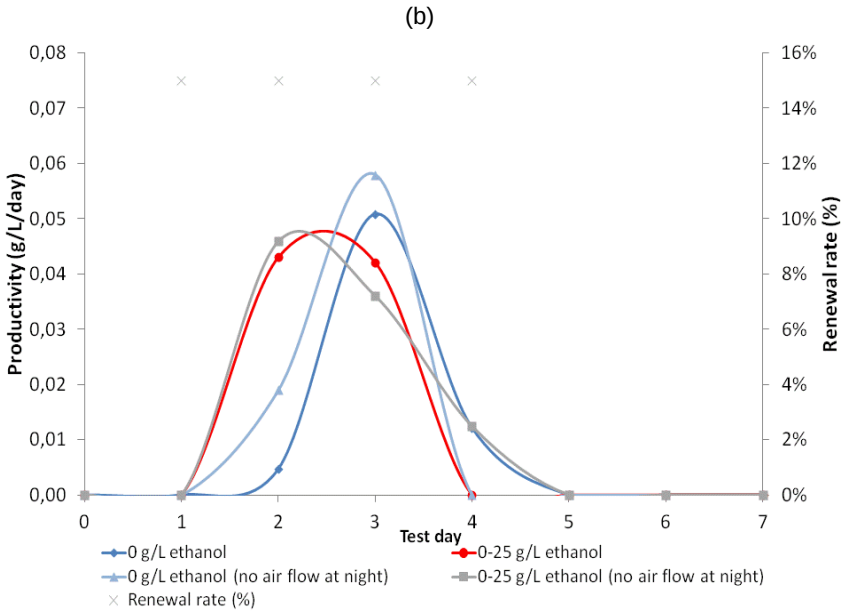


Figure 3.1. (a) Optical density (at 730 nm) evolution; (b) Daily productivity of cell cultures, expressed in g L^{-1} dia^{-1}. OD was measured through UV-Vis Genesys Spectrophotometer (Annex 7.2.)

The average productivity of the different cell cultures were similar (Figure 3.1.b) (Equation 7.3). These values showed that ethanol toxicity in the cyanobacteria does not have major impact in productivity, when cultures are submitted to daily renewals and photoperiod. However, the lower number of renewals and the short duration of the test may also influence this conclusion. Table 3.3 resumes the influence of aeration and ethanol in the average productivity.

Table 3.3: Average productivity of WT *Synechocystis* 6803 between the different conditions tested.

Average Productivity (g L^{-1} day^{-1})		Aeration	
		With	Without
Ethanol	With	0.028 ± 0.025	0.031 ± 0.017
	Without	0.023 ± 0.025	0.026 ± 0.030

The productivity values determined above are corroborated with the nitrate consumption calculations (Equation 7.5). As it is shown on Figure 3.2, the nitrate (NO_3^-) consumption of cultures with ethanol was 1.6 mM day^{-1} during the renewals and then, increased to about 1.8 mM day^{-1} when cultures are submitted to stronger aeration and 24 h of light whereas for those without ethanol, the nitrate consumption was around 0.1 mM day^{-1} until day 3 and then, when renewals stopped, increased to about 1.2 mM day^{-1}. This increase in consumption is due to the absence of renewals and increase in illumination period to 24 h which are better conditions to cellular growth and results in higher cellular concentrations (Figure 3.1.a). The higher values of nitrate consumption by cultures with ethanol may be due to the presence of bacteria which reduce the NO_3^- in the medium. Ethyl alcohol is a carbon source for bacteria that consumes nitrate, therefore, alcohol additions result in bacterial growth andproliferation. During this process, bacteria assimilate the nitrate leading to nutrient depletion in the culture medium. In the cultures without ethanol, there was an increase in consumption related to the increase in cell concentration.

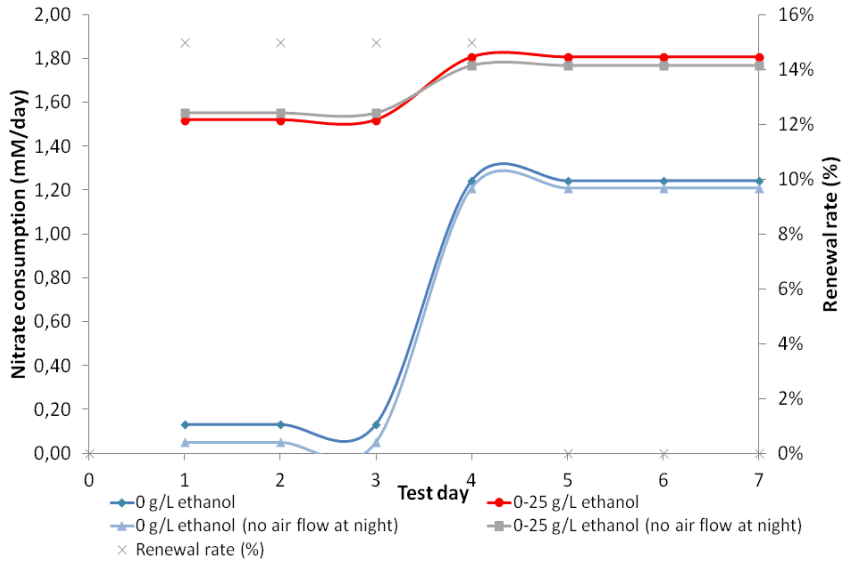


Figure 3.2. Daily nitrate consumption and renewal rate over the test duration. Nitrate set-point was 6mM and was measured through UV-Vis Genesys Spectrophotometer (see Annex 7.2.)

Despite achieving cellular growth, sustained by daily productivity and nitrate consumption, microscopic observation showed that, at the beginning of the test (Figure 3.3), there was a visible aggregation of cells due to poor aeration – 5 mL min^{-1} – during daytime. By the end of the test (day 7), the agglomeration had significantly increased, in particular, in cultures with no aeration during the night and in presence of ethanol. Table 3.4 resumes the influence of aeration and ethanol to the occurrence of bacterial contamination.

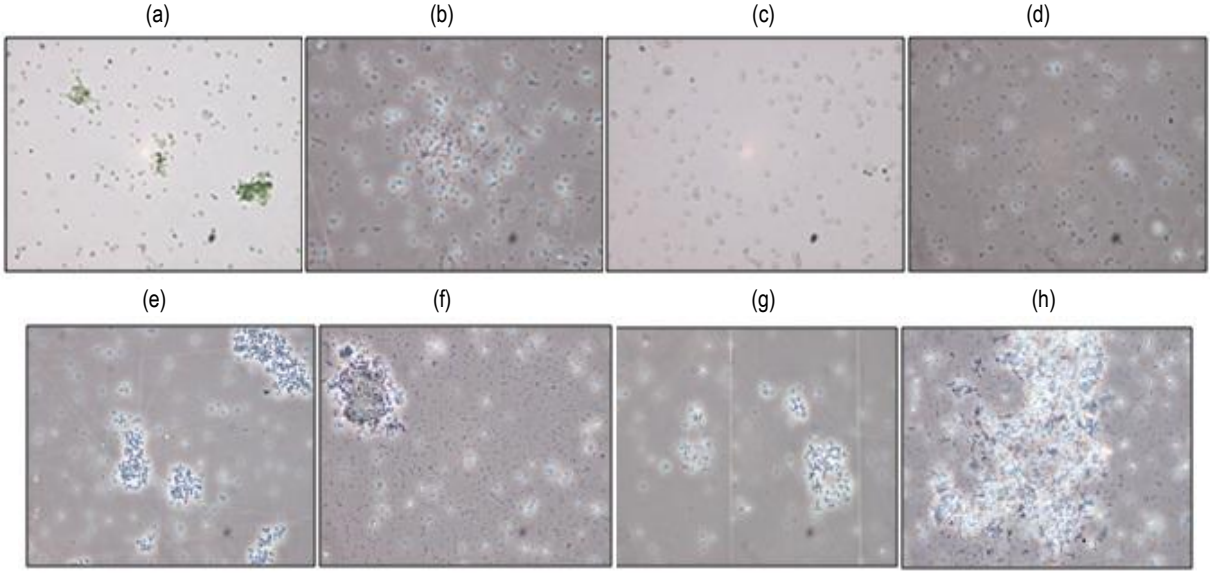


Figure 3.3. Photographic record of cultures at day 2 and day 7 respectively:
(a),(e) AL7 – 0 g L^{-1} of ethanol;
(b),(f) AL8 – 0-25 g L^{-1} of ethanol;
(c),(g) AL9 – 0 g L^{-1} of ethanol (no air flow at night);
(d),(h) AL10 – 0-25 g L^{-1} of ethanol (no air flow at night).
Photographs (a) and (c) are bright field images; all other are phase contrast. Magnification: 80x.
***Synechocystis* 608 is typically a slightly oblong spheriod, approximately 1.5 microns in diameter.**

According to the results obtained, the progressive increase of ethanol concentration led to intense proliferation of bacteria on the culture, while the presence or absence of aeration appear to be unrelated to the occurrence of bacterial contamination. This indicates that the WT *Synechocystis* 6803 cell culture is much more sensitive to bacterial proliferation when ethanol is added, however, no relation was found between bacterial contamination and aeration conditions.

