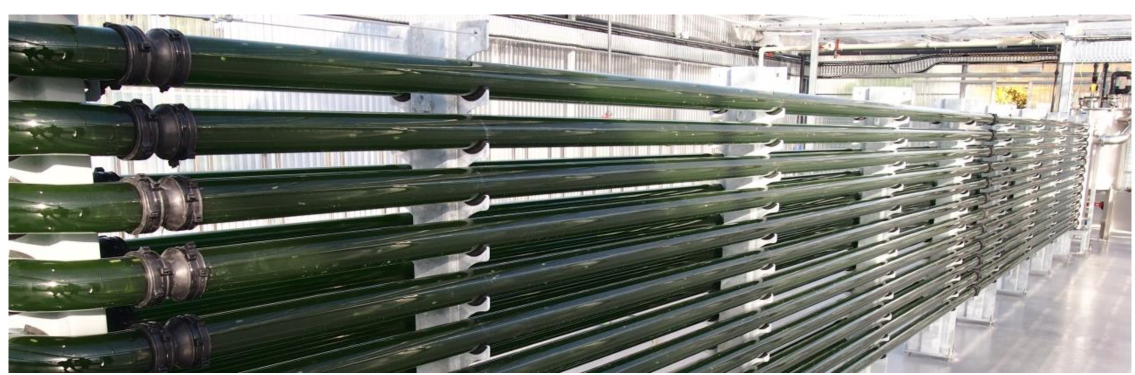

Development of methodologies for fast analysis of ethanol in cyanobacteria cultures

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Thesis to obtain the Master of Science Degree in

Chemical Engineering

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June 2014

Acknowledgements

I'd like to thank A4F and its staff for providing the conditions, training and constant support for the conclusion of this work. Additionally, I'd also like to thank the LNEG (Laboratório Nacional de Engenharia e Geologia) for the support provided so that the tests could be done. Finally, I'd like to thank the European Commission whose Framework Project 7 financed the work done.

I'd like to thank my thesis supervisor, Edgar Santos, for all the help and guidance towards its conclusion.

And for last, I'd like to thank my family and friends whose psychological support was not small at all.

Abstract

English

Production of third generation biofuels from genetically modified cyanobacteria is, currently, subject of great attention from the scientific community, industry and policy makers throughout Europe and the World. The possibility to produce bioethanol as a by-product of *Synechocystis* sp. PCC 6803 metabolism has been target of large investment, research and development in the World.

However, in order to be able to produce bio-ethanol at industrial scale through the indicated method, it becomes necessary to optimise procedures for *Synechocystis* sp. PCC 6803 cultivation – with and without ethanol – in photobioreactor and to investigate simple and fast-application methods of determining the amount of ethanol in a culture.

Having these needs in mind, the following work reports the investigation done on the production procedures of *Synechocystis* sp. PCC 6803 (general culture, nutritive media, tolerance to ethanol and pilot scale production), on the methodologies available to test the amount of ethanol in a culture and how can these methodologies be applied to *Synechocystis* production.

This investigation was done with resource to laboratory and pilot unit testing prepared with basis on prior investigation of physical, chemical and biological conditions for microalgae cultivation.

The general conclusions achieved are that *Synechocystis* sp. PCC 6803 can be properly cultivated in industrial scale with industrial nutritive media, that the presence of ethanol has an impact on its productivity and that the ideal methodology for fast analysis of ethanol quantification is the refractometry – even though it's limited down to a concentration of 2 g/L of ethanol.

Keywords: *Synechocystis, biofuels, bioethanol, pilot scale, chemical analysis, photobioreactor*

Portuguese

A produção de biocombustíveis de terceira geração a partir de cianobactérias geneticamente modificadas é, actualmente, tema de grande atenção da parte da comunidade científica como das entidades políticas europeias e mundiais. A possibilidade de produzir bioetanol como sub-produto do metabolismo da *Synechocystis* sp. PCC 6803 tem sido alvo de grande investimento, pesquisa e desenvolvimento na europa e no mundo.

No entanto, para ser possível produzir bioetanol à escala industrial através do método indicado, torna-se necessário optimizar procedimentos para o cultivo – com e sem etanol – de *Synechocystis* sp. PCC 6803 num fotobioreactor e investigar métodos simples e de aplicação rápida com vista a determinar as quantidades de etanol na cultura.

Tendo isto em mente, o presente trabalho reporta a investigação feita sobre procedimentos de produção de *Synechocystis* sp. PCC 6803 (cultura geral, meio nutritivo, tolerância ao etanol e produção à escala piloto), sobre as metodologias disponíveis para testar a quantidade de etanol numa cultura e como podem estas metodologias ser aplicadas à mesma cultura.

As conclusões gerais foram que a *Synechocystis* sp. PCC 6803 é cultivável à escala piloto com meios nutritivos adequados; que a presença de etanol tem impacto na produtividade da cultura e que o método mais adequado para a determinação de etanol é a refractometria – apesar de estar limitado até acima de 2 g/L de etanol na cultura.

Palavras-chave: *Synechocystis, biocombustíveis, bioetanol, escala piloto, análise química, fotobioreactor*

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PBR – Photo-bioreactor

EU – European Union

MMF – Macro, Micro e Ferro

OD – Optical Density

WT – Wild Type

GMO – Genetically Modified Organisms

Lge – Litter of gasoline equivalent

IEA – International Energy Agency

1. Introduction

Microalgae are a multidisciplinary area. Working with these microorganisms implies dealing with a complex and intertwined understandings and notions of chemical and biological engineering and analytical chemistry. Because of that, microalgae are also a field filled with growth potential, both for the subject itself and for the field of researchers involved.

Biofuels have the potential to significantly reduce transportation's output of carbon and, therefore, reduce its impact on climate change. Using microalgae to produce biofuels has many advantages over other forms of biomass, for instance, it occurs naturally, are fast growing organisms, and microalgae production results in a much less significant land footprint due to the higher productivity than all other crops.

From the European Union 7th Framework Program, the *Direct Ethanol from MicroAlgae* (DEMA) project focus is the producing bioethanol as a secretion of cyanobacteria. The aim of the project is to introduce the capacity to produce ethanol in *Synechocystis* sp. PCC 6803 through metabolic engineering and then establish the technology for the industrial production of bioethanol. This project provides the context for the work done in this thesis.

In the past, work has been developed regarding metabolic engineering: first by Coleman (11) with *Synechococcus* sp. PCC 7942 and then by Pengcheng Fu (12) with *Synechocystis* sp. PCC 6803. Both researches developed methods to genetically modify microalgae however were not successful to optimise the cultivation process.

To further develop the topic, it became necessary to understand the functioning of the wild type species, how it can be cultivated, which nutritive medium, how does the microalgae behaves at pilot scale and what is its tolerance to ethanol concentrations. However, it also became necessary to identify methods and techniques that would allow the quick identification of the amount of ethanol present in the culture.

As such, this thesis reports the study done on all the previously mentioned needs by studying follow-up procedures for *Synechocystis* sp. PCC 6803 culture, comparing three different nutritive media (BG-11, MMF and Hubel 7), testing its tolerance to ethanol and cultivating in a 1,1 m^3 photobioreactor. In parallel, it also reports the study of different methods to detect and quantify ethanol and how can they be applied to pilot scale production of *Synechocystis* sp. PCC 6803.

2. Literature review

2.1 Biofuels

Human activity is intrinsically connected to fuels. As a general definition, a fuel is something that can store potential energy which can be posteriorly released in the form of heat energy. Daily human actions have become entirely dependent on the availability of energy, thus fuels. There are multiple types of fuels that are used in the most different ways with the sole purpose of releasing that stored energy. Within these types, we have the biofuels which are members of the hydrocarbon fuel family and are produced from organic matter in a shorter period of time. This fact contrasts immediately with fossil fuels which take millions of years to form.

Biofuels are generated through a carbon fixation process which takes inorganic carbon and converts it into organic compounds. As such, biofuels have been used since the very first moment man discovered how to extract energy from materials – the wood they burned to make fire. Up until the early XXth century biofuels were the mainstream source of energy until the discovery of large supplies of crude oil. Due to the increased supply of fossil fuels, the geopolitical and economic interest in biofuels faded away. In the beginning of the XXIth century the oil prices and greenhouse emissions raised – and they brought awareness of biofuels as an alternative.

Current definitions break biofuels down into three different categories (1):

- First generation biofuels – These are also known as conventional biofuels. They are made from sugar cane, starch, corn or vegetable oil – the definition of first generation biofuel is any biofuel made from feedstock that can be also consumed by humans;

- Second generation biofuels – These are produced from sustainable feedstock. This sustainability is defined by its availability, its impact in greenhouse gas emissions, its impact in land used and the potential to threaten food supply. Food crops cannot be considered second generation biofuels but they can become so if they already fulfilled their food purpose. Second generation biofuels are often known as “advanced biofuels;

- Third generation biofuels – This definition is rather recent and it’s exclusively referring to any biofuel derived from algae. These biofuels are given their separate category due to their unique production mechanism and potential to mitigate most of the issues with the 1st and 2nd generations.

Their categories can be further subdivided by type of biofuel as resumed in Table 1.

Table 1 - Biofuels classification summary.

	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

It is generally agreed that biofuels generate large benefits upon comparison with fossil fuels. However this advantage is not necessarily obvious neither in terms of primary fossil energy consumptions nor in terms of greenhouse gas emissions – in fact, in the worst case, the can both exceed them, (2) (3). The way to properly evaluate the benefits of biofuels is through assessment of their life cycle which will depend on the feedstock, choice of location, by-products generation, process technology and use of the fuel (4).

Regarding the gas emissions, while the carbon emission is considered neutral, the critical point for its influence on gas emissions is the feedstock – because it determines the energy yield per unit of land, the use of fertilizers, the co-products of its use and if the feedstock production is replacing any green area. Other environmental impacts associated with the production of biofuels are acidification, eutrophication, photo smog, health hazards, ozone depletion due to N_2O, loss of biodiversity and impact on ground source water.

As of now, the relatively high production costs still remain a critical barrier to commercial development despite the fact there have been continuous improvements. Independently of that, the competitiveness of biofuels will increase as prices for crude oil and other fossil sources raise and overstep the critical point. To the day, in the European Union, the production of biofuels still depends largely on legislative framework and subsidies (5). It is expected that, in the medium-long term, biofuels will have a large socio-economic impact as it will open new market opportunities. The dimension of the impact is difficult to assess as no studies have been conducted on the topic yet. Holistically, the biofuels offer large economic advantages over fossil fuels but direct comparisons are difficult as negative externalities associated with fossil fuels (such as military expenditures, costs for environment or health) tend to be poorly quantified.

Overall, the general consensus is that biofuels are a technology worth developing with the purpose of overcoming fossil fuel limitations. In the European commission framework there is currently a strategy for biofuels with the following vision (6):

By 2030, the European Union 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.

For the transport sector in particular, the EU has been supporting biofuels with the purpose of reducing greenhouse gas emissions, sustaining European competitiveness and diversifying fuel supply sources by developing long term replacements for fossil fuels. During the end of the first decade of the XXIth century, the EU enacted the Framework Programmes 6 and 7 which included extensive support for biofuel research.

It is widely expected that globally production of biofuels will continue growing in the coming years. Indeed, the investment and production capacity of biofuels has known extensive development in the recent years (Figure 1) (7). However, the growth will decrease reflecting the downturn in global economic activities in 2008; concerns about biofuels economic and environmental sustainability, food prices and other aspects.

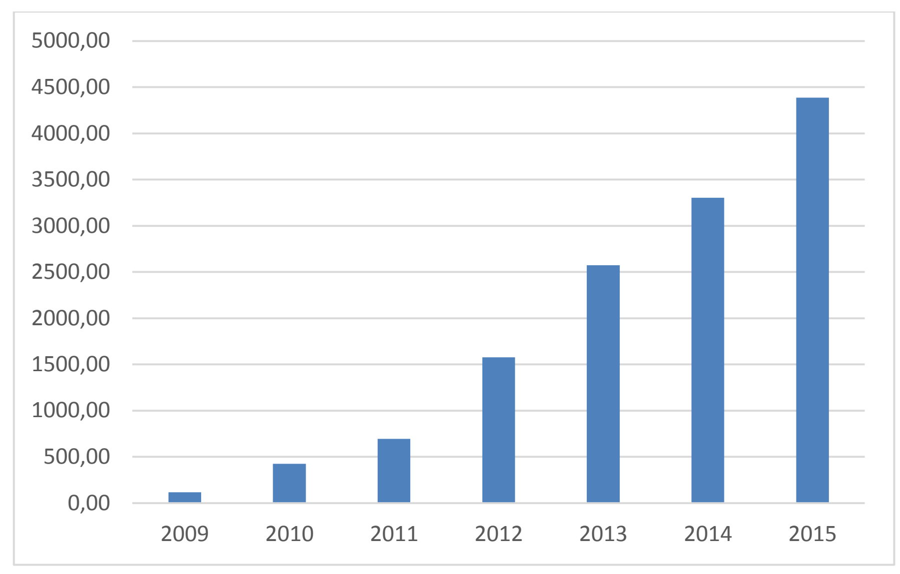


Figure 1 – Worldwide production capacity of Biofuels (millions of gallons per year)

However, the International Energy Agency (IEA) estimates an average annual growth rate of 7% (Table 2) (7) (5). The USA, who already are the world's largest consumer of biofuels, will increase its consumption even further. Europe will lead the global growth in demand in the coming years, and bioethanol will account for the dominating share of this growth.