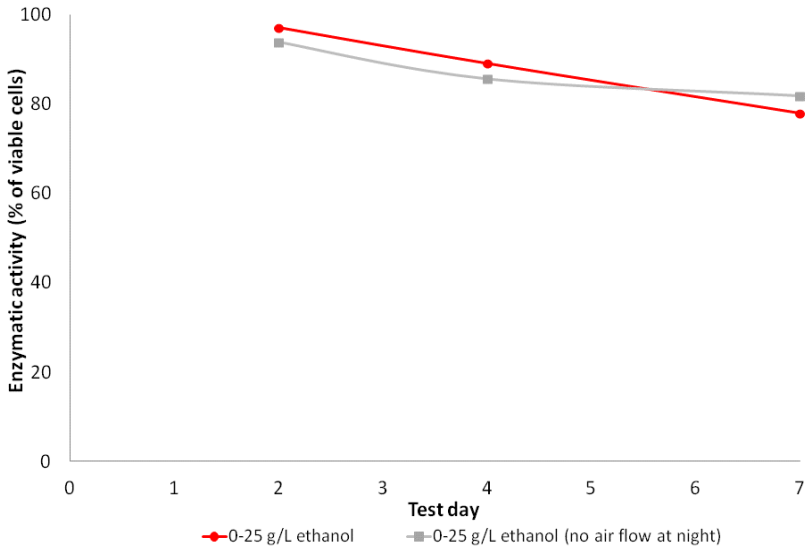
In addition, a more yellowish pigmentation of ethanol-treated cells was observed when compared to the reference blue-green color, which might imply changes in the amount of chlorophyll and other pigments.

Table 3.4. Bacterial contamination quantitative evolution between the different conditions tested.

Bacterial contamination		**Aeration**	
		With	**Without**
Ethanol	**With**	Day 2: + Day 7: ++	Day 2: + Day 7: ++
	Without	Day 2: - Day 7: -	Day 2: - Day 7: -

Flow cytometry measurements were performed in order to assess the culture "well-being" in presence of ethanol and its implications to the cell enzymatic activity and membrane integrity. According to Figure 3.4, there was a slight decrease in enzymatic activity (below 80 %) for WT *Synechocystis* 6803 cells under ethanol treatment, which may reveal that the cell culture was not perfectly healthy during the test. This may be associated with the increase of ethanol concentration in the medium, producing a harmful effect on the cyanobacteria. However, since there are no results for cultures without ethanol, no comparisons can be made. Nevertheless, results from previous tests with healthy cultures showed a percentage of 92 % viable cells growing without ethanol after 40 days (47). On the other hand, the presence of ethanol appeared not to affect the membrane integrity. In addition, the absence of aeration during the night had no negative effect on the culture health.

(a)



(b)

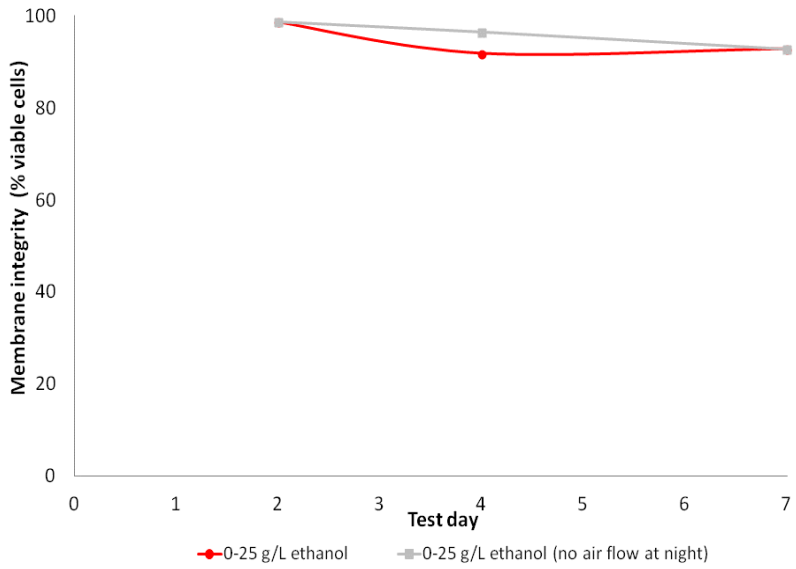


Figure 3.4. Flow cytometry results during the test: (a) enzymatic activity; (b) membrane integrity. Flow cytometer analysis was performed according to the protocol from P. Hyka et al (48).

3.3 Characterization of genetically modified strains: PCR

Sequence determination through PCR was used in order to assess the genetic stability of the culture and confirm that during different stages of scale-up the gene for *adh* and *pdc* expression was still inserted in the cyanobacteria.

DNA Preparation for culture collection sample analysis

A loopful (2 to 3 isolated colonies from agar plates) of *Synecochystis* 6803 cells was suspended in 30 µL PCR water in 1.5 mL reaction tubes with a few zirconium beads (Ø = 0.8 mm). The tubes were on vortex (Vortex Genie 2, Scientific Industries, Inc., Bohemia, NY) for 5 minutes, and then frozen for 10 minutes at -20 ºC and thawed once for DNA extraction.

DNA Preparation for scale-up and production culture analysis

In order to extract the cell DNA, the mineral medium was removed from cell suspension by centrifugation of 10 µL of the culture in 30 µL of PCR water for 3 minutes at 14 000 rpm (Centrifuge Minispin). The cell pellet was rinsed with 30 µL of PCR water and centrifuged again under the same conditions. After discarding the supernatant, the cell pellet was mixed with an equal volume of zirconium beads (Ø = 0.8 µm) and a total volume of 10 µL of PCR water and cells was on vortex for 5 min. After disruption, cells were frozen for 10 minutes at -20 ºC and thawed once for DNA extraction.

DNA Amplification

DNA amplification of the targeted sequence was carried out in accordance with the protocol provided with 2x Dream Taq Green Master Mix (Termo Scientific, US). Each amplification reaction required 2

µL of DNA, 1 µL of each primer (10 µM) and 12.5 µL of Master Mix up to a final volume of 25 µL. Primers were provided by the University of Limerick and their sequences are listed in Table 3.5.

Table 3.5. Primer sequences used for PCR – f, forward; r, reverse.

Primer	Sequence
DEMA 9 f	5'GTCAGTTCCAATCTGAACATCGA
DEMA 13 r	5'CAATTTGCAGATTATTCAGTTGGCAT

PCR amplification was run on a thermocycler (Termo Scientific, US) with the following program: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 49.9 °C for 30 seconds and extension at 72 °C for 6 minutes with a single final extension at 72 °C for 15 min. After the run finished, the thermocycler cooled the reaction to 4 °C, so that the PCR products can be stored at -20 °C.

Gel Electrophoresis

The PCR products were analyzed by agarose gel electrophoresis, running 3 µL of PCR product on 0.8 % (w/v) agarose gel in 1x TBE (with GreenSafe Premium as nucleic acid stain) for 1 h at a constant voltage of 100 V. A 1 kb molecular weight ladder (O'GeneRuler, Thermo Scientific, US) was also loaded onto the gel as a marker. PCR products were then visualized through a UV light and photographed using a UV transilluminator (UVP). Since primers bind to the recombination region at either end of the *pdc-adh* kanamycin, the desired product size is about 1 kb and 5 kb, for the WT and GMO strain, respectively.

Application

In this study, PCR technique was used to confirm the presence of the ethanol producing gene insertion in the GMO. The construct was amplified by PCR with specific primers DEMA 9 f and DEMA 13 r. No template control reactions (NTC) and positive control (with WT) were included to ensure that the sample was not contaminated and that there were no errors during the execution of the protocol, respectively. For each batch of GMO microalgae that arrived at the laboratory the PCR sequencing was performed.

An example is presented in Figure 3.5. The agarose gel showed one DNA band in each pore, indicating successful amplification of the targeted sequence. No WT bands were detected in pore 3 – PCR product of the GMO strain; meaning that the ethanol construct insertion remained on the GMO strain. Also, the bands observed in pore (2) and (3) had the expected size: 1 kb for WT and approximately 5 kb for GMO, which correspond to the ethanol insertion.

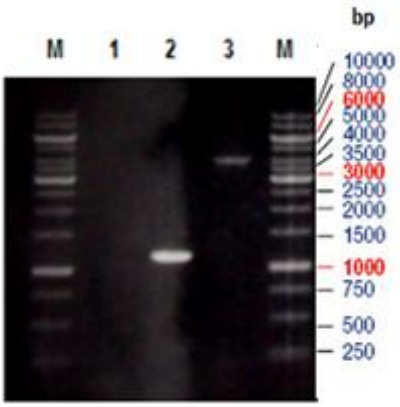


Figure 3.5. Agarose gel electrophoresis of PCR-amplified DNA fragments of GMO *Synechocytis* 6803. M: PCR markers, 1: NTC, 2: Positive control- Synechocytis WT (plates), 3: PCR product of GMO Synechocytis 6803 (plates).

3.4 Scale-up strategy

Typical *Synechocystis* 6803 WT and GMO strain were cultivated in round glass flasks (Figure 3.6). The apparatus consists in a round glass flask of 5000 mL with a gas distributor at the bottom. The gas, air enriched with 0.5 % in CO_2, is sprinkled in the form of bubbles into the liquid phase. At the air system inlet and outlet air filters (Ø = 0.2 µm, Midisart 2000 Sartorius, Germany) were placed to maintain sterile conditions and prevent cross contamination, respectively.