Table 2 - World consumption of biofuels and forecast (Mtoe).

	2004	2010	2015	2030
OECD	**8.90**	**30.50**	**39.00**	**51.80**
North America	7.00	15.40	20.50	24.20
United States	*6.80*	*14.90*	*19.80*	*22.80*
Canada	*0.10*	*0.60*	*0.70*	*1.30*
Europe	2.00	14.80	18.00	26.60
Pacific	0.00	0.30	0.40	1.00
Transition Economies	**0.00**	**0.10**	**0.10**	**0.30**
Russia	*0.00*	*0.10*	*0.10*	*0.30*
Developing Countries	**6.50**	**10.90**	**15.30**	**40.40**
Developing Asia	0.00	1.90	3.70	16.10
China	*0.00*	*0.70*	*1.50*	*7.90*
India	*0.00*	*0.10*	*0.20*	*2.40*
Indonesia	*0.00*	*0.20*	*0.40*	*1.50*
Middle East	0.00	0.10	0.10	0.50
Africa	0.00	0.60	1.10	3.40
North Africa	0.00	0.00	0.10	0.60
Latin America	6.40	8.40	10.40	20.30
Brazil	*6.40*	*8.30*	*10.40*	*20.30*
World	**15.50**	**41.50**	**54.40**	**92.40**

2.2 Bioethanol

Bioethanol is the most common biofuel and accounts for nearly 90 % of the biofuel usage worldwide (7). The conventional bioethanol production process is based on enzymatic conversion of starchy biomass into sugars and the fermentation of 6-carbon sugars with final distillation of ethanol to fuel grade (8). The world's largest conventional producers of bioethanol are United States (corn ethanol) and the Brazil (sugar cane ethanol).

Currently, research and development focus on developing advanced processes for bioethanol production such as processes that use lignocellulosic materials as feedstock or microalgae for production.

Ethanol production from lignocellulosic feedstock (9) includes biomass pre-treatment to release the cellulose and hemicellulose, hydrolysis to release fermentable 5- and 6-carbon sugars, sugar fermentation, separation of solid residues and non-hydrolysed cellulose and distillation to fuel grade. Research happens especially at new chemical and enzymatic processes to provide for better conversion. Solid residues and co-products such as lignin and other components, particularly from forest materials, can inhibit the hydrolysis – however they can be extracted and used as fuel in the production process reducing cost and emissions.

Another model for ethanol production is through the usage of microalgae. Microalgae, recognised as one of the oldest living organisms, are thallophytes (plants lacking roots, stems, and leaves) that have chlorophyll a as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive cells. Microalgae are able to fix carbon dioxide efficiently from different sources, including the atmosphere, industrial exhaust gases, and soluble carbonate salts.

The idea of microalgae (1) as biofuel source started to gain ground due to increasing oil prices. The usage of microalgae offers the following advantages over other plants:

- higher growth rates;
- capable of all year round production;
- need less water than terrestrial crops;
- does not require herbicide or pesticide use;
- sequester CO_2 emitted from the usage of fossil fuels;
- can be cultivated on non-arable land thus not competing with agriculture resources.

Each model of production has its own limitations and advantages with regards to fossil energy input, co-products generation, production cost and environmental impact (Table 3 - Comparison of biofuel production methods).

Table 3 - Comparison of biofuel production methods (7).

	Feedstock			
	Corn	Sugar Cane	Ligno-cellulosic	Microalgae
Fossil energy input (%)	60-80	10-12	100	-
Co-products	-	Heat and Power	Heat and Power	Biomass
Production cost ($/lge)	0,6-0,8	0,3-0,5	1	0,6
CO_2 reduction (%)	15-25	90	70	100
Pollutant abatement	CO	CO	CO, NO_x	CO
Land use (lge*/ha)	1500-3000	3000-6000	-	-

*** – lge stands for litter of gasoline equivalent.**

The data available in Table 3 (7) allows us to observe that each model of production has benefits and disadvantages towards each other. Bioethanol is a fuel with a high octane number and a low tendency to create knocking – premature combustion – in internal combustion engine. The oxygen present in the biofuel allows low-temperature combustion with reduction of CO and NO_x emissions. Fuels with low ethanol percentage (5 %-10 %) can be used in conventional internal combustion engines with almost none technical modification. Flex-fuel engines (of which there are 6 million running, mainly in Brazil, USA and Sweden) can ran on up to 85% ethanol blends only having few modifications made during production. Thus, ethanol combustion offers fuel and emissions savings due to the high octane number, the high compression ratio and the combustion benefits from ethanol vapour cooling which partly offsets its lower energy content per litre.

It is expected that ethanol can overcome the traditional barriers generally faced by biofuels, mostly in Europe. Indeed, ethanol production has been expanding consistently and both Brazil and the USA continue to maintain a consistent lead on its production (10).

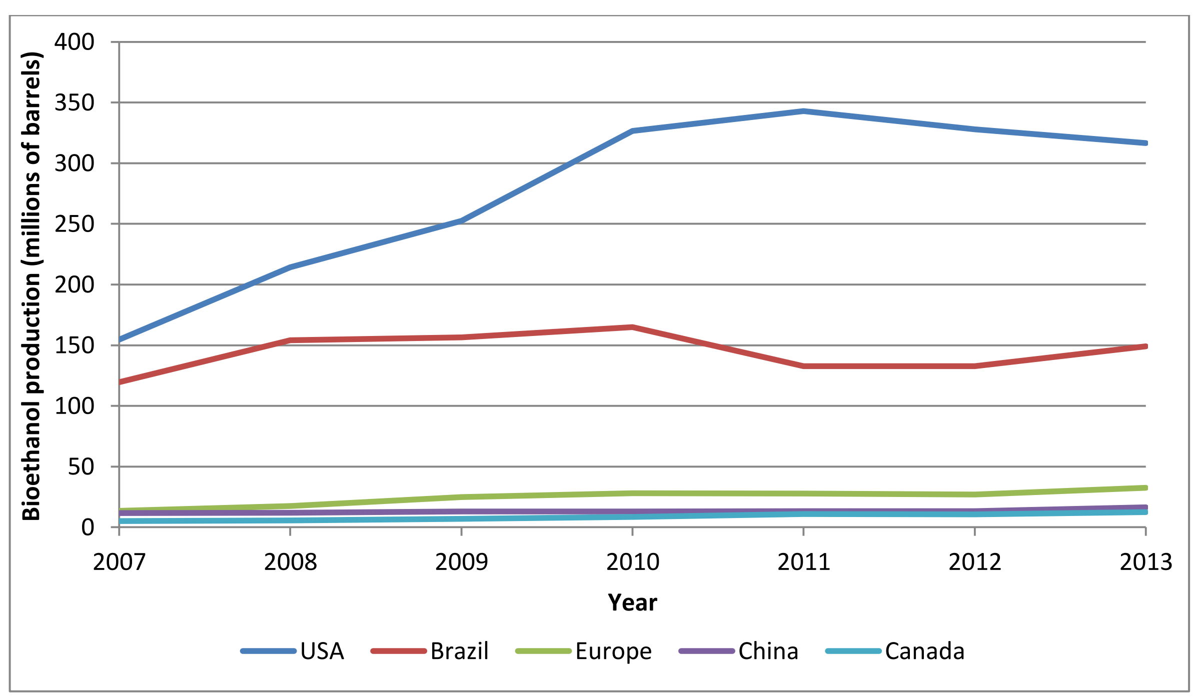


Figure 2 - Production of bioethanol between 2007 and 2013 (millions of barrels).

2.3 Ethanol production through the use of microalgae

As mentioned in previous sub-chapters, current production of bioethanol happens through the fermentation of crops. As demand for bioethanol increases, so do the concerns over the excessive use of agricultural land and feedstock for its production. Additionally, experts have argued such processes are neither cost nor energy efficient. Thus, it becomes necessary to develop alternative paths for bioethanol production in order to decrease the costs associated to labour, land and time of fermented crops. The concept of the 3rd generation of biofuels was created through the production via microalgae.

The first approach considered was the genetic engineering of photosynthetic prokaryotes which would combine the conversion of solar energy, carbon sequestration and ethanol production in a single organism. One of the first experiments was done by Deng and Coleman (11) and consisted in the genetic modification of *Synechococcus* sp. PCC 7942 for the uptake of CO_2 by a photosynthetic organism via photoautotrophic metabolism. The transformed cyanobacteria synthesized ethanol, which diffused from the cells into the culture medium. As these cyanobacteria rely only on light, CO_2, and inorganic elements to grow, production of ethanol by cyanobacteria was demonstrated to be a potential solution for bioconversion of solar energy and CO_2 into a valuable resource.

The creation of the pathway for ethanol production in cyanobacteria is based on the insertion of the pyruvate decarboxylase and alcohol dehydrogenase from the ethanolgenic bacteria *Zygomona mobilis* (12). However, the focus of current work for bioethanol production is the *Synechocystis* sp. strain PCC 6803.

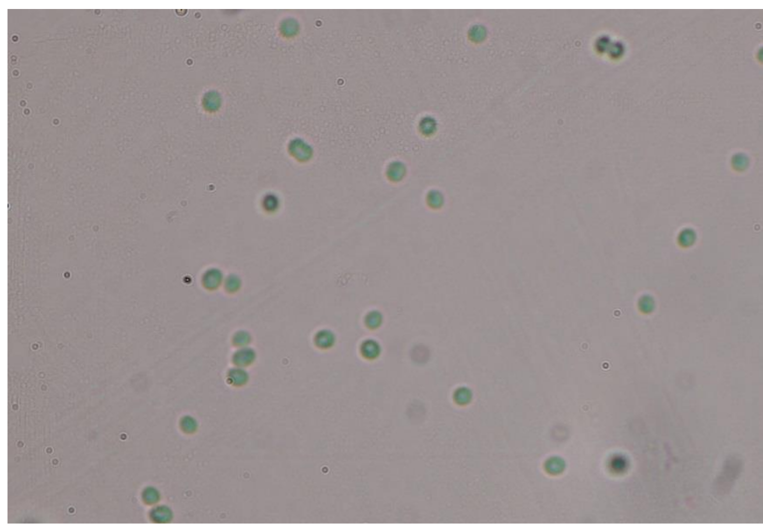


Figure 3 - *Synechocystis* sp. PCC 6803 strain.

Synechocystis sp. PCC 6803 strain, a freshwater, non-filamentous, non-nitrogen fixing cyanobacteria capable of heterotrophic growth, was the first photosynthetic autotrophic organism that was completely sequenced and annotated. Additionally, this cyanobacterium is naturally easy to transform and has allowed the establishment of techniques for precise genome manipulation. Mutation

of *Synechocystis* sp. PCC 6803 is induced through transformation with a plasmid vector targeted to the genome via recombination and selection in appropriate conditions (12). This has allowed creating a strain of freshwater cyanobacteria *Synechocystis* sp PCC 6803 that is the basis of an efficient process of ethanol production by photoautotrophically converting CO_2 to bioethanol.

The autotrophic growth of *Synechocystis* is based on two specific processes (13):

- Cells using the light as the energy source to drive the electron transfer reactions which enable the movement of electrons from chlorophyll to move along the electron transport chain which leads to the reduction of NADP to NADPH (Figure 4 - NADPH production.).

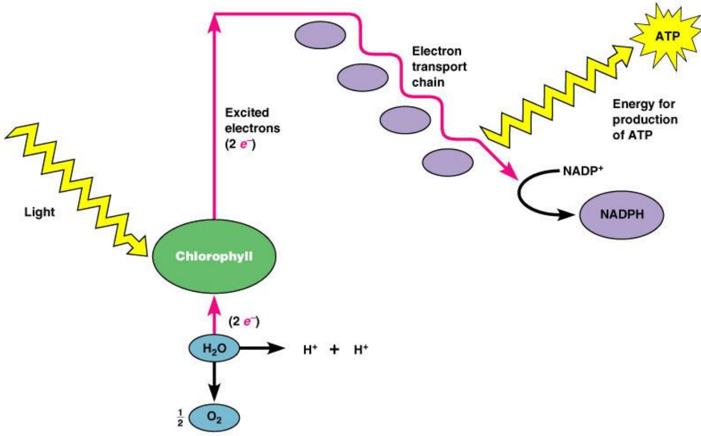


Figure 4 - NADPH production.