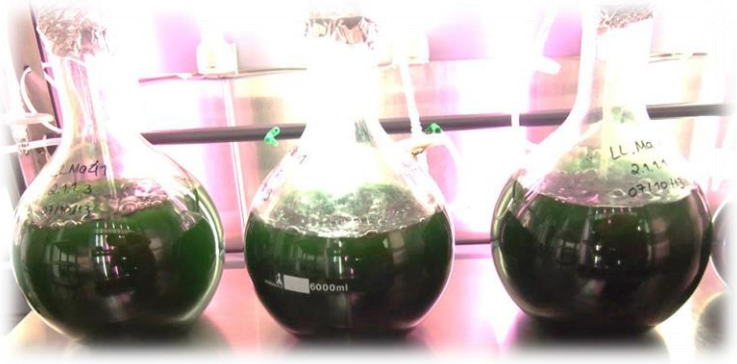


Figure 3.6. Typical 5 L round glass flasks cultivating the WT strain.

The 1.1 m^3 pilot scale cultivation of a GMO ethanol producing *Synechocystis* 6803 requires the production of at least 100 L of inoculum in the laboratory while ensuring the genetic stability of the strain; production of ethanol and contaminant control. To this regard, the use of kanamycin is mandatory for imposing the selective pressure to the transformed cyanobacteria. However, at pilot scale, antibiotic treatment is not cost effective and therefore it is important to analyse the genetic stability of GMO strains when cultivated in absence of kanamycin. For this purpose, specific growth rates of the GMO strains with and without kanamycin were established and compared to the WT. Moreover, from all the carbon fixed by GMO strains only a portion is used to produce biomass since the ethanol production is a competing process. The quantification of such parameter is extremely relevant to understand current process limitations.

3.4.1 Specific growth rate determination

The specific growth rate (μ) of the cyanobacteria cultures was determined by the natural logarithm (Ln) of the cell concentration versus time. The slope of the linear regression of the early exponential growth phase corresponds to the μ (Equation 3.1), where N_1 and N_2 are the concentration of cells at the beginning (t_1) and at the end (t_2) of the exponential growth phase, respectively. Generation time (G) was calculated according Equation 3.2.

$$\mu = \frac{Ln\ (N_2 - N_1)}{t_2 - t_1} \qquad \text{Equation 3.1}$$

$$G = \frac{Ln\ 2}{\mu} \qquad \text{Equation 3.2}$$

Experimental conditions

The engineered strain of *Synechocystis* 6803 (with and without the presence of kanamycin) and the WT strain were cultivated autotrophically in triplets for 11 days in 250 mL Erlenmeyer flasks containing 100 mL of culture media supplemented with sodium bicarbonate (carbon source) at a pH of 8.0 The Erlenmeyers were closed with cotton plugs and aluminum foil and placed on an orbital incubator (Orbital incubator Agitor 200, Aralab) at 170 rpm and 25 ºC under continuous light intensity of 30 μmol $m^{-2}s^{-1}$ (Table 3.6). Culture parameters: pH and temperature were monitored twice a week. Cell growth was monitored over the cultivation period by sampling with 24 h intervals and measuring the optical density at 730 nm using a UV-Vis spectrophotometer (± 0.005 A) (Genesys 10S UV-Vis, Thermo Scientific, US) (see Annex 7.2).

Table 3.6. Experimental conditions, specific growth rate and generation time. The specific growth rates were determined by linear regression of the early exponential growth phase data. Each value represents the average of three cultures. Correlation coefficient (R^2) = 1.000 (WT); 0.9857 (GMO without kanamycin); 0.9121 (GMO with Kanamycin).

Species	Volume	CO_2 source	Light intensity	Temp. / Pressure	pH	Agitation	Specific growth rate (h^{-1})	Doubling time (h)
WT	100 mL	100 mM sodium bicarbonate	30 μmol/m^2/s	25ºC/ Ambiental pressure	~8	170 rpm	0.03	23.62
GMO without kanamycin							0.02	32.75
GMO with kanamycin							0.01	66.25

Results

The specific growth rate (Table 3.6) was calculated during the exponential growth phase, which occurred during the initial four days of the test (Figure 3.7). Moreover, the end of the exponential growth phase was determined with a half log plot.

Among the different cultures tested, the maximum specific growth rate of *Synechocystis* 6803 was observed for the WT strain. GMO strain culture cultivated without kanamycin had a lower specific growth rate and GMO strain maintained with kanamycin – selective pressure – revealed the lowest value among the different strains. These results are in agreement with expectations since, as said, the carbon fixed by the GMO strains is not entirely converted into biomass but also in ethanol.

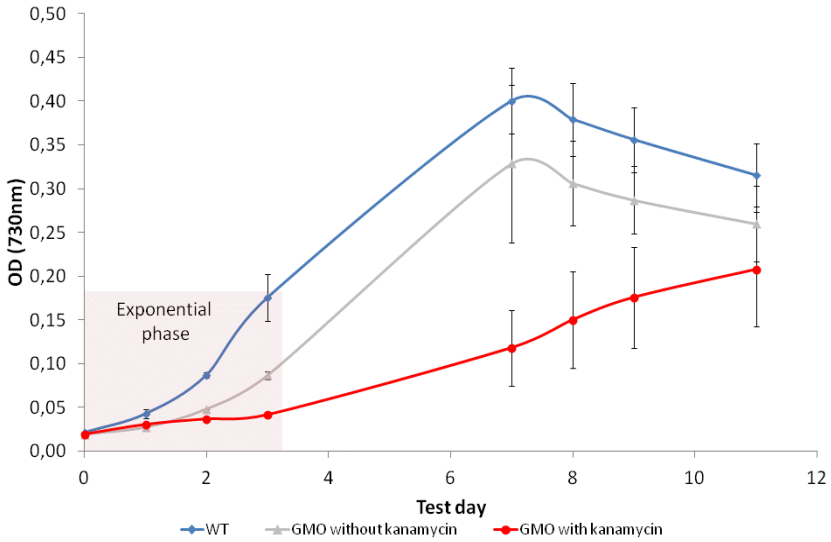


Figure 3.7. Optical density evolution with time.
The error bars denote standard deviations. Each data point represents the mean of biological triplicates.

3.4.2 Use of fixed carbon by the microalgae: biomass vs ethanol

The quantification of the fixed carbon that is converted into biomass or into the production of ethanol (Figure 3.8.a) may be a parameter to determine the genetic stability of the culture. Indeed, if changes in the ratio between the carbon to biomass and carbon to ethanol exist, then it is most likely that the cell is genetically compromise and some mutations or gene suppressions may occur. In the scope of this work, a simple test was developed to quantify the ratio between these two carbon outputs (Figure 3.8.b).

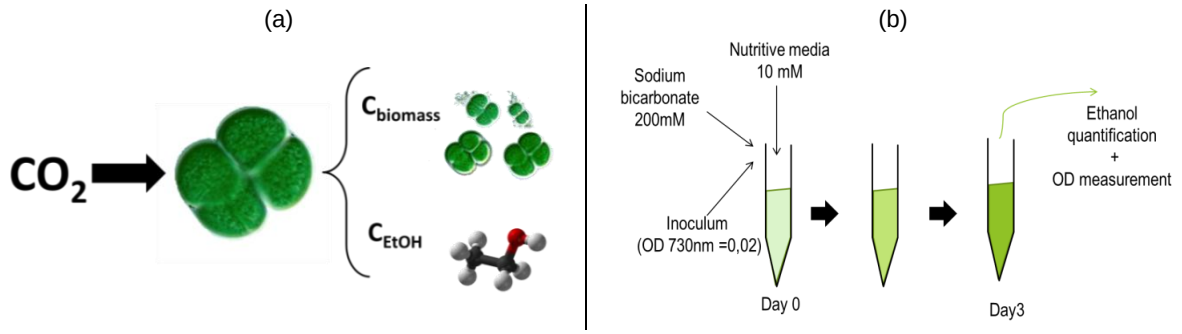


Figure 3.8. (a) Distribution of carbon outputs from *Synechocystis* 6803 GMO strain; (b) Procedure of the reference test for biomass and ethanol carbon output quantification.

The amount of carbon (in mmol) converted into biomass ($C_{Biomass}$) is calculated based on DW concentration (g L^{-1}) and the wt% of carbon present in the biomass. The DW concentration was determined through a correlation between the DW and OD (see Annex 7.2 - Equation 7.1).

The wt% of carbon in the biomass was found to be 51.4 % and was determined using the molecular formula of the biomass:

$$C\ H_{1.62}O_{0.4}N_{0.22}P_{0.01}\ (47) \qquad \text{Equation 3.3}$$

The amount of carbon (in mmol) used to synthesized ethanol was determined with the ethanol concentration (g L^{-1}) measurement (see Annex 7.3) and then converted to mmol of C (C_{EtOH}) considering the volume used, through the following equation:

$$C_{EtOH}(mmol) = \frac{[EtOH](g\ L^{-1}) \times \%\ C\ \times Volume\ (L)}{MM_{EtOH}} \qquad \text{Equation 3.4}$$

Performing the balance between the initial and final measurements leads to the distribution of carbon outputs.