- Carbon fixation reactions in which the ATP and the NADPH produced by the previous process are used to convert CO_2 into carbohydrates and other metabolites.

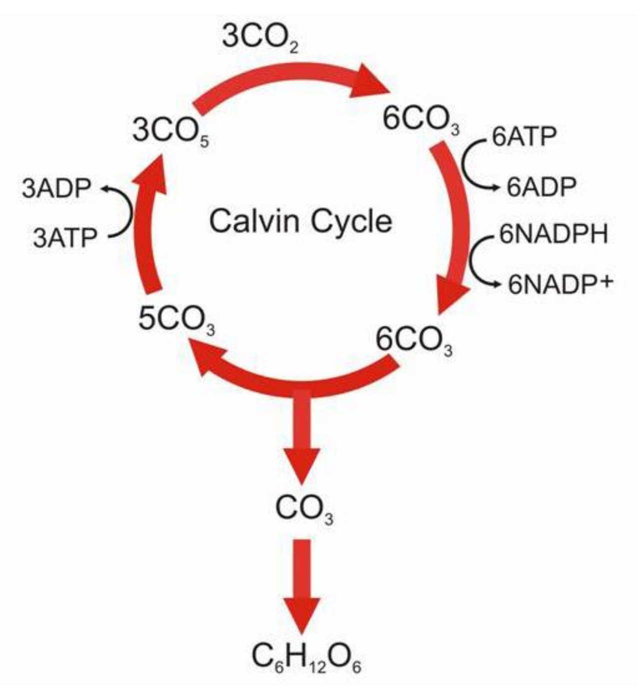


Figure 5 - Carbon fixation cycle (Calvin Cycle).

Genetic engineering (14) allowed introducing an ethanol-producing pathway into the *Synechocystis* metabolic network. The end result is that the carbon flow from the pyruvate was directed to the formation of the end product – ethanol – instead of biomass.

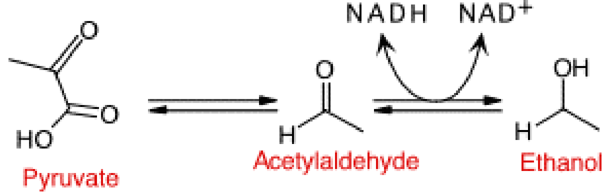


Figure 6 - Pyruvate conversion to ethanol.

The introduction of this new pathway raised a few concerns. First of all, it was observed that the microalgae growth rate was halved (Figure 7 - Comparison of biomass development of WT vs GMO *Synechocystis* sp. PCC6803.) while photon flux through culture remained the same in comparison with the wild type. That can be explained by the competing metabolic pathways – the microalgae is producing ethanol and thus using less carbon for biomass formation though absorbing the same amount of light.

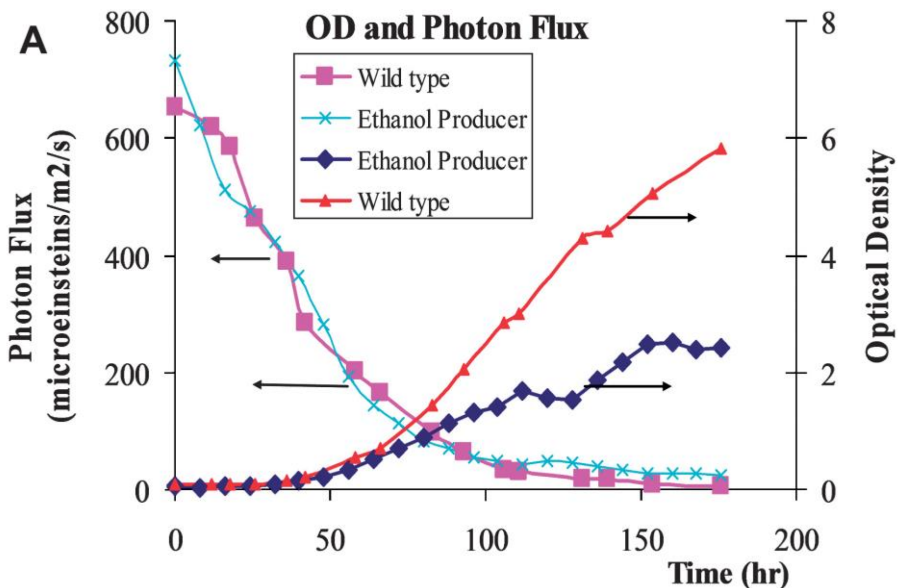


Figure 7 - Comparison of biomass development of WT vs GMO *Synechocystis* sp. PCC6803.

The situation where the concentration of ethanol would reach toxic levels and inhibit *Synechocystis* growth was also considered; however, experiments have concluded it requires up to 1 % v/v (~35 g/L) of ethanol in the media to have negative impacts on cell growth (12). Considering the studied range of cellular concentration, that probably will not be an issue. Overall, the species will be expected to have a similar behaviour to the one of the wild type but have a slower growth.

2.4 Pilot unit scale cultivation methodologies

As with most plants, each microalgae species grow best in specific conditions of pH, temperature, nutrient mix, light intensity and other factors. Microalgae have been grown in industrial scale for many years now – mainly for application in food and feed. Overall, there are three main cultivation methodologies for pilot scale units: raceways, tubular photobioreactors and green wall panels.

2.4.1 Raceways

Open ponds or raceways are shallow, annular channels where mixing can take place using paddle wheels or not happen at all. These are considered the most common cultivation systems worldwide. The key factor that plays in their favour is the low cost associated. Since the biggest advantage of these open ponds is their simplicity, it results in low production costs and low operating costs.

However, it also brings notorious disadvantages as open ponds include poor light use by the cells, evaporative losses, diffusion of CO_2 to the atmosphere, and requirement of large areas of land (1). Furthermore, contamination by predators and other fast growing heterotrophs have restricted the commercial production of algae in open culture systems to only those organisms that can grow under extreme conditions. Also, due to inefficient stirring mechanisms in open cultivation systems, their mass transfer rates are very poor resulting in low biomass productivity. Overall, open ponds are not considered the most effective cultivation systems. In average, 10 % of the solar light can be converted into chemical energy through photosynthesis due to limited light penetration into the turbid fluid. (15)

This system is at higher effectiveness in sunnier regions where the penetration rate of light in the pond reaches higher levels. Many companies have been trying out closed ponds where the control over the environment is much better than in open ponds. As a variation of the open pond system, the idea is to close the pond by covering it or placing it in a greenhouse. While this results in a smaller system, it reduces many of the disadvantages associated with the open system and allows more species to be grown.

Figure 8 - Open pond raceway type photobioreactor.

2.4.2 Tubular photobioreactors

Microalgae are also commonly cultivated in tubular photobioreactors. In general they are constructed with either glass or plastic tubes and the cultures are re-circulated either with a centrifugal pump or an airlift system. Tubular photobioreactors consist of straight, coiled or looped transparent tubing arranged in different ways to maximize sunlight incidence. Properly designed tubular photobioreactors are able to reasonably isolate the culture from potential contaminants and allow extended cultures duration.

Tubular photobioreactors are very suitable for both indoor and outdoor mass cultivation of microalgae since they have a large illumination surface area. However, this is also a disadvantage because it makes photo inhibition – lack of cell growth due to excess of light – much more frequent (16). The technology has the advantages of (i) not having limitations regarding the cultivation of microalgae; (ii) more efficient use of land, (iii) easier harvest procedure and (iv) better operation control. They are also relatively easier to clean once the cultivation is concluded. (17)

Common limitations are the mass transfer and temperature control. The first is caused by very high dissolved oxygen levels and the second is due to the lack of capacity to have control over temperature over the extension of the tubes as a whole.

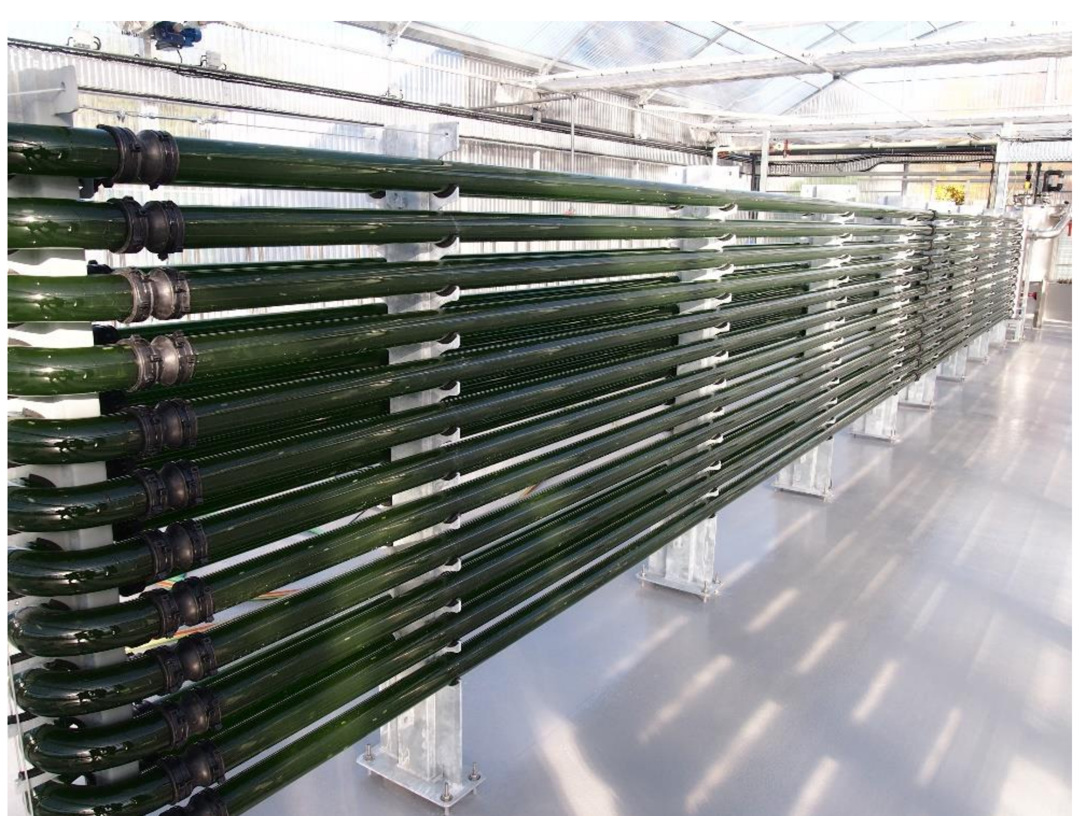


Figure 9 - Tubular photobioreactor.

2.4.3 Green wall panels

Green wall panels consist on thin polyethylene bags supported by a rigid metal framework. The air is supplied at the bottom which provides good overall mixing, a good supply of CO_2 and an efficient removal of O_2. This technology is commonly used because it has a low cost, high transparency and acceptable levels of contamination at start-up. The high surface of exposure to illumination provides for the growth of high densities of photoautotrophic cells. (17)

Although microalgae cultivation in this kind of systems is simple and widely employed, the corresponding technology is somehow primitive with the obvious constraints derived from high fragility and low versatility of the material used. Additionally, the scale-up reveals to be a problem as experiments have shown that increases in culture volume decrease bag productivity.

Figure 10 - Greenwall photobiorreactor

2.5 Monitoring methods in a microalgae cultivation

Microalgae cultivation requires close monitoring to ensure the proper growth and development of the cultures and to make sure the results of the process are the ones desired. For that, there are a series of methods employed to monitor microalgae cultures.

2.5.1 Optical density

Measuring the optical density (OD) of growing cultures is a common method to quantify culture growth. Online photometry allows continuous real time analysis of those parameters without any laboratorial work. Continuous measuring of optical density is the most basic and powerful tool for providing optimal yields and controlling reproducibility in many fermentation strategies. Measuring the OD is a common method to quantify the concentration of substances (Beer-Lambert law), since the absorbance is proportional to the concentration of the absorbing species in the sample. Photometers quantify the optical density of liquid samples by comparing the intensity of light that has passed through (I) and the intensity of the light before it enters the sample (I_o) (Equation 1 – Absorbance definition.). In spectroscopy the absorbance A is usually defined as:

$$A = -\log(\frac{I}{I_0})$$

Equation 1 – Absorbance definition.

Optical density can be measured through the use of different types of spectrophotometers equipment (microplate reader; cuvette reader). In the specific case of cyanobacteria, the optical density is measured at a wavelength of 730 nm (12).