Experimental conditions

For this test, the inoculum origin was 5 L glass flasks cultures growing with air enriched in CO_2 in a controlled temperature of 25 ºC. Optical density and ethanol concentration were measured at the beginning of the test. Afterwards, in presence of sodium bicarbonate (carbon source) and nutritive media, the cultures were cultivated in an orbital incubator for 3 days. Then, ethanol and OD measurements were made and the subsequent mathematical treatment described above.

Results

Table 3.7 summarizes the reference test performed with the WT and GMO strains with and without kanamycin – selective pressure. As it was expected, and it is observed, GMO cultures perform better – towards ethanol production – in the presence of kanamycin. This should be adopted in for the scale-up strategy.

Table 3.7. Results from the reference test.

Strain	Inoculum reference	C -> Ethanol (%)	C -> Biomass (%)
WT	Flask (5L) scale-up	-	100
GMO without kanamycin	Flask (1L) scale-up	15	85
		16	84
GMO with kanamycin	Flask (1L) scale-up	30	70
		33	67

3.5 Disinfection and sterilization methods

Since A4F is certified to cultivate GMO cultures, it is crucial to guaranty that no organism/DNA is released to the environment. Thereby, the disinfection method was validated for *Synechocystis* 6803 GMO. At lab-scale, autoclave sterilization is the practicable standard method; however, at pilot scale

such methods are not viable. Therefore, lab-scale tuning of disinfection methodologies will provide the conditions that guarantee the culture production and handling safety. Controlled conditions such as exposure time and disinfectant concentration were tested in order to avoid the spread of GMO microorganisms in the environment.

Experimental conditions and settings

GMO *Synechocystis* 6803 culture was treated with 0.005 % (50 ppm) of sodium hypochlorite in order to inactivate/destroy the microalgae before wastewater disposal and different exposure times were tested. The experimental conditions are presented in Table 3.8.

Table 3.8. List of experimental conditions to determine the effectiveness of the disinfection method.

Volume culture (mL)	200
DW (g L^{-1}) (3 conditions)	1 ; 0.45 and 0.23
Disinfectant concentration	Sodium hypochlorite 50 ppm
Microorganism	*Synechocystis* 6803
Sampling times (h)	After 1 ; 2 and 7

Results

The culture pigmentation decreases with the time of exposure, as it is possible to observe in Figure 3.9. Moreover, for the lowest biomass concentration tested, it was possible to observe the complete destruction of microalgae after 2 h treatment with a concentration of 50 ppm (Figure 3.9.c). The absence of cell growth was assessed by plating aliquots of treated culture in agar plates and liquid growth media. Tests demonstrated that after 7 days of incubation none GMO cyanobacteria had grown (data not shown).

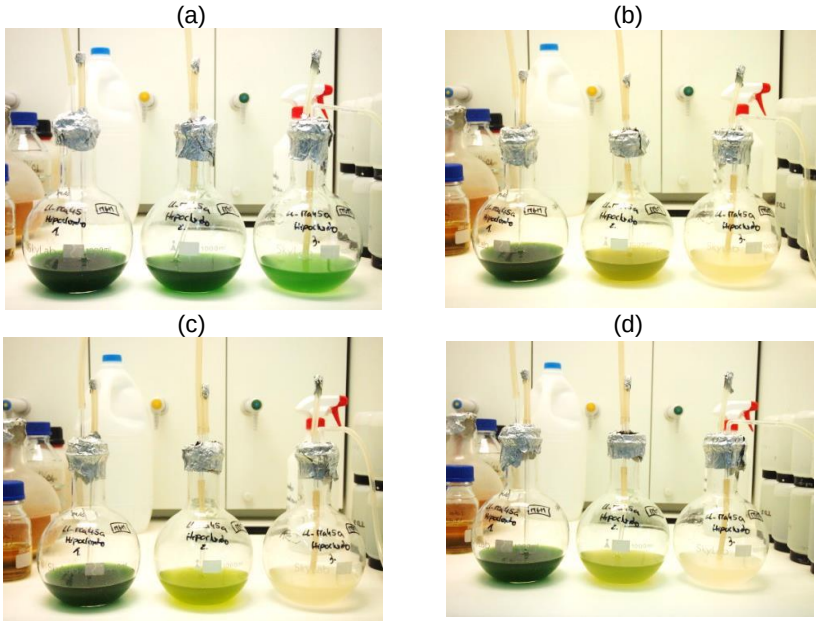


Figure 3.9. Evolution of different biomass concentration of *Synechocystis* GMO at time: (a) zero; (b) 1 h; (c) 2 h and (d) 7 h after disinfectant addition. From right to left there is an increase in concentration.

3.6 Conclusion

This chapter provides all the information regarding the strains used and cultivation conditions as well as cryopreservation for genetic stability maintenance. The methods were adapted from previous knowledge at A4F and cryopreservation was successfully achieved. In addition, characterization of the genetic transformation by PCR was optimized during this thesis using primers supplied by DEMA project partner University of Limerick. This represents a tool for controlling the pilot scale cultures for the genetic transformation. Moreover, in these cultures no kanamycin is used and therefore cells are not subjected to selective pressure and mutations can occur.

Scale-up strategies were addressed by analysing the effect of selective pressure; the distribution of fixed carbon between biomass and ethanol production; and WT S*ynechocystis* 6803 tolerance to ethanol concentrations of 25 g/L. It was found that the scale-up strategy should consist in (1) using kanamycin during scale-up – from cryopreservation stocks; (2) well aerate culture flasks and (3) with cultivation conditions for faster growth, *e.g.* 24 h illumination. This would favour the maintenance of the genetic integrity and ethanol production until the PBR.

Finally, the disinfection method procedure (hypochlorite concentration and disinfection duration) were evaluated, resulting in a protocol for disinfection of biomass effluents in pilot scale before being discarded to the municipal wastewater collector: addition and homogenization of 50 ppm of hypochlorite for 2 hours.

Chapter 4.
Pilot Scale Cultivation of *Synechocystis* 6803

4. Pilot scale cultivation of *Synechocystis* sp. PCC 6803

The A4F experimental pilot plant, located at Lisbon, is a compliant GMO pilot unit with a total capacity of 4.3 m^3. This existing infrastructure accommodates a 1.1 m^3 PBR – a DEMA project dedicated production line (Figure 4.1).

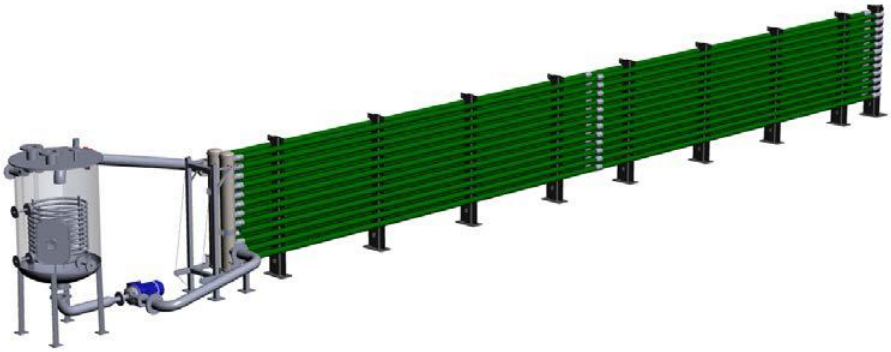


Figure 4.1. PBR set-up.

4.1 Pilot plant: systems description and main features

A4F Experimental Unit in Lisbon (Portugal)

The A4F Experimental Unit was constructed and commissioned in 2012. In order to define the pilot plant technical details a process engineering study – including a material and energy balance – was performed to define the characteristics of the plant (e.g. GMO compliant). Such information became the basis for engineering design, which defines both the detailed plant layout and the mechanical characteristics of the process units and ancillary facilities. All of these studies resulted in design drawings, which were elaborated to produce fabrication drawings (construction isometrics). The further elaboration of the characteristics was captured by "as built" drawings. The A4F Experimental Unit in Lisbon (Portugal) has the following characteristics:

- 1000 m^2 of implantation area;
- GMO compliant;
- 2 PBR of 1.1 m^3 with acrylic (PMMA) tubes and 1 PBR with glass tubes;
- 3 independent production lines;
- refrigeration of the tubular photobioreactors with a compression chiller;
- production of saline solution;
- culture concentration through centrifuge technology and dried through spray drying;
- culture medium treatment and recirculation;
- storage of the final biomass in a refrigeration chamber at -20 ºC.

Location

The A4F Lisbon's Experimental Unit is located within the campus of a national R&D institute for energy and geology shared with many technological SMEs, situated northwest of Lisbon city center. The unit is installed in an open area with few to none obstacles on the Sun path allowing maximum solar radiation use by the microalgae (Table 4.1).

Table 4.1. General information regarding A4F experimental unit.

Implantation: Campus do Lumiar Estrada do Paço do Lumiar 1649-038 Lisbon Portugal	
Location	Lat. 38° 46.2239' N, Long. 9° 10.6322' W
Average radiation	4.8 kWh/m^2/day
Obstacles on sun path	Shadow effect during winter at early day hours from trees located south of the unit.

4.2 Pilot scale PBR

The PBR is installed in a greenhouse facility which was equipped with temperature, radiation and humidity control. The PBR, with the main features detailed in Table 4.2, was set to the cultivation conditions summarized in Table 4.3.

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Table 4.2. PBR features summary.

Pump Type	Centrifugal pump - ITT Lowara 500/30/P
Power	3 kW
Frequency	41 Hz
PBR total volume	1300 L
Tank culture volume	350 L
Tubes material	Glass
Tank material	Stainless Steel 316

Table 4.3. Cultivation conditions summary.

Species	*Synechocystis* sp. PCC 6803
Volume	1.15 m^3
Inoculum	18 x 6L balloons from scale-up cultures
Salinity	0 g/L
Renewals	20 % (WT); 20 -30% (GMO)
Temperature and Pressure	Atmospheric (inside greenhouse)
Light	Natural
Culture Medium	Industrial medium (1 M)
Nitrate concentration set-point	8 mM
pH set point	8.3
CO_2 source	Pure CO_2

4.3 Pilot scale PBR - Results and Discussion

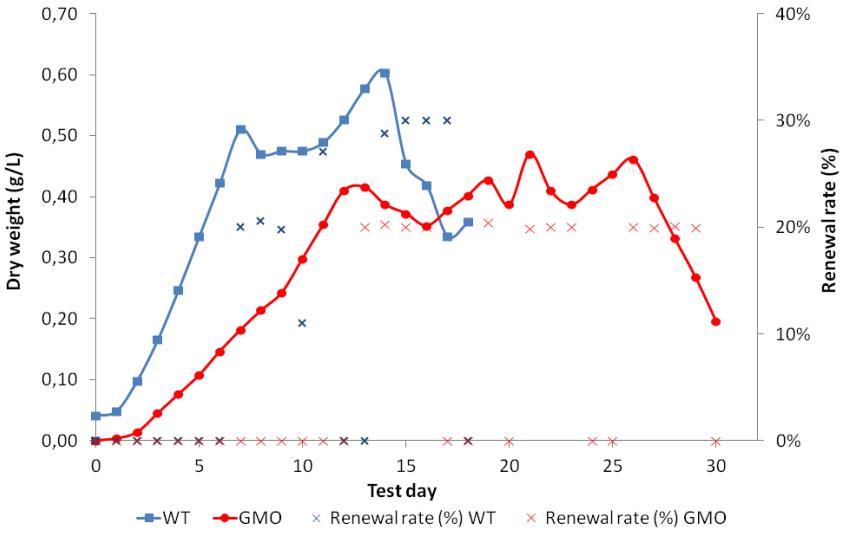
Cultivation in the PBR was carried out for *Synechocystis* 6803 strains – WT and GMO – under the same operating conditions. As stated above, cyanobacteria production in the PBR was carried out at the optimum growth temperatures (30 ± 1 °C, pH at 8.3 ± 0.5) (47), while the radiation supplied was natural light inside the greenhouse.

Overall Performance

The biomass concentration evolution for both strains is represented in Figure 4.2. The curves exhibited 3 distinct phases: a lag phase, which lasted for less than 2 days in the case of WT strain; an exponential growth phase from day 2 to the 10^{th}; followed by a 2-week semi-continuous operating regime. The GMO strain required a longer adaptation period as it exhibited a latency period of 3 days. During this period, which was due to the lower initial cell concentration, the PBR was shaded in order to minimize the stress in the cultures – which could eventually lead to photo-bleaching. The exponential phase, where linear biomass growth is observed due to light limitations, lasted for two weeks after which the cultured was subjected to a semi-continuous operating mode.

During semi-continuous operation with the WT strain, the PBR was operated with 20 % dilution of the culture during the first week and 30 % during the second week. With 20 % renewal rate the culture maintained a concentration of 0.47 ± 0.003 g/L, with daily biomass generation matching the amount removed from the system at this concentration. During the second week the concentration in the PBR stabilized at 0.42 ± 0.08 g/L, approximately. However and as expected, considering this concentrations and dilutions rates, we conclude that biomass production is identical for both conditions, approximately 0.1 g/L/day. Therefore, aspects such as potential for culture contamination; ethanol concentration and extraction or biomass concentration will drive the operation mode of the PBR and the daily renewal amount. These results led to the choice of a renewal rate of 20 % for the ethanol producing strain taking into account, among others, that it has a slower growth rate.

(a)



(b)

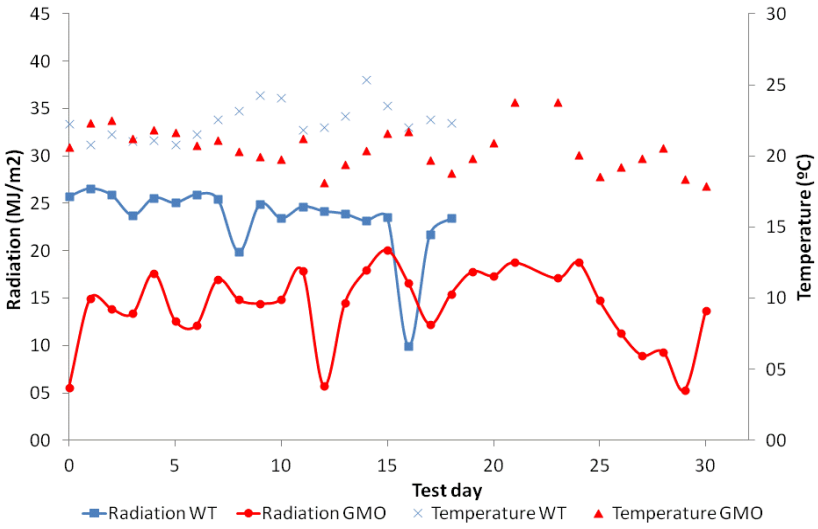


Figure 4.2: (a) Evolution of biomass concentration of *Synechocystis* 6803 strains expressed in g L^{-1}; (b) Weather conditions during PBR cultivation. The DW concentration was determined through a correlation between the DW and OD (see Annex 7.2 - Equation 7.1).

During the semi-continuous operating regime at 20 % renewal rate, the average concentration of the PBR with the GMO strain was 0.36 ± 0.07 g/L (Figure 4.2.a).The lower value is observed since ethanol production uses part of the fixed carbon which decreases the amount of carbon available for biomass production – the GMO strain carries the ethanol genes under *psbA2* promoter which uses the carbon available to produce both biomass and ethanol.

Although the GMO culture was growing favorably (Figure 4.2.a), there was a pronounced decrease of biomass concentration in the final week of the trial (days 26 to 30). This was due to poor radiation with high renewal rate and a contamination that led to culture degradation and decrease in the culture productivity – biomass production rate did not match the biomass removed from the PBR (Figure

4.2.b). The poor condition of the culture after contamination led to the proliferation of more contaminants (bacteria, fungi, cists, ciliates and other microalgae (specifically *Chlorella*)).

The average biomass concentration and the productivity of each operating conditions is presented in Table 4.4. A loss of daily volumetric productivity of about 50 % is verified between the GMO and WT strains, at 20 % daily renewal.

Table 4.4: Average productivity and biomass concentration of *Synechocystis* 6803 strains.

Species	Renewal rate (%)	Average Biomass Concentration ($g\ L^{-1}$)	Average Productivity ($g\ L^{-1}\ day^{-1}$)
WT *Synechocystis*	20	0.47 ± 0.003	0.09 ± 0.02
	30	0.42 ± 0.08	0.07 ± 0.04
GMO *Synechocystis*	20	0.36 ± 0.07	0.04 ± 0.03

The productivity values determined above are corroborated by the nitrate consumption calculations. The nitrate consumption of WT and GMO strains, at 20% daily renewal, was around 0.9 and 0.4 mM day^{-1}, respectively.

Macro - Miccroscopic Observation

Figure 4.3 illustrates the macroscopic evolution of the reactor where WT and GMO *Synechocystis* 6803 strains were cultured, respectively. In general, both *Synechocystis* 6803 strains had similar appearance, showing an intense blue-green color, characteristic of cyanobacteria. At inoculation, cultures showed a yellowish aspect due to the nutrients solution which has a typical brownish color. Once the PBR was concentrated the blue-green color was dominant. At the end of the test the GMO strain showed a brownish color due to the poor state of the culture and contaminants present, soon after the trial was terminated.