2.5.2 pH and conductivity

The pH range has a large impact in most microalgae cultures. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. For the specific case of *Synechocystis* sp. PCC 6803 the optimal pH value is, approximately, 8.3 (14). In general, the addition of carbon dioxide (with consumption) allows adjust the values of the pH, which may reach limiting values of up to pH of 10,5 - 11 or under 7 during microalgae growth. In specific cases where the pH is lower than 7, it might be necessary to add sodium bicarbonate to increase the pH, provide a carbon source and avoid culture death.

Conductivity is a measure of the ability of water to conduct electric current. Conductivity can be used as a relative measure of general quality changes within a culture with time, in response to fresh water additions or nutrient supply. It is sensitive to variations in dissolved solids, mostly mineral salts and the degree to which these dissociate into ions. The amount of electrical charge on each ion; the ion mobility; and the water temperature all have an influence on conductivity. Conductivity is related to concentration of total dissolved solids plus major ions and is expressed as microsiemens per centimetre ($mS.cm^{-1}$).

2.5.3 Nitrogen measurement

Nitrogen measurement is one of the most important monitoring techniques in microalgae cultivation. Indeed, nitrogen is, next to carbon, a major element in microalgae nutrition. An essential factor of the preparation and administration of nutritive media is the form and amount in which the nitrogen is supplied to the culture. As such, the guiding values to nutritive media addition are the amount of nitrogen in the medium and the need of the culture. Nitrogen can be supplied either as nitrate (NO_3^-) or ammonia (NH_4^+). Moreover, and since other nutrients (apart from carbon) are given proportionally to nitrate, the availability of nitrogen (thus nutritive media) much be accounted.

Nitrates are measured by a spectrophotometric screening method following Lambert-Beer's law. The method consists in measuring the absorption at 220 nm which enables rapid determination of NO_3^-. However, and because dissolved organic matter also may absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement is made at 275 nm and used to adjust the NO_3^- value. The extent of the empirical correction is related to the nature and concentration of organic matter. Additionally, acidification with 1 M HCl is designed to prevent interference from hydroxide or carbonate concentrations up to 1000 mg $CaCO_3$/L. Chloride has no effect on the determination.

Upon determination of the amount of nitrate in the sample, the required amount of nutritive media to be added can be calculated.

2.5.4 Dry weight

Dry weight is an important parameter for the determination of biomass concentration, and further, productivity and quantification of biomass biochemical profile. It consists on the measurement of the mass of a specified volume of culture whose water was completely removed. The results allow obtaining the concentration of microalgae in g/L.

2.5.5 Cellular concentration

There are two main methods to determine the cellular concentration of microalgae samples: haemocytometer and electronic particle counter. The haemocytometer (Neubauer chamber) was developed for counting cells in blood samples. It consists in two mirrored surfaces which have a grid etched upon the surface. Each grid has 9 squares of 1 mm along the sides. These squares are further divided into smaller areas. Counting the cells per square allows calculating the density of cells in the sample.

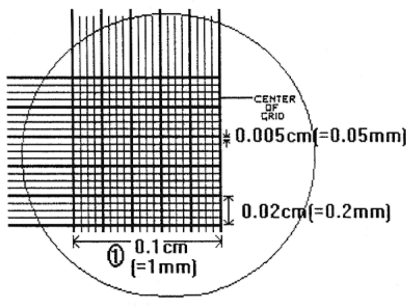


Figure 11 - Grid of Neubauer chamber.

The electronic particle counter operates under the principle that particles, suspended in an electrolyte solution, are sized and counted by passing them through an aperture having a particular path of current flow for a given length of time.

2.6.7 Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity.

These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

- The fluidics system transports particles in a stream to the laser beam for interrogation.
- The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.
- The electronics system converts the detected light signals into electronic signals that can be processed by the computer. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis.

When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them.

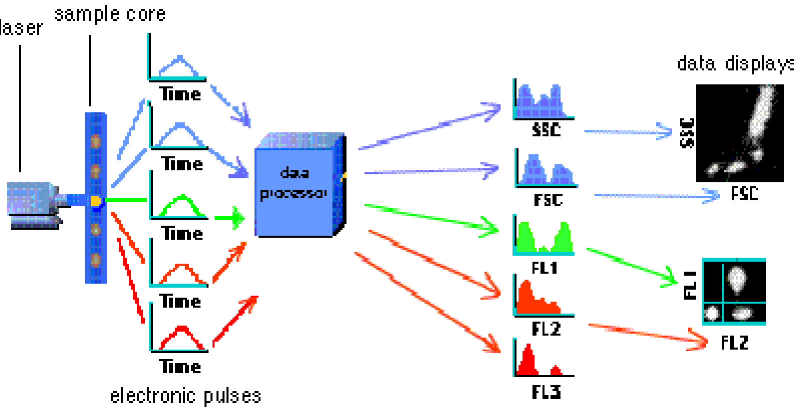


Figure 12- Scattered and emitted light signals are converted to electronic pulses that can be processed by computer.

2.6 Analytical methods for ethanol quantification in aqueous solutions

For the purpose of determining the amount of ethanol present in a microalgae culture, several analytical methods were identified and evaluated.

2.6.1 Enzymatic method for ethanol determination

This method is based in a two-step principle where ethanol is oxidized to acetaldehyde in the presence of alcohol dehydrogenase and NAD^+ (nicotinamide adenine dinucleotide) (Equation 2). The reaction equilibrium however lies on the ethanol side so it is necessary to trap the products. For that, aldehyde dehydrogenase is added to convert the acetaldehyde to acetate (Equation 3).

The method relies on the spectrophotometric measurement of the NADH product of the reaction at 340 nm. The values obtained allow to, stoichiometrically, calculate the amount of ethanol present in the sample.

$$Ethanol + NAD^+ \xrightarrow{ADH} Acetaldehyde + NADH + H^+$$

Equation 2 - Enzymatic method first reaction.

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

Equation 3 - Enzymatic method second reaction.

This method is only linear when the cuvette has between 0.25 and 12 µg of ethanol which implies that dilution is required as described in Table 4 - Dilution factor for different ethanol concentrations.

Table 4 - Dilution factor for different ethanol concentrations.

Estimated concentration of ethanol (g/L)	Dilution factor
< 0,12	1
0,12 – 1,2	10
1,2 – 12	100
12 – 120	1 000
>120	10 000

The test procedure consists in first detecting the absorbance of a mixture of water, NAD^+, the sample (or water in the case of the blank) and aldehyde-dehydrogenase (A_1) and then adding the alcohol-dehydrogenase to start the reaction and measure the absorbance once more after a period of time (A_2). The result obtained from the difference of the differences (A_2-A_1) of the sample and the blank (Equation 4 - Enzymatic method absorvance calculation.) is used to determine the ethanol concentration (Equation 5 - Enzymatic method concentration calculation.).

$$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$$

Equation 4 - Enzymatic method absorvance calculation.

$$c = \frac{V \times MG}{\varepsilon \times d \times v \times 2 \times 1000} \times F \times \Delta A$$

Equation 5 - Enzymatic method concentration calculation.

where, V = final volume (mL); v = sample volume (mL); MW = molecular weight of ethanol (g/mol); d = light path (cm); ε = extinction coefficient of NADH at 340 nm (m^2/mol); F = dilution factor applied.

Note that, despite the method reliability and its widely usage as a mean to determine the amount of ethanol present in liquids for a large range of concentrations, its highly sensitive which means that it has to be performed in an ethanol free atmosphere. Any ethanol manipulation happening nearby may have an impact in the results of the test. This method does not suffer the interference of aldehydes and ketones due to the order of reagent addition. Methanol is also not an issue because of unfavourable K_m values. The only interferences possible come from n-butanol and n-propanol. Secondary and tertiary alcohols do not react at all.

2.6.2 Redox titration

This method is the basic redox titration and it allows finding the concentration of ethanol in an aqueous solution. The ethanol is oxidised to acetic acid by reacting with an excess of potassium dichromate in acid.

$$2Cr_2O_7^{2-} + 16H^+ + 3C_2H_5OH \xrightarrow{yields} 4Cr^{3+} + 11\,H_2O + 3CH_3COOH$$

Equation 6 - Chromium oxidation of ethanol.

The amount of unreacted dichromate is then determined by adding potassium iodide solution which is also oxidised by the potassium dichromate forming iodine.

$$Cr_2O_7^{2-} + 14H^+ + 6I^- \xrightarrow{yields} 2Cr^{3+} + 7\,H_2O + 3I_2$$

Equation 7 - Iodine reduction.

The iodine is then titrated with a standard solution of sodium thiosulfate and the titration results are used to calculate the ethanol content of the original solution.

$$2S_2O_3^{2-} + I_2 \xrightarrow{yields} S_4O_4^{2-} + 6I^-$$

Equation 8 - Iodine oxidation.

Because alcoholic beverages such as wine or beer contain other oxidizing substances that could interfere with the titration, the dichromate solution is placed in a flask and the alcoholic beverage sample is suspended in a small container above it. The water and ethanol slowly evaporate and as the ethanol becomes in contact with the dichromate it first dissolves, and only after it is oxidised. More ethanol evaporates until eventually all the ethanol from the beverage has reacted with the dichromate. Since this mass transfer is time consuming, it is necessary to place the flask with the suspended sample (Figure 13 - Flask of ethanol with the dichromate recipient.) in a warm place overnight.

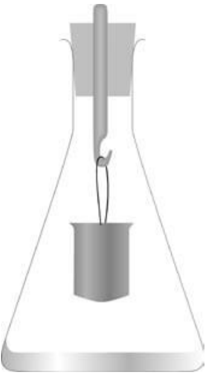


Figure 13 - Flask of ethanol with the dichromate recipient.

This method, as mentioned, is not very practical due to the extended time necessary to carry out an analysis. It also has the additional flaw of interfering with other volatile components susceptible of being oxidized.

2.6.3 Refractive index

In optics, the refractive index or index of refraction of a substance (optical medium) is a dimensionless number that describes how light, or any other radiation, propagates through that medium. It is the ratio between the velocity of light going through a medium and the velocity of light in vacuum. For example, the refractive index of water is 1.3330 which means the light travels 1.3330 slower in water than in vacuum.

The refractive index determines how much light is distorted, or refracted, when entering a material. Therefore, different materials have different refractive indexes and, in the event of material combination, the refractive index will present a value in between the refractive indexes of the two pure substances.

Figure 14 - Portable refractive index apparatus.

Table 5 - Refractive indexes of water and ethanol.

Medium	Refractive Index
Water	1,3333
Ethanol	1,3571

There is a reasonable difference between refractive indexes of ethanol and water which allows determining the ethanol concentration based in this principle. However, a handicap to this method may be the presence of other elements in the sample (such as dissolved salts) which may generate appreciable deviations from the correct analysis. While in a controlled environment this can be handled by controlling the amount of dissolved salts and adjusting a calibration curve to them, in larger scales it can become an issue due to diversity of the sample.

2.6.4 Pycnometry

Pycnometry is based on the principle of relative density. Relative density, or specific gravity, is the ratio of the density (mass of a unit volume) of a substance to the density of a given reference material.

Pycnometry is done with a pycnometer or specific gravity bottle (Figure 15 - Glass pycnometer.). This device allows determining the density of the liquid. A pycnometer is usually made of glass, with a close-fitting ground glass stopper with a capillary tube through it, so that air bubbles may escape from the apparatus. This device enables a liquid's density to be measured accurately by reference to the appropriate media fluid, such as water or mercury.

If the flask is weighed empty, full of water, and full of a liquid whose relative density is desired, the relative density of the liquid can easily be calculated.

Pycnometry allows the calculation of the composition of a mixture by considering that the volume proportion to the mass is constant. That makes the density of the substance proportional to mass addition which allows the calculation of the mass fractions of the mixture.

Figure 15 - Glass pycnometer.

Pycnometry is a fairly simple and straightforward method. However, it is rather sensitive to manipulations and not very efficient at sample preservation.

2.6.5 Gas chromatography

Gas chromatography (GC) is a common type of chromatography used to separate compounds that do not decompose when vaporized. GC is done with a carrier gas (usually helium or nitrogen) which acts as the mobile phase and a liquid stationary phase. The gaseous compounds under analysis interact with the walls of the column - which cause the different retention times.

The main differences between GC, High-Performance Liquid Chromatography (HPLC) and Thin-layer Chromatography (TLC) are in the stationary phase which is usually solid and the temperature control of the gas column. GC is considered to be similar to fractional distillation in regards to separation.

Headspace gas chromatography with flame-ionization detection (HS-GC–FID, Figure 16 - Gas Chromatography set-up.) has, for years, become the standard for ethanol analysis because of its ease of automation, sensitivity, accuracy, and specificity.

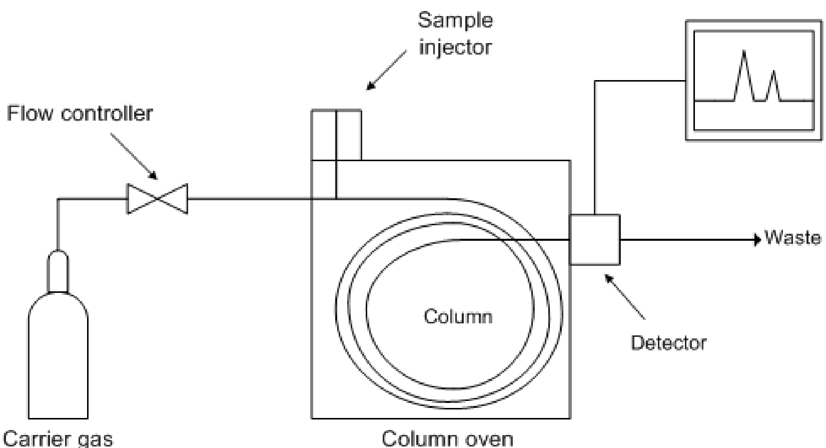


Figure 16 - Gas Chromatography set-up.

As limitation, not every sample is fit for GC – especially if is a culture of microalgae – additional processing is required, filtering at 0.22 μm.

2.6.6 Near infra-red spectroscopy

Near-infrared spectroscopy (NIR) is a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum - from about 800 nm to 2500 nm. In recent years, infrared spectroscopy has become an important analytical tool in the context of complex bioprocess fluids. NIR allows near real-time bioprocess monitoring and permits simultaneous analysis of several components within the same sample.

The main advantage of the technique is that NIR penetrates much further into a sample than mid infrared radiation. Near-infrared spectroscopy is very useful in probing bulk material with little or no sample preparation. NIR allows creating a calibration curve which makes the amount of ethanol in any substance easily measurable.

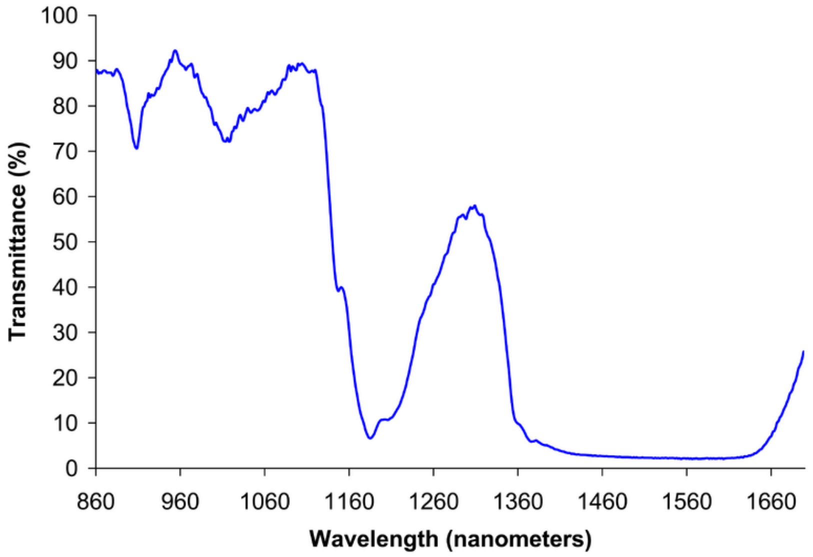


Figure 17 - Near Infrared ethanol band.

3. Microalgae Production

3.1 Optimisation of routine follow-up procedures for *Synechocystis* cultivation

Each type of microalgae culture has its own particular characteristics. While the monitoring methodologies apply to most cultures, using one single result such as OD to determine the other parameter (dry weight, cellular concentration…), the correlation for each parameter needs to be optimised for each specific microalgae. Doing so, it generates the possibility of interpreting data more efficiently and expanding the understanding of the microalgae behaviour. This means establishing correlations between monitoring methodologies that allow for better understanding of culture growth, productivity and how can that relate to the microalgae products. The three methods that were correlated are: OD (using a microplate reader) at 730 nm, cellular concentration and dry weight are summarized in Table 6. The extended results are presented in 7.1 Annex 1.

Table 6. Correlations for *Synechocystis* sp. PCC 6803 Kaplan summarized.

Dry weigth (g/L)	$DW = 0{,}277 \times OD_{730}\ (g/L)$
Cellular concentration (cel/mL)	$CC = 5{,}7067 \times 10^{7} \times OD_{730}\ (cel/mL)$

3.2 Optimization of nutritive media for *Synechocystis* sp. PCC 6803

Nutritive medium is defined as media type that support the growth of microorganisms. Also, nutritive media consists in general a combination of nitrogen, phosphate and trace metals which have a role in the microalgae growth and development. Nutritive media are prepared according to recipes, put into appropriate culture vessels and stored until needed. Before storing, the media were sterilized through filtration.

Nitrate is the nitrogen source used in culture media and the point of primary intracellular nitrogen assimilation into the organic linkage. Inorganic (ortho)phosphate, the P form preferentially used by microalgae, is added to culture media. Most microalgae are capable of producing cell surface phosphatases which allow them to utilise this as a phosphorus source. The trace metals which are essential for microalgae growth are incorporated into essential organic molecules, particularly a variety of coenzyme factors which enter into photosynthetic reactions. Of these metals, the concentrations (or, more accurately, the biologically available concentrations) of Fe, Mn, Zn, Cu and Co (and sometimes Mo and Se) in natural waters may be limiting to algal growth.

The tested nutritive media were:

- BG-11 – Castenholz (1988) Methods in Enzymology 167, 68-93;
- MMF – industrial recipe prepared at laboratory scale (composition is in Annex 2);
- Hubel_7– industrial recipe acquired externally.

3.2.1 Experimental setting and conditions

The setting consisted of 6 bubble columns with duplicates of the 3 nutritive media at test (Figure 18 - Airlift set up with different nutritive media.). The objective of the setting was, as mention above, to study the growth and behaviour of *Synechocystis* sp. PCC 6803 in the nutritive media (BG-11, MMF and Hubel) and to determine the growth rates for each nutritive medium.

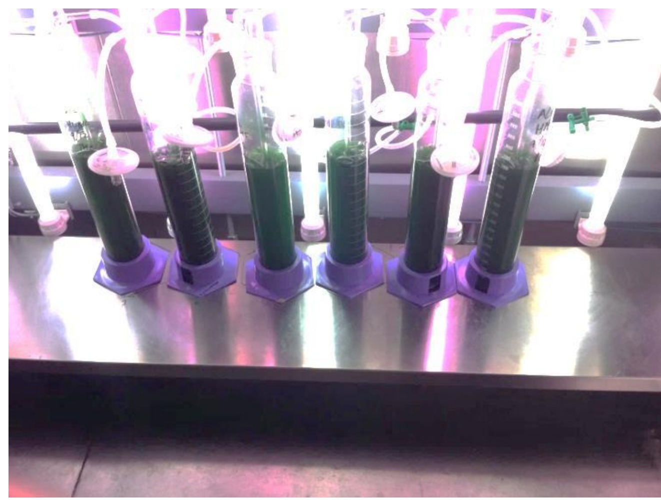


Figure 18 - Airlift set up with different nutritive media.

The experimental conditions for the essay are described in Table 7 - Experimental conditions. On a daily basis, before collecting samples, the volume of the bubble columns was adjusted to compensate the volume losses due evaporation.

Table 7 - Experimental conditions.

Species	Synechocystis sp. PCC 6803
Inoculum	Scale-up culture in 6 L round flasks
Initial cell concentration	OD_{730} > 0,2
Culture volume	700 mL
Carbon source	Air enriched with 0,5% of CO_2
Daily Renewal rate	25 %
Nitrate concentration	6 mM (Mon – Thu); 8 mM (Fri)
Temperature	25 ºC
Pressure	atmospheric
Light source (intensity)	Fluorescent lights (170 µmol/m²/s)
Photoperiod	Continuous
Nutritive media	BG-11; MMF; Hubel_7

Since BG-11 is used as a cultivation medium rather than a nutritive medium, it was concentrated 40 times in order to be used as such. Moreover, and due to this fact, the water used in the beginning of the test was sterilized and demineralized. After a first collection of samples for elemental analysis, a second phase of the test was started on the 18th day of the test, by initiating the use of tap water to supplement the cultures after renewal/evaporation. After another 3 weeks, new samples were collected for elemental analysis.

Additionally, it was observed that the bubble columns should be directly inoculated from the culture in scale-up and through culture re-suspension in demineralized water, as the sudden change in medium may result in culture death due to low pH – caused by lack of growth and CO_2 dissolution in the medium. This caused the test to be restarted after 1 week.

3.2.2 Experimental results

Figure 18 depicts the culture evolution in stationary phase.

Figure 19 - Culture evolution in stationary phase.

3.2.2.1 Productivity

Making use of the correlations and monitoring methods established, it became possible to track and evaluate the productivity of the culture in different nutritive media over time. Biomass concentration is assessed through the use of the correlation between OD_{730} and dry weight (Table 6). Productivity is then determined by the daily renewal rate (Figure 22).

Table 8 - Productivity correlation

Productivity (g/L/day)	$Pr = 0{,}277 \times OD_{730} \times Renewal\ rate\ (\%)$ (g/L/day)

It is observed that the BG-11 medium has a much superior performance during the phase 1 (demineralized water) in opposition to the other two nutritive media. Also, considering that the inoculum was not washed and therefore carried some nutrients, the decrease in productivity of MMF and Hubel_7 observed after the 10th day is an indicator that one or more nutrients may have been exhausted. Once the tap water replaced the demineralized water we started observing an equality of productivities (Figure 22).

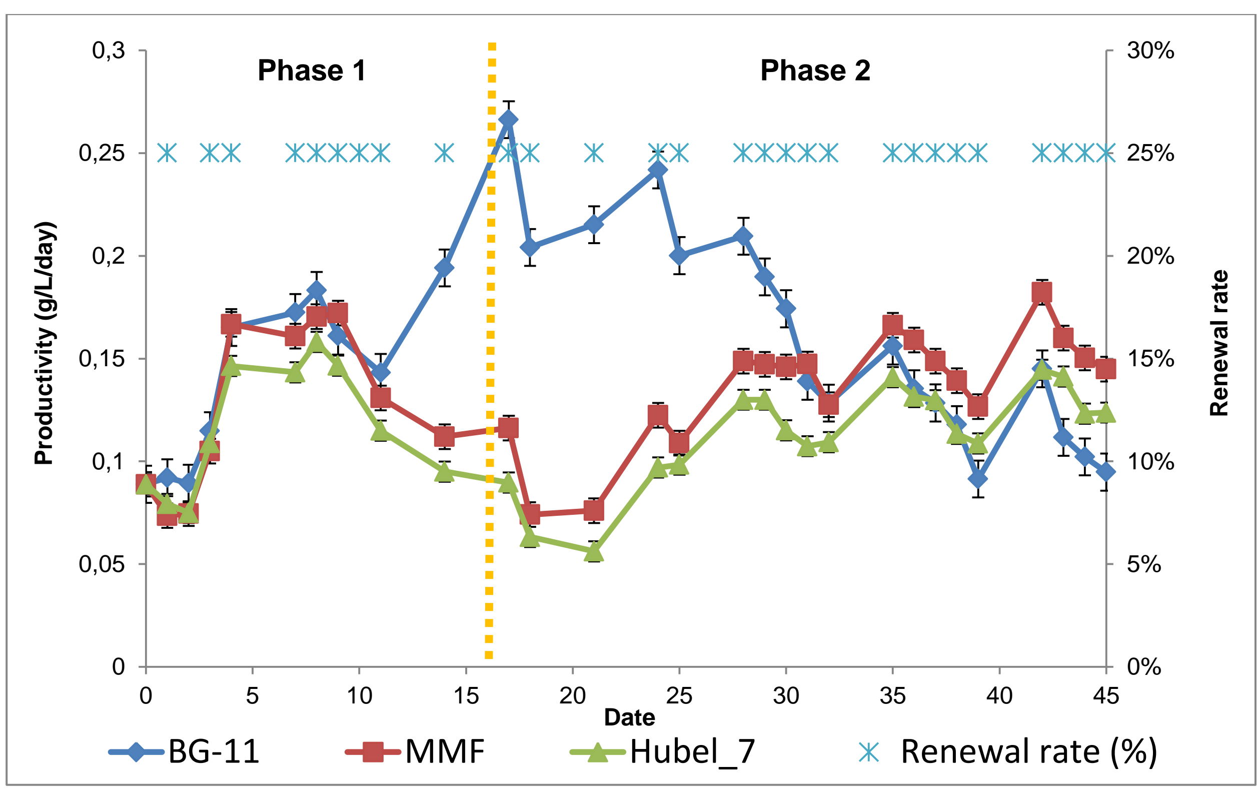


Figure 20 - Productivity throughout the duration of the test.

The medium Hubel_7, even though not as productive as the MMF, revealed itself to be very consistent throughout the test showing few variations past the point the tap water replaced demineralized water. The average value of the biomass production was calculated once the stationary phase initiated (Day 28) and is reported in Table 9 - Average biomass concentration and productivity of each medium from day 29 to day 45.

Table 9 - Average biomass concentration and productivity of each medium from day 29 to day 45.

Nutritive Medium	Average biomass concentration (g/L)	Average productivity (g/L/day)
BG-11	0,550 ± 0,027	0,137 ± 0,007
MMF	0,598 ± 0,051	0,150 ± 0,013
Hubel_7	0,500 ± 0,030	0,125± 0,014

Results of elemental analysis carried out to the nutritive medium, the laboratory tap water and the supernatant of both phase 1 and 2 are compiled in Tables 9 to 12. From the comparison between nutritive media it is concluded (Table 9):

- MMF and Hubel_7 formulation is similar apart from the magnesium and the calcium concentration values which are significantly higher in the Hubel_7 formulation. This is due to impurities in the industrial raw materials used to produce the nutritive media.
- BG-11 has a completely different nutrient concentration profile. Main differences from the other 2 media are (i) presence of only 1/3 of the phosphorus concentration from the recipe; (ii) almost no iron is present in this media; (iii) high Mg, Ca and Co concentrations.
- Tap water main ions contributing to the nutritive media are Mg and Ca.

Table 10 – Elemental analysis comparison between nutritive media and tap water.

Medium	BG-11	MMF	Hubel_7	Tap water
N (mM)	1000			
Nutrient	(mM)	(mM)	(mM)	(mM)
P	11,55	34,91	30,50	0,00003
Mg	15,26	0,61	4,24	0,07760
Zn	0,12	0,41	0,34	0,00148
Mn	0,09	0,36	0,62	0,00002
Mo	0,54	0,38	0,10	0,00001
Co	0,22	0,04	0,05	0,00002
Cu	0,06	0,04	0,05	0,00002
Fe	0,04	5,69	4,65	0,00013
B	6,87	0,03	0,16	0,00194
Ca	3,68	0,80	4,78	0,26900

The elemental analysis of the supernatant at the end of phase 1 and phase 2 shows that the decrease in productivity of BG-11 was most likely due to lacking phosphorus, iron and cobalt (Table 10). Indeed, BG-11 media is typically a culture media buffered to 8.3. However, it was prepared a concentrated solution to be used as a nutritive media. This concentrated solution showed formation of precipitates and when sterilization through filtration at 0.22 μm was carried out most of precipitates (most certain iron phosphate) were removed in the filter which leads to a depletion of these vital nutrients in the media. Also, the calcium concentration reflected the change from demineralized to tap water increasing from 0.27 mM to 0.51 mM. However, the magnesium concentration did not reflect an increase since during phase 1 an accumulation was already observed.

Table 11 – Comparison between BG-11 supernatant from phase 1 and phase 2.

BG-11	Supernatant					
	End of phase 1			End of phase 2		
N (mM)	4,19			6,86		
Nutrient	Recipe (mM)	Supernatant (mM)	Δ (%)	Recipe (mM)	Supernatant (mM)	Δ (%)
P	0,0484	0,0205	-58	0,0792	0,0033	-96
Mg	0,0640	0,2751	330	0,1047	0,2365	126
Zn	0,0005	0,0045	812	0,0008	0,0040	398
Mn	0,0004	0,0061	1475	0,0006	0,0007	9
Mo	0,0023	0,0025	11	0,0037	0,0044	18
Co	0,0009	0,0002	-78	0,0015	0,0001	-93
Cu	0,0002	0,0008	259	0,0004	0,0009	140
Fe	0,0086	0,0046	-46	0,0140	0,0015	-90
B	0,0288	0,0839	191	0,0472	0,1244	164
Ca	0,0154	0,2722	1667	0,0252	0,5077	1913

Regarding the MMF and Hubel_7 nutritive media, the decrease in productivity observed from test day 8 to 15 when using demineralized water is mainly attributed to lack of magnesium. Indeed, the

concentration of magnesium in MMF is the lowest of the three nutritive media and the absolute value of Mg concentration in phase 1 in both MMF and Hubel_7 is extremely low, < 0.03 mM. After the replacement for tap water with a Mg concentration of 0.08 mM, the concentration of Mg increase so the productivity (Table 12 - Comparison between MMF supernatant from phase 1 and phase 2.and Table 13 - Comparison between Hubel_7 supernatant from phase 1 and phase 2.).

Table 12 - Comparison between MMF supernatant from phase 1 and phase 2.

MMF	**Supernatant**					
	End of phase 1			End of phase 2		
N (mM)	6,96			6,5110		
Nutrient	Recipe (mM)	Supernatant (mM)	Δ (%)	Recipe (mM)	Supernatant (mM)	Δ (%)
P	0,2430	0,3045	25	0,2273	0,1320	-42
Mg	0,0042	0,0274	550	0,0839	0,0300	-64
Zn	0,0028	0,0099	250	0,0026	0,0041	57
Mn	0,0025	0,0046	82	0,0024	0,0025	6
Mo	0,0027	0,0046	72	0,0025	0,0029	16
Co	0,0003	0,0004	54	0,0003	0,0006	144
Cu	0,0003	0,0012	358	0,0002	0,0004	50
Fe	0,0396	0,0511	29	0,0371	0,0305	-18
B	0,0002	0,0394	-	0,0002	0,0412	-
Ca	0,0055	0,1531	-	0,0052	0,2718	-

The difference in productivity observed between MMF and Hubel_7 is believed to be due to (i) the lack of manganese in the Hubel_7 formulation and (ii) the presence of large amounts chromium in the Hubel_7 formulation, more 1000x in Hubel_7 than MMF. The two recipes are very similar and so the behaviour of the species growth to the nutritive media.

Table 13 - Comparison between Hubel_7 supernatant from phase 1 and phase 2.

Hubel_7	**Supernatant**					
	End of phase 1			End of phase 2		
N (mM)	6,05			7,23		
Nutrient	Recipe (mM)	Supernatant (mM)	Δ (%)	Recipe (mM)	Supernatant (mM)	Δ (%)
P	0,1846	0,2145	16	0,2206	0,3043	38
Mg	0,0256	0,0163	-36	0,1106	0,0989	-11
Zn	0,0021	0,0048	130	0,0025	0,0064	159
Mn	0,0037	0,0038	0	0,0045	0,0030	-34
Mo	0,0006	0,0014	142	0,0007	0,0016	134
Co	0,0003	0,0003	4	0,0004	0,0005	42
Cu	0,0003	0,0008	171	0,0004	0,0010	169
Fe	0,0281	0,0471	68	0,0336	0,0688	105
B	0,0010	0,0352	-	0,0012	0,0648	-
Ca	0,0289	0,1276	-	0,0345	0,4832	-

It is possible to conclude that *Synechocystis* sp. PCC 6803 Kaplan can grow under industrial nutritive medium without requiring major adjustments. However, and towards an industrial application is it mandatory to perform the same test with water recycling and an optimized recipe for the nutritive media. Results from such test such translate into a full optimization of the nutritive media for an industrial large scale application.

3.3 *Synechocystis* tolerance to different types of ethanol

Ethanol is commercially available in different grades and types of purity. Additionally, different types of ethanol have different specifications – specially related to being denatured or not. The denaturing process, which depends on country legislation, was created to prevent ethanol consumption as a spirit. In Portugal, the law defines that denaturing must occur partially on 0.25 % (m/V) with an anti-septic and corrosive substance named cetrimide (cetyltrimethylammonium bromide). Alternatively, non-denatured ethanol is heavily taxed and much more expensive.

In this scenario, and in order to minimize costs in purchasing ethanol, two tests were set to adequately evaluate *Synechocystis* tolerance to denatured ethanol and then to ethanol itself.

3.3.1 Experimental conditions

Table 14 - General experimental conditions.As it was necessary to test both the tolerance to denatured ethanol and to ethanol itself, two different test conditions were set – which are expressed in Tables 14, 15 and 16.

Table 14 - General experimental conditions.

Species	*Synechocystis* sp. PCC 6803
Nitrate concentration	6 mM
Nutritive medium	Hubel_7
Initial cell concentration	OD_{730} nm > 0,2

The first test consisted in two parallel systems – a flask and a bubble column – with respective duplicates as control. Both systems were kept at 10 g/L concentrations in the specified conditions in Table 15.

Table 15 – Experimental conditions of test 1.

System	Ethanol concentration (commercial 96 % v/v)	Final volume	Agitation	Inoculum	Light intensity	CO_2 Source	Temperature / Pressure	Photoperiod
AL2 (control)	0 g/L	700 mL	Aeration	LL12.5.1d	170 µmol/m2/s	Air + 0,5 % CO_2	25 ºC	24 h
AL5	10 g/L							
B1 (control)	0 g/L	100 mL	170 rpm	LL12.5.1d	30 µmol/m2/s	Air diffusion	25 ºC	
B2	10 g/L							

The second test consisted in three flasks each having a different concentration of ethanol as expressed in table 16:

Table 16 - Test 2 conditions.

System	Ethanol concentration (99.5 %)	Final volume	Inoculum	Agitation	Light intensity	Temperature	Photoperiod
B1	0 g/L	100 mL	scale-up culture in 6 L round flasks	170 rpm	30 µmol/m2/s	25 ºC	24 h
B2	5 g/L						
B3	10 g/L						

3.3.2 Discussion

The first test resulted in short-term death of cultures with the flasks with commercial ethanol losing pigmentation and cell activity after 3 days and the bubble columns after 4. Results from flow cytometry indicate that the flask and airlift with commercial ethanol have no cells with enzymatic activity – thus concluding the culture death.

Figure 21 - Evolution of the Erlenmeyrs cultures (control and with ethanol 96%).

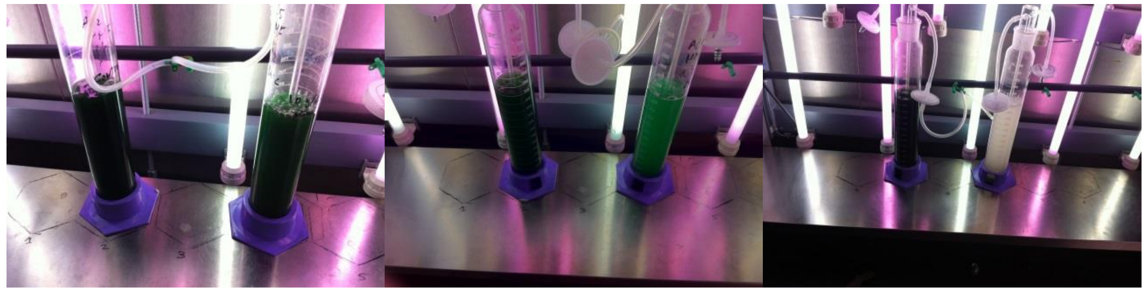


Figure 22 - Evolution of the airlift system cultures (control and with ethanol 96%).

Table 17 - Flow Cytometry results

Sample	Cells with enzymatic activity (%)	Cells with intact cell membrane (%)
AL2 (control)	94,6	98,6
AL5	0	0
B1 (control)	94,5	99
B2	0	1,5

The second test did not result in the death of cultures as before but a severe contamination was observed in both the flasks containing ethanol.

3.3.3 Conclusion

This test allowed drawing a few conclusions regarding *Synechocystis* behaviour when subject to ethanol – commercial and not commercial. The first conclusion obtained is that the tests in the PBR cannot be done with commercial ethanol as the presence of cetrimide is likely to kill the culture.

The second and main conclusion indicates that ethanol will probably support the development of contaminants in the culture (bacteria and fungi) as it represents an additional carbon source for them to develop. This conclusion might imply the need of antibiotic addition to the process or a tight control of the ethanol concentration in the PBR.

3.4 Pilot-scale Production

As part of the operational tests, it becomes necessary to evaluate the behaviour and development of *Synechocystis* sp. PCC 6803 Kaplan in a conventional tubular PBR. This experiment aimed to evaluate the culture's productivity over time, to evaluate the culture conditions regarding contamination and cell morphology and the behaviour of the culture in a PBR projected for this purpose.

3.4.1 Experimental conditions

The PBR was installed in a greenhouse facility which was equipped with temperature, luminosity and humidity control. The PBR, with the main features detailed in Table 16, was set to the cultivation conditions summarized in Table 17.

Table 18 - PBR features summary

Pump Type	Centrifugal pump – ITT Lowara 500/30/P
Power	3 kW
Frequency	30 Hz
PBR total volume	1,305 m^3
Tank culture volume	350 L
Tubes material	Glass
Tank material	Stainless Steel 316

Table 19 - Cultivation conditions summary.