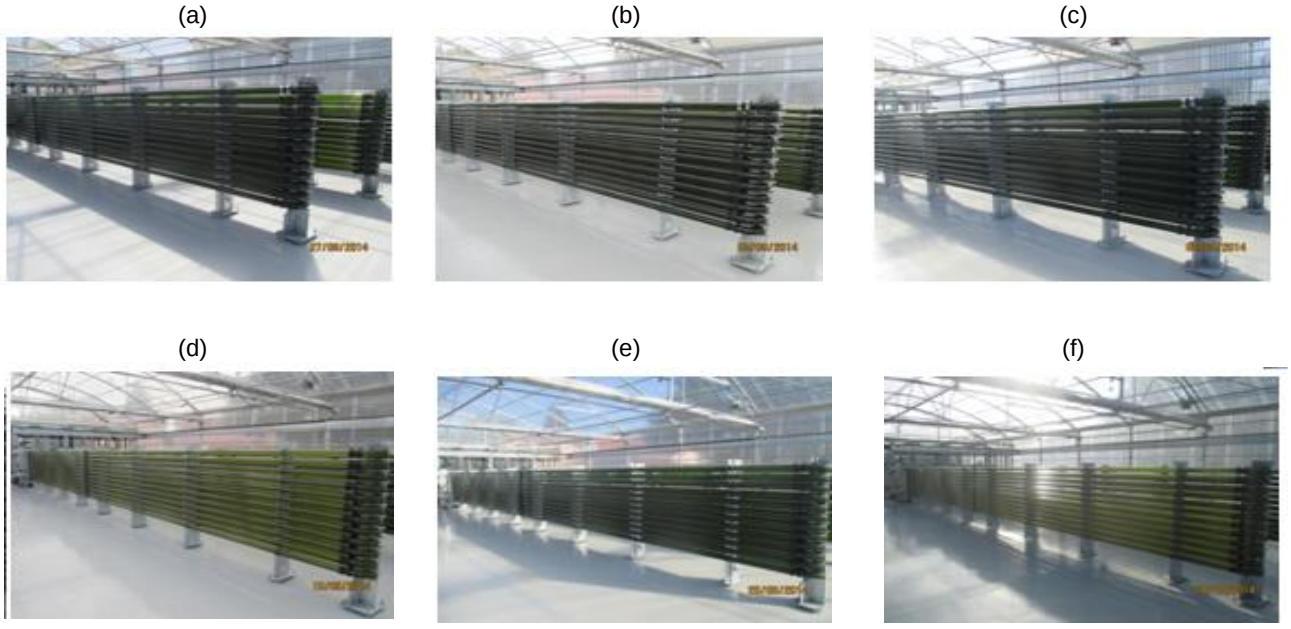


Figure 4.3: Photographic record of WT (a)-(c) and GMO (d)-(f) cultures growing in PBR:

(a)-(c): day 9; day 16; day 18;

(d)-(f): day 5; day 15; day 30.

The general aspect of cultures was evaluated using the optical microscope (BX53-Olympus, Japan) at a magnification of 80 x. Freshly collected culture samples were observed and photographs were taken (Figure 4.4). At an early stage, both cultivations revealed a healthy condition, exhibiting very few bacterial and fungi contaminations. However, predatory protozoans (such as microflagellates, dinoflagellates, ciliates and amoebae) began to emerge along with the inoculated microalgae. However, the renewal rate and frequency of PBR conferred a significant advantage for contaminants mitigation and with 30 % daily renewal, it was visible a decrease of contaminants in the WT culture in comparison to a 20 % dilution rate. This strategy of delaying the onset of contamination was applied in GMO cultures: to dilute the culture as frequently and as heavily as the algae culture allows (i.e. growing at a concentration of cells sufficient to allow the biomass production to match the removed quantity). However, due to the reduced growth rate of the GMO strain, daily renewals of 20 % were imposed. As previously stated, at day 26, a contaminant microalgae (*Chlorella*) was noticed in GMO cultivation. Although this contamination was not very intense, daily dilutions were not able to eliminate it and led to terminate the test.

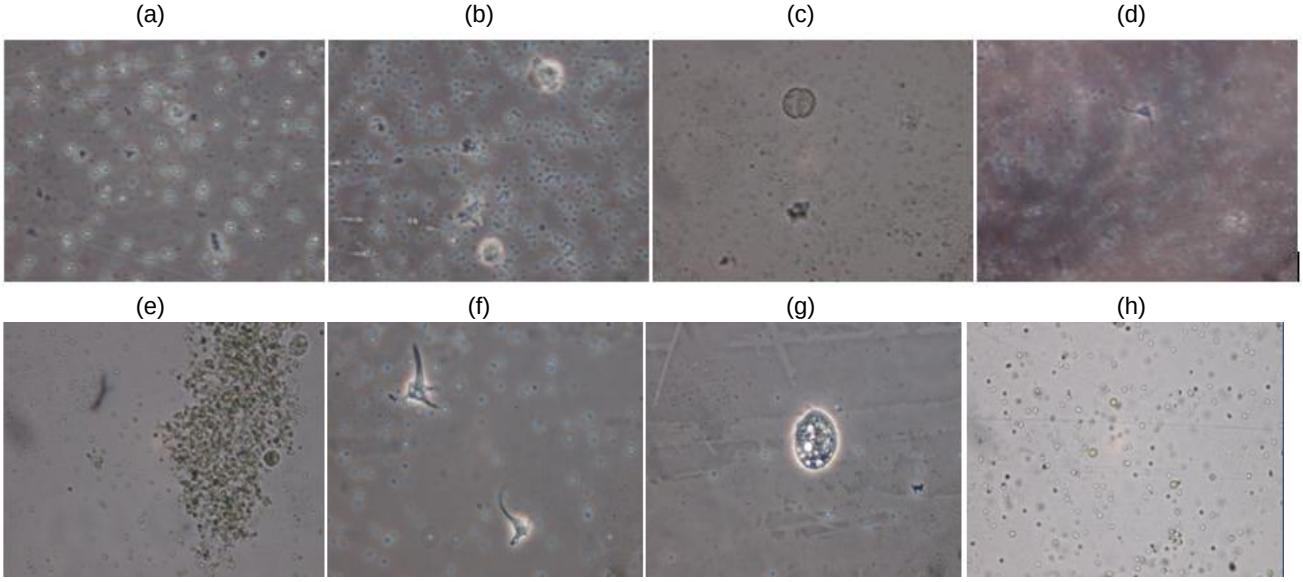


Figure 4.4: Microscopic observation record of contaminants in PBR with WT and GMO strain, respectively:
(a) Bacteria; (b) Amoebae; (c) Cyst; (d) Dinoflagellate.
(e) Agglomerate with cysts and ciliates; (f) Dinoflagellate; (g) Ciliate; (h) Contaminant microalgae.

Photographs (c), (e) and (h) are bright field images; all other are phase contrast. Magnification: 80x. Synechocystis is typically a slightly oblong spheriod, approximately 1.5 microns in diameter.

Ethanol production

Ethanol culture concentration and respective OD were measured twice a day, at 9 am and 5 pm (Annex 7.3). During GMO cultivation no significant ethanol concentrations were detected in the PBR. Ethanol measurement in the first two days of the test was of 4.09 and 10.40 mg L^{-1}, respectively, and only residual values were measured for the remaining days (see Figure 4.5). From the measurement of ethanol production, in the previous reference tests, it was possible to calculate the correspondent

ethanol concentration. The experimental data, however, revealed much lower values than those estimated.

Prior to the inoculation of the PBRs, scale-up cultures growing in 5 L round flasks were examined for the presence of contaminants and ethanol concentration was measured (ca. 25 to 50 mg L^{-1}). The most likely explanation for the non-detection of ethanol on the PBRs is related with ethanol consumption by contaminants such as bacteria (alcohol is a viable carbon source for bacteria growth). The ethanol evaporation from the PBR may also be a hypothesis; however, this is unlikely to occur since the PBR is a closed system.

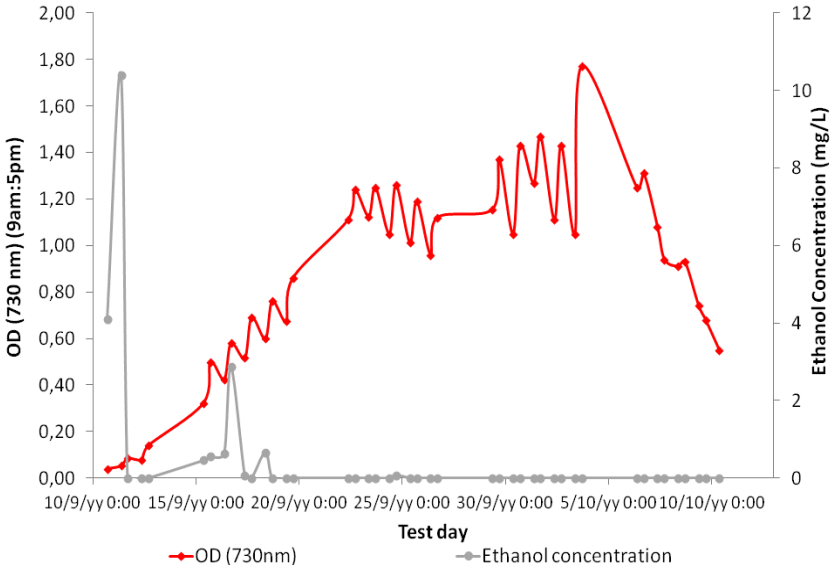


Figure 4.5: Optical density (730 nm) and ethanol concentration over time.

The GMO culture was maintained without any selective pressure, therefore, antibiotic resistance may be lost and then GMO would revert to a WT population. At this point, PCR was performed to confirm if the ethanol construction was still present in the GMO strain and if it was conserved throughout production cycles.

The ethanol construct was amplified by PCR with specific primers developed in the scope of the project. No template control reactions (NTC) and positive control (with WT) were included to assure that the sample was not contaminated and that no errors were made during the execution of the protocol, respectively.

The agarose gel showed one DNA band in each pore, indicating successful amplification of the target sequence. No WT bands were detected in pores 3, 4 and 5, meaning that the construct insertion remained on the GM strain. Also, DNA bands had the expected size: 1 kb for WT and approximately 5 kb for GMO, which corresponds to the ethanol insertion (Figure 4.6).

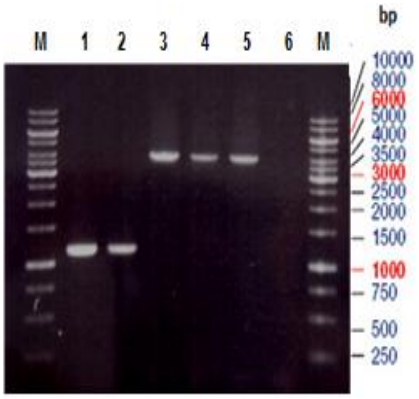


Figure 4.6: Agarose gel electrophoresis of PCR-amplified DNA fragments of GMO *Synechocytis* using Dream Taq PCR Master Mix (Thermo Scientific, USA), stained with GreenSafe Premium and photographed under UV light - M: PCR markers; 1: Positive control- Syn WT (plates); 2: Positive control- Syn WT (liquid); 3: PCR product of GMO Synechocytis (plates); 4: PCR product of GMO Synechocytis (scale-up); 5: PCR product of GMO Synechocytis (PBR) and 6: NTC.

Total load and variability of cultivable bacterial contaminants was also determined at different growth stages for GMO strain. For this, a diversity of media was tested to stimulate different bacterial communities. Samples were collected in the beginning of the test; immediately before the start of renewals; and in the last day of the test. They were collected in a sterilized 50 mL syringes and plated in nutrient agar (NA), TY, laboratory formulated A4F nutritive media + 0.5 % glucose and in A4F industrial nutritive media + 0.5 % agar media using no dilution and 10^2, 10^4 and 10^6 dilution. Plates grew at 25 ± 1 ºC for 2 to 3 days and colonies were then counted to assess total bacterial counts (CFU/mL) (Figure 4.7). As it is shown in Figure 4.7, contaminants growth increased in all the media tested between the beginning and the end of the test. In particular, the total amount of CFU/mL increased over 100-fold in NA medium.

Colonies from each medium were isolated taking into account their macromorphological differences and, according to the results obtained, a greater bacterial diversity was noticed at the end of the GMO test (Table 4.5).

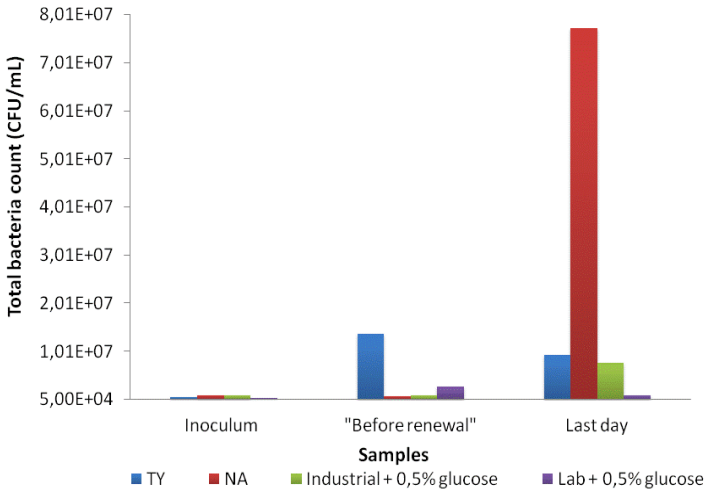


Figure 4.7: CFU representation for inoculums, "before renewal" and last day samples.

Table 4.5: Isolation of individual bacterial colonies from PBR.

Medium plates /Sample	Inoculum	"Before renewal"	Last day
TY	2	4	6
NA	3	3	9
MMF + 0,5 % Glucose	2	4	5
Hubel + 0,5 % Glucose	2	4	7

In an attempt to understand whether the GMO cells were still ethanol producers, lab scale tests were done with PBR culture. However contaminants overcame the cyanobacteria leading the culture to its death.

Chapter 5. Conclusions and Future Work

5. Conclusions and Future Work

The main goal of this research was to develop a competitive technologic approach that allows the production of bioethanol from microalgae with low-cost scalable PBR. In order to achieve that purpose, the photosynthetic capability of genetically modified *Synechocystis* 6803 was used to convert CO_2 into ethanol by the assembly of an ethanol-producing pathway.

Before the growth of the genetically modified ethanol producing strain, at pilot scale in the PBR, some tests were carried out in order to characterize and preserve the cyanobacteria in study.

Although the use of long-term preservation is widespread in laboratory and clinical settings, few data is available for algae cryopreservation and even less for cyanobacteria. In this study, a standardized methodology for freezing, thawing and assessment of viability was tested in WT and GMO *Synechocystis* 6803. Dense cultures were transferred in aliquots and stored under -70º using DMSO at a final concentration of 8 % (v/v) as cryoprotectant. The post-thaw recovery was evaluated after 7 days. The cell growth was analyzed in both liquid and solid cultures, with and without the antibiotic by microscopic observation (optical microscopy). It was clear that both strains had tolerance to freezing stress resulting in the development of a satisfactory and acceptable cryopreservation protocol valid for the cyanobacteria in study.

Characterization of the genetic transformation was carried out by PCR using primers supplied by DEMA project partner University of Limerick. This technique revealed itself as a powerful tool in the assessment of pilot scale cultures for the genetic transformation and in cultures where no kanamycin is used (therefore cells are not subjected to selective pressure).

The procedure used for disinfection (hypochlorite concentration and disinfection duration) for complete elimination of genetically modified *Synechocystis* 6803 was also evaluated, resulting in a protocol for disinfection of biomass effluents in pilot scale before being discarded to the municipal wastewater collector. The method used is as follows: 1) addition of 50 ppm of sodium hypochlorite to the biomass effluent; 2) homogenization of the resulting mixture for 2 hours.

Finally, scale-up strategies were addressed by analyzing the effect of selective pressure; the distribution of fixed carbon between biomass and ethanol production; and *Synechocystis* 6803 tolerance to ethanol concentrations of 25 g L^{-1}. It was found that the scale-up strategy should consist in: (1) using kanamycin during scale-up – *Synechocystis* 6803 used comes directly from cryopreservation stocks; (2) assuring good aeration of the culture flasks and (3) cultivating conditions should be the most favorable possible (optimal) in order to obtain higher growth rates, *e.g.* 24 h radiation. This would favor the maintenance of the genetic integrity and ethanol production until the PBR inoculation.

Pilot Scale Cultivation of *Synechocystis* 6803

The daily volumetric productivity of GMO was ca. 50 % lower when compared to the WT strain: 0.04 ± 0.03 g L^{-1} day^{-1} in GMO and 0.09 ± 0.02 g L^{-1} day^{-1} in WT. It was expected since the carbon flux in GMO culture is partially redirected to a preferred product, i.e, ethanol. However, during GMO cultivation, no significant ethanol concentrations were detected in the PBR. Since ethanol production does not constitute an advantage for cyanobacteria, and the selective pressure was not maintained, a single cell within the population containing a mutation that suppresses the ethanol production has an advantage over the remaining cells and overgrows the remaining cells. At this point, PCR was performed to assess if the ethanol construction was still present in the GMO strain and if it was conserved throughout the production cycles. The DNA bands obtained had the expected size: 1 kb for WT and approximately 5 kb for GMO, which corresponds to the ethanol insertion.

No phenotype of ethanol stress was observed in the PBR, namely a more yellowish pigmentation characteristic of the ethanol producer compared to the rather blue-green of the reference strain. This change in cellular pigment composition was observed in the seven-day ethanol exposure test, where WT culture started with an ethanol concentration of 2 g L^{-1} and reached 25 g L^{-1} after 7 days.

The proliferation of bacteria in *Synechocystis* 6803 cultures began to emerge along with the inoculated microalgae as well as other predatory contaminants (such as microflagellates, dinoflagellates, ciliates and amoebae). The strategy of daily renewals (20-30 % for WT and 20 % for GMO) was applied in order to delay the onset of contamination. Nonetheless, in the last week of the GMO test, a contaminant microalgae was noticed in cultivation. The differences registered in terms of bacterial contamination for both strains were not significant when compared to the ethanol tolerance test, where the increase of ethanol concentration led to intense propagation of bacteria on the culture.

In face of the results, bioethanol production from microalgae still faces several challenges that need to be addressed in order for it to become a reality, since the ethanol production of engineered *Synechocystis* 6803 strains are still low as compared with that from the biomass fermentation or agricultural crops and residues. Under all the circumstances, more research is needed in order for it to become a reality, namely maximize the ethanol productivity of cyanobacteria through metabolic engineering; increase culture ethanol tolerance in order to obtain higher ethanol concentrations (>1-2%) and maximize biomass productivity and ethanol production through enhanced culture robustness.

Future work

Since in the PBR test with GMO species no ethanol production was detected, some procedures should be implemented and optimized in the next cultivation. Some of the main procedures/operations are described below:

- Improve disinfection/sterilization procedures. PBR and all the main equipment should be sterilized with steam since it has the largest margin of safety due to its reliability, consistency and lethality.
- PBR inoculation should be carried out with kanamycin in order to maintain the selective pressure of the cells;

- Perform tests in order to understand if there might be ethanol evaporation from the PBR;
- Evaluate the effect of the photoperiod in ethanol production

Some of the further questions which should be addressed are, if the ethanol construct is still intact throughout the production cycle (by sequencing the PCR products) and if the contaminants are ethanol consumers (by sequencing the isolated bacteria of GMO culture using 16S and 18S rRNA gene sequencing (DGGE)).