Species	*Synechocystis* sp. PCC 6803
Volume	1,155 m^3
Inoculum	10 6L balloons from scale-up cultures
Salinity	0 g/L
Renewals	25%
Temperature and Pressure	Atmospheric (inside greenhouse)
Light	Natural
Culture Medium	Phase 1 - MMF (2 M) Phase 2- Hubel_7 (1 M)
Final Nutrient concentration	6 mM (Mon-Thru); 8 mM(Fri)
pH set point	8.3
CO_2 source	Pure CO_2

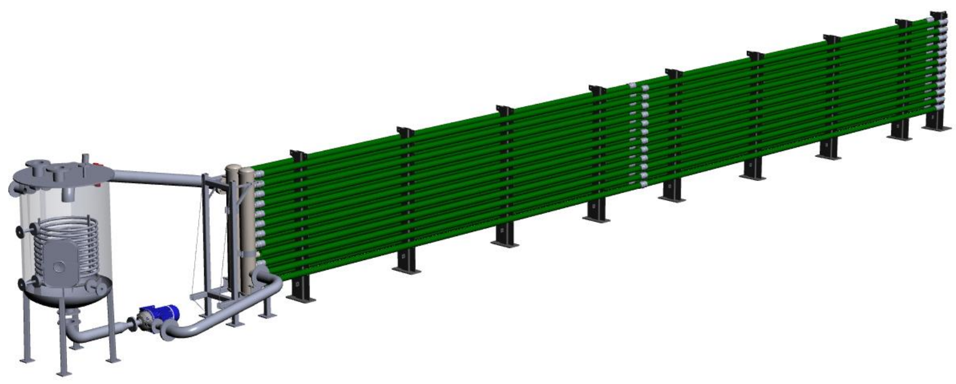


Figure 23 - PBR set-up

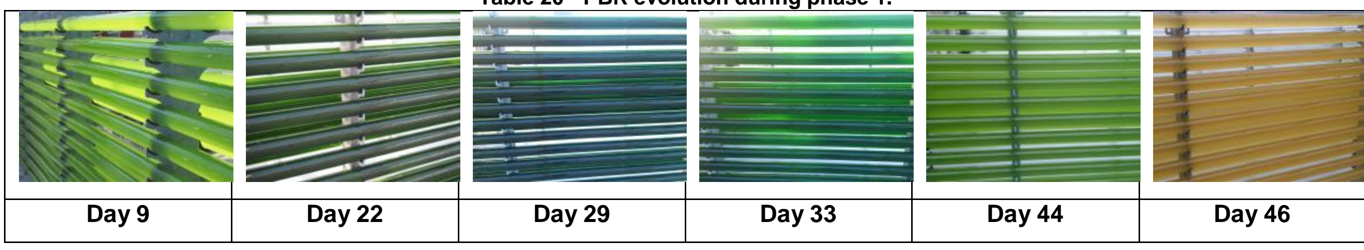
3.4.2 Results

The test was divided into 2 phases: (1) batch growth until stationary state was reached and afterwards renewals in order to determine the productivity rate; (2) renewals started once the set concentration value was achieved. Also, in phase 1 it was tested MMF as the nutritive media whereas in phase 2 the industrial Hubel_7 media was used.

3.4.2.1 Phase 1 – MMF nutritive media

The test lasted for 46 days with the batch period cultivation for 30 days and a semi-continuous operation during the remaining 16 days. Table 18 depicts the PBR macroscopic evolution throughout the essay.

Table 20 - PBR evolution during phase 1.



Day 9	Day 22	Day 29	Day 33	Day 44	Day 46

The biomass concentration over time is represented in Figure 24. Typical evolution is observed during the batch phase: (1) initial exponential evolution followed by (2) a linear increase of biomass result of moderate light limitations due to microalgae self-shading. The stationary state was reached at a concentration of 0.75 g/L. Although the dry weight is not significantly higher when compared to other species, since the microalgae size is approx. 1 to 2 μm, the cellular concentration was 1,5 x 10^8 cells/mL.

Once renewals started - approximately 25 % of the PBR volume -, the culture concentration decreased, with the culture not being able to have a productivity rate to match the amount of biomass removed from the PBR on a daily basis. As the culture concentration decrease, a contamination of ciliates, feeding of the microalgae, were developed and lead to culture death.

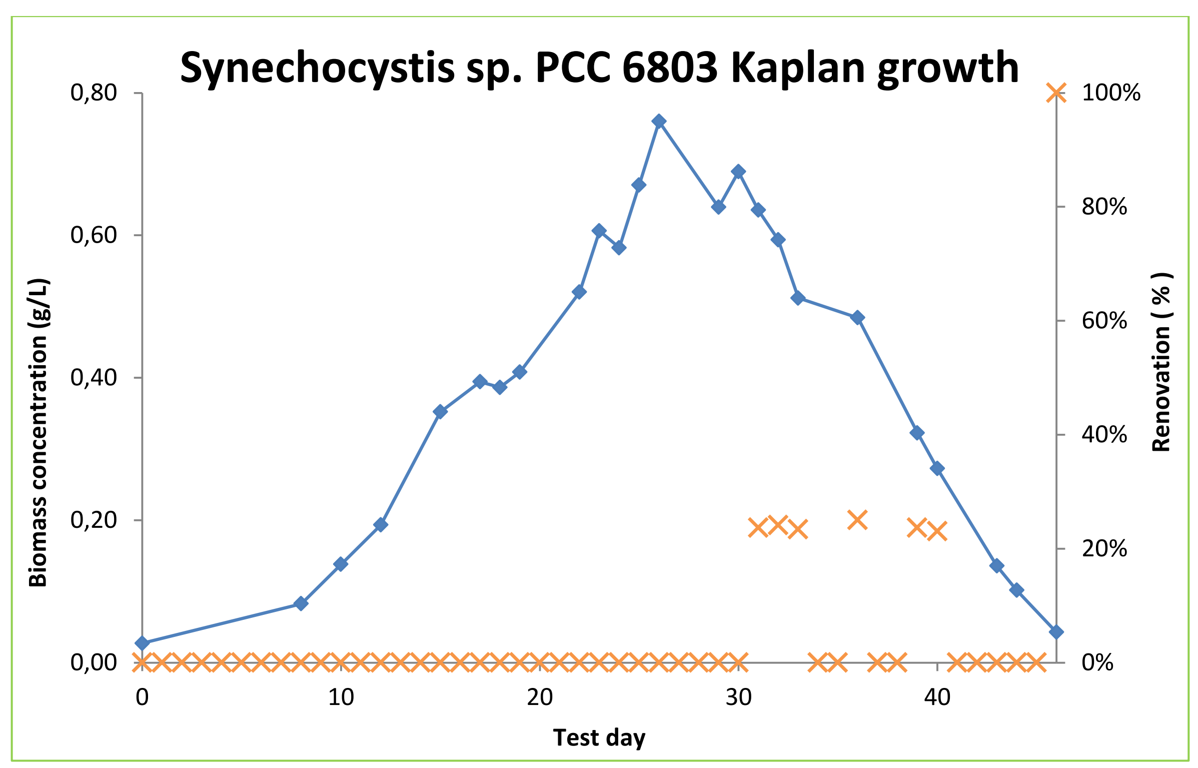


Figure 24 – Biomass concentration evolution over time during batch phase and semi-continuous phase.

3.4.2.2 Phase 2 – Hubel_7 nutritive media

The test lasted for 51 days with the batch period cultivation for 20 days and a semi-continuous operation during the remaining 31 days. Table 19 depicts the PBR macroscopic evolution throughout the essay. Renewal rate was reduced to 15% at an initial stage to reduce its impact in culture growth.

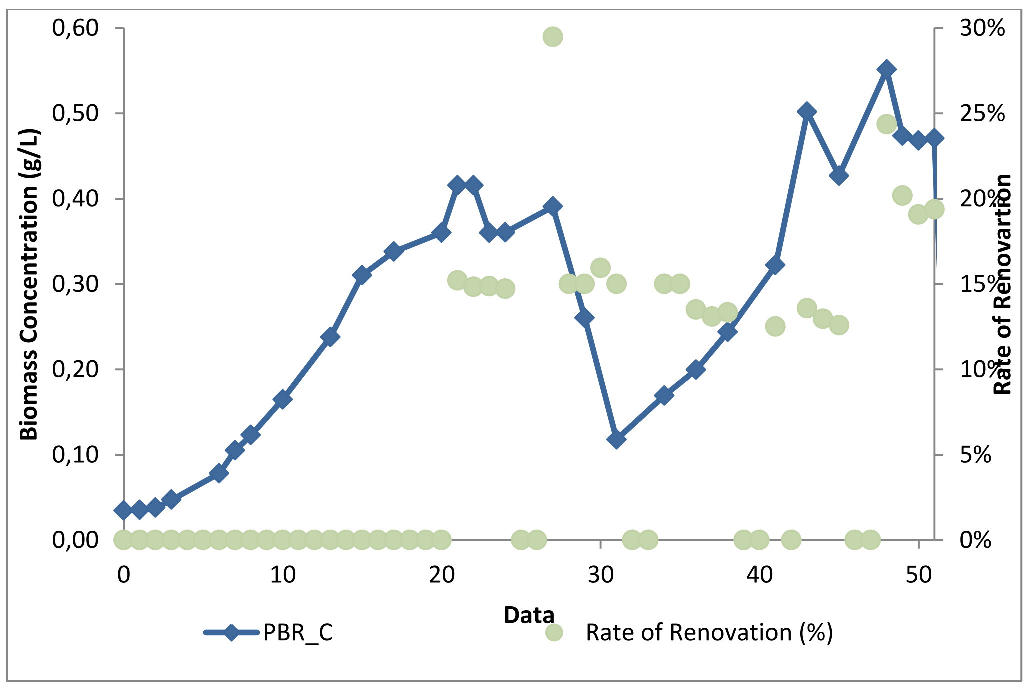
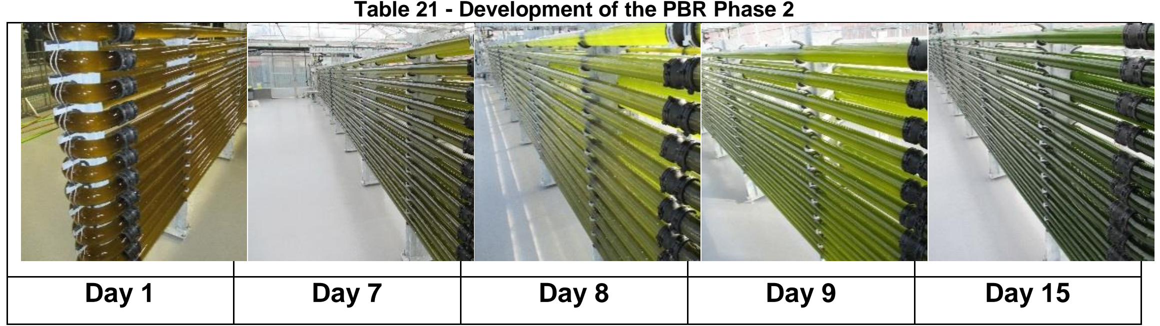


Figure 25 - Productivity of the PBR during the Phase 2

The biomass concentration over time is represented in Figure 26. Once again, the evolution is typical during the batch phase: (1) initial exponential evolution followed by (2) a linear increase of biomass result of microalgae self-shading. The stationary state was reached at a concentration of 0.37g/L. To respond to the sudden drop in productivity the nitrate set point was raised to 8 mM. This caused the culture to respond to that raising productivity and it allowed raising the renovation rate with success to 20%. However, with time, further contamination was noticed and by day 40 there was contaminated with fungi, bacteria, ciliates and other microalgae (specifically *chlorella*) – although not very intense.

Table 21 - Development of the PBR Phase 2



Day 1	Day 7	Day 8	Day 9	Day 15

3.4.3 Conclusion

This test allowed concluding that the production of *Synechocystis* in a pilot scale PBR is viable with both MMF and Hubel 7 as nutritive media. It allowed identifying diverse contamination focuses (ciliates, other microalgae, etc) which will allow implementing anti-contamination measures. Finally, it also allowed understanding the ideal set point for renovations and culture productivity – 20 %. However, it is noticed that further optimization of the nutritive media will be needed at industrial scale.

4. Methodologies

4.1 Tested techniques

4.1.1 Near-Infrared Spectroscopy

The NIR (Near Infra-Red) spectroscopy test was done recurring to a piece of equipment named Spectralizer™.

Figure 26 - Spectralizer™

This equipment is specialized in quality control functioning based on references stored in its memory – specifically a calibration curve – which it uses to evaluate diverse parameters of a solution at the same time (ethanol, organic acids, sugars and pH estimations). Contrarily to most NIR spectrophotometers, the Spectralizer does not provide a continuous spectrum – it uses a serious of filters to measure each wavelength. The equipment is ready to analyse both solid and liquid samples. These are aspirated into the machine through a tube and analysed during 1 minute. The equipment software provides the data in the form of graphics and figures but does not provide the actual numbers – they need to be collected manually.

The features of the equipment are the following:

Table 22 - Spectralizer™ features

Analyses dust, solids, pastes and liquids
Uses 6 to 19 different filters with different wavelengths
Spectrum band between 1445 and 2348 nm
Ratio Signal/Noise de 10000:1
Operational temperature range: 5 a 40ºC

4.1.1.1 Experimental set up

There were two groups of tests done. The first was done by analysing samples of culture supernatant liquid with the following concentrations of ethanol: 1 g/L, 2 g/L, 2,5 g/L, 5 g/L, 10 g/L, 15 g/L, 25 g/L, 40 g/L e 50 g/L.

The supernatant fluids were obtained through the centrifuge of 400 mL plastic recipients with culture – which meant there was still some biomass suspended independently of the effort made to centrifuge. The wavelengths used to test these samples were:

Table 23 - Ethanol absorption wavelengths

Wavelength (nm)
1445
2139
2208
2270

The second test consisted in the preparation of a calibration curve based on the previous samples.

4.1.1.2 First test

The first test consisted on the analysis of the ethanol concentrations 1 g/L, 2 g/L, 2,5 g/L, 5 g/L, 10 g/L, 15 g/L, 25 g/L, 40 g/L e 50 g/L.

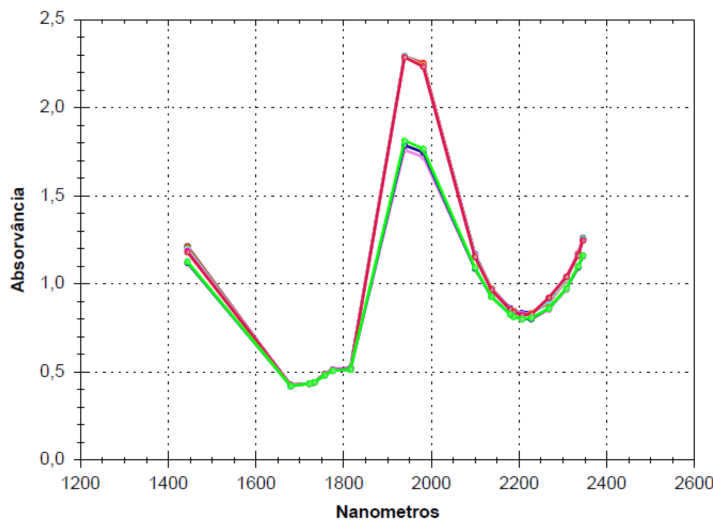


Table 24 - Spectre legend

Concentration (g/L)	Colour
0	Black
1	Dark blue
2	Light green
2,5	Red
5	Blue
10	Dark Green
15	Orange
25	Light blue
40	Pink
50	Dark Red

Figure 27 - Absorvance spectre of samples.

The graphic provided by the program is neither useful nor readable without the software. It was possible to distinguish differences but not to make an accurate reading about ethanol concentrations.

4.1.1.3 Second Test

The second test consisted on creating the calibration curve. The curve was generated by the equipment software and then validated by excel calculation.

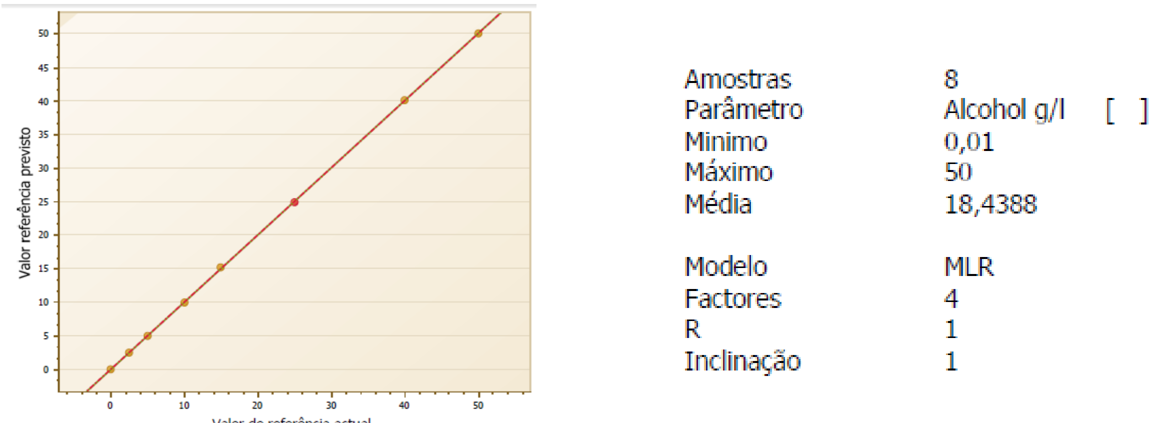


Figure 28 - Calibration curve of ethanol.

The curve seems robust however; software instructions indicate that any less than 30 points is not statistically robust. The consistency of the points was tested in excel for the different wavelengths:

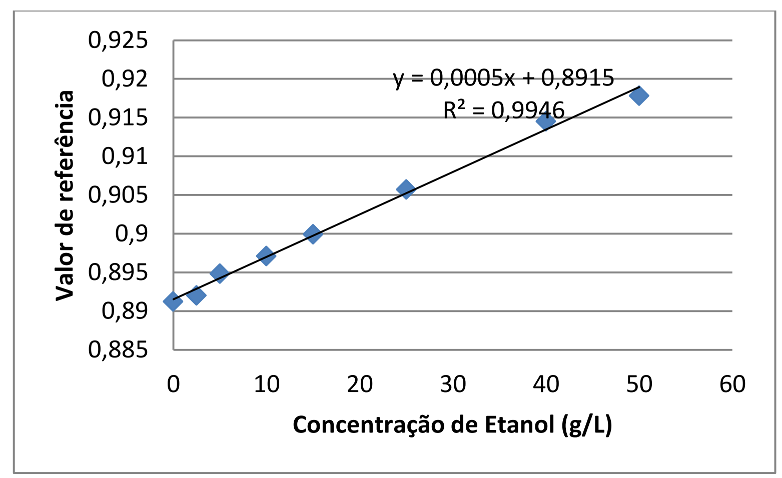


Figure 29 - Calibration curve at 2139 nm

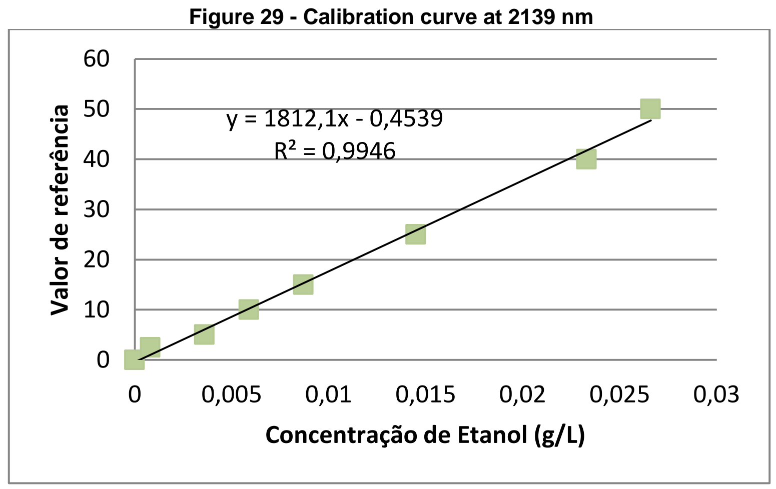


Figure 30 - Calibration curve at 2270 nm

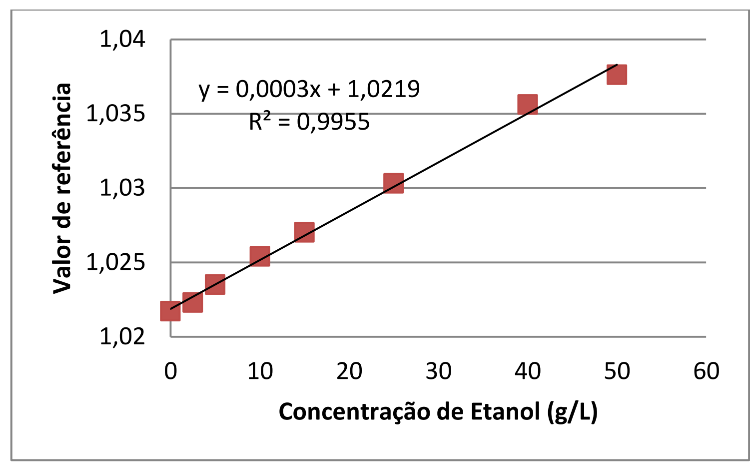


Figure 31 - Calibration curve at 2208 nm

In all the calibration curves it was observed quite some consistency for the amounts of ethanol.

4.1.1.4 Conclusion

The conclusion is that the NIR spectroscopy is a very valid and relative secure way to analyse and quantify the presence of ethanol in any medium including microalgae. However, this equipment is rather expensive and its acquisition requires more than a single set of use. While very useful to detect ethanol, it is not economically viable to acquire this piece of equipment exclusively to analyse ethanol.

4.1.2 Refractometry

The refractometry test was done recurring to a portable refractometer with the purpose of verifying if it was possible to establish a correlation between the measured refractive index and the amount of ethanol in the sample – of water and culture – and if that correlation allowed to identify amounts of ethanol. One special concern is the fact that dissolved salts have an impact in the refractive index which might disturb the measurements. Additionally, it was unknown if the culture itself would interfere with the measurements either.

4.1.2.1 Experimental setting

There were two sets of tests done. The first was aimed at establishing a calibration curve of ethanol in water and the second to test the impact of culture and nutritive media in the measurements. In the first test samples with 0 g/L, 1 g/L, 2,5 g/L, 5 g/L, 10 g/L, 25 g/L, 40 g/L and 50 g/L of ethanol were measured directly. In the second test the following samples were measured:

Table 25 - Tested samples in refractometry.

Sample #	Sample
1	Mixed culture
2	9,7 mL of mixed culture + 0,3 mL ethanol (20 g/L)
3	Supernatant of mixed culture
4	9,7 mL of supernatant + 0,3 mL ethanol (20 g/L) added before centrifuge
5	9,7 mL of supernatant + 0,3 mL ethanol (20 g/L) added after centrifuge
6	Culture with MMF medium
7	Culture with Hubel 7 medium

4.1.2.2 Results

The results of the first test were the following:

Table 26 - Relative index for each concentration.

Concentration (g/L)	Index
0	1
2,5	1,001
5	1,0015
10	1,0025
25	1,007
40	1,0115
50	1,014

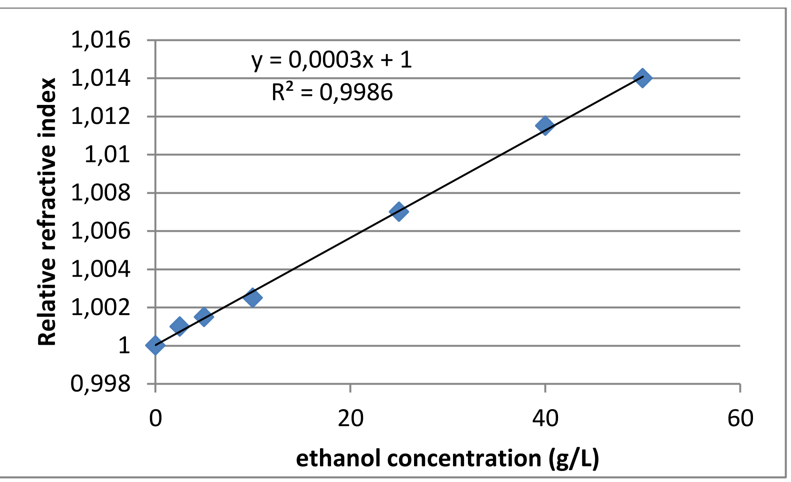


Figure 32 - Correlation between relative index and concentration.

The second test had the following results:

Table 27 - Tested samples & refractive indexes.

Sample #	Sample	RI
1	Mixed culture	1,0015
2	9,7 mL of mixed culture + 0,3 mL ethanol (20 g/L)	1,007
3	Supernatant of mixed culture	1,0015
4	9,7 mL of supernatant + 0,3 mL ethanol (20 g/L) added before centrifuge	1,007
5	9,7 mL of supernatant + 0,3 mL ethanol (20 g/L) added after centrifuge	1,007
6	Culture with MMF medium	1,0015
7	Culture with Hubel 7 medium	1,0015

4.1.2.3 Conclusions

This test allowed concluding that the ethanol concentration is, indeed, correlated with the refractive index through the expression:

$$C\ (g/L)\ =\ 3552{,}1 \times RI - 3552{,}2$$

Equation 9 - Correlation between Refractive Index and ethanol concentration (demineralized water).

However, and