Regarding cryopreservation, since the viabilities of the resting stages generally decrease with time, thawing should be done after 6 months to 1 year in -70 ºC, to assure that the cells are capable of recovering activity.

Finally, expression of pdc and adh in cyanobacterial cells could be analyzed performing Western blot analysis.

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7. Annexes

7.1 Annex 1. Biofuels by fuel and feedstock

Table 7.1 - Biofuels by fuel and feedstock.
Adapted from (8).

Classification	Fuel	Feedstock	Observations
1st generation	Ethanol	Starches from wheat, corn, sugar cane, molasses, potatoes, other vegetables	
	Propanol		
	Butanol		
	Biodiesel	Oils and fats	
	Vegetable Oil	Unmodified	
	Fat		
	Bio ethers	Dehydration from alcohols	
	Biogas	Methane made from waste crop material through anaerobic digestion or bacteria	Same properties as methane from fossil fuels
	Wood	Natural materials	Wide variety of materials
2nd generation	Cellulosic Ethanol	Made from wood, grass or inedible parts of plants	
	Bio hydrogen	Made from algae breaking down water	Used in place of the hydrogen produced from fossil fuels
	Methanol	Made from inedible plant matter	More toxic and less energy dense than ethanol
	Dimethyl furan	Made from fructose found in fruits and some vegetables	Energy density close to that of gasoline. Toxic to respiratory tract and nervous system
3rd generation	Microalgae and seaweed based biofuels	Multiple fuels made from microalgae	More expensive, but may yield 10-100X more fuel per unit area than other biofuels

7.2 Annex 2. Analytic methods

7.2.1 Estimation of Microalgal Biomass Concentration

One of the basic parameters for monitoring the performance of microalgal production systems is the estimation of the biomass produced. The growth of microalgal cultures can be expressed in several ways such as the increment of biomass, the number of cells, the amount of proteins, pigments, etc over a given period of time. In this study, biomass was estimated throughout optical density (OD) measurements using a wavelength selected according to the cyanobacterium pigments. Thereafter, the OD values were correlated with dry weight and microscopic cell counts.

7.2.1.1 Optical density

Cell growth was monitored by measuring the OD at a wavelength of 730 nm using UV-Vis spectrophotometer (SG) (Genesys 10S UV-Vis, Thermo Scientific, US). Each sample was read in duplicate in plastic cuvettes, with 1 cm of path, against distilled water, to obtain accurate results. Microplate reader (MR) (SpectroStar Nano – BMG Labtech) was also used to measure the OD and samples were measured in quadruplicate. Given the linear range of the spectrophotometers, dilution of the samples was required in the later phase of *Synechocystis* 6803 cell cultures to respect the linearity of the Beer-Lambert law.

When OD was not measured, a linear interpolation was performed in order to determine the missing values.

7.2.1.2 Dry weight (DW) method

The growth and development of the culture in test were also assessed through the biomass dry weight, where a direct correlation between the light absorption and dry weight at different concentrations were established. The correlations OD_{730} and dry weight (g L^{-1}) obtained for *Synechocystis* 6803 were Equation 7.1 and Equation 7.2.

$$DW\ (g\ L^{-1}) = 0.3838 \times OD\ (730\ nm) SG - 0.0164 \quad \text{Equation 7.1}$$

R2=0,9886

$$DW\ (g\ L^{-1}) = 0.3127 \times OD\ (730\ nm) MR - 0.0258 \quad \text{Equation 7.2}$$

R2=0,9927

7.2.1.3 Determination of Daily volumetric productivity

The biomass parameter measures the extent of growth and it is used to evaluate the productivity of the cultivations, expressed in g L^{-1} day^{-1}. Therefore, the daily volumetric productivity of cultures was determinate through the multiplication of the renewal rate by the cell concentration of that day (Equation 7.3).

$$Productivity = Renewal\ rate \times DW$$ Equation 7.3

7.2.2 Estimation of Nitrate Concentration - Nitrate determination

The concentration of the nitrate ion in the inorganic medium of *Synechocystis* 6803 cultivation was determined by ultraviolet absorption spectrometry.

Culture samples with a volume of 1.5 mL were centrifuged at 14000 rpm for 10 minutes in the Centrifuge Minispin. The supernatant obtained was diluted using distilled water and HCl (1M) was added at a final concentration of 3 % (v/v) to prevent interferences from other absorbing compounds (such as hydroxide or carbonate anions).

Nitrate was estimated by measuring the sample absorbance at 220 and 275 nm with a spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, US), against distilled water, using quartz cuvettes with 1 cm optical path.

The measurement of the UV absorption at 220 nm allows for a rapid determination of nitrate; however dissolved organic matter can also absorb at this wavelength. Therefore a correction was made by using a second absorption value at 275 nm (Equation 7.4); at this wavelength, nitrates do not absorb, but dissolved organics do.

$$Abs\ {NO_3}^- = Abs\ (220\ nm) - 2 \times Abs\ (275\ nm)$$ Equation 7.4

The absorbance value was then converted to nitrate concentration using the calibration curve obtained with the nitrate standard solutions (KNO_3).

7.2.2.1 Determination of nitrate consumption

The nitrate consumption of day x (mM day^{-1}) was based on the daily adjustment carried out in the previous day $x - 1$ and in the measure performed in the present day (Equation 7.5).

$$Consumption\ {NO_3}^-{}_x = [{NO_3}^-]_{x-1} - [{NO_3}^-]_x$$ Equation 7.5

When nitrate was not measured, a linear interpolation was performed in order to determine the missing values.

7.3 Annex 3. Analytical Method for Ethanol Quantification in Aqueous Solutions

7.3.1 Enzymatic Method for Ethanol Determination

For the determination of ethanol concentration a 7mL of culture sample was taken, spun down at 3500 rpm (centrifuge Nahita, model analogic 2655,) for 15 min and the supernatant was placed in a 15 mL sterilized tube. The tube filled with supernatant was then placed in a freezer, at -20 ºC, until the assay was performed.

The measurement of ethanol was carried out according to the protocol provided with the enzymatic ethanol assay kit – UV method (Nzytech, Portugal). This method is based on a coupled enzymatic reaction: ethanol oxidation to acetaldehyde, reducing NAD^+ to NADH, catalyzed by alcohol dehydrogenase (ADH) (Equation 7.6) followed by the conversion of acetaldehyde to acetate by aldehyde dehydrogenase (Al-DH) (Equation 7.7).

$$Ethanol + NAD^+ \xrightarrow{ADH} Acetaldehyde + NADH + H^+ \qquad \text{Equation 7.6}$$

$$Acetaldehyde + NAD^+ + H_2O \xrightarrow{Al-DH} Acetate + NADH + H^+ \qquad \text{Equation 7.7}$$

The amount of NADH formed through the combined action of ADH and AL-DH is measured at 340 nm, and is proportional to the ethanol present.

Procedure

Each reaction took place in a plastic cuvette, with 1 cm light path, and require 2.00 mL of distilled water, 0.10 mL of supernatant previously thawed at room temperature (or distilled water for the blank), 0.20 mL of buffer (1.5 M, pH=9), 0.20 mL of NAD^+ (12.5 g L^{-1}) and 0.02 mL of AL-DH (EC 1.2.1.3; 75 U mL^{-1}). The cuvettes were sealed with cuvette cap and parafilm M® to prevent the assay mixture from adsorbing ethanol present in the surrounding air. The solutions were homogenized by gently inversing the cuvettes and the absorbance, of the mixtures, was measured after approx. 2 min (A1) using a UV-Vis spectrophotometer (± 0.005 A) (Genesys 10S UV-Vis, Thermo Scientific, US). The second reaction started when 0.02 mL of ADH (EC 1.1.1.1; 167 U mL-1) was added to the mixtures and the absorbance of the final solutions was read after approx. 5-10 minutes – when the reaction is complete (A2). All the solutions used were included in the utilized kit.

The result obtained from the difference of the differences (A_2-A_1) from the sample and the blank (Equation 7.8) is used to estimate the ethanol concentration (Equation 7.9). where V is the final volume (2.54 mL); v is the sample volume (0.1 mL); MW is the molecular weight of ethanol (46,05 g mol^{-1}); d is the light path (1 cm) and ε is the extinction coefficient of NADH at 340 nm (6300 L x mol^{-1} x cm^{-1}).

$$\Delta A_{\text{Ethanol}} = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank} \qquad \text{Equation 7.8}$$

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2 \times 1000} \times \Delta A_{Ethanol} \quad [g\ L^{-1}]$$ Equation 7.9

The amount of ethanol present in the assay should range from 0.25 to 12 µg in 0.10-2.00 mL sample volume. Therefore, given the linear range of the spectrophotometer and the sensitivity of the ethanol assay, the dilution of the sample (with distilled water) or a different sample volume can be required. That way the result must be multiplied by the corresponding dilution/concentration factor.

This enzymatic method is very sensitive so, in order to prevent the contamination with unknown sources of ethanol, all the procedures were performed in an ethanol free atmosphere. The determination is relatively specific for ethanol since methanol, aldehydes, ketones, secondary and tertiary alcohols as well as glycerol do not interfere with this methodology. The only interferences possible come from n-butanol and n-propanol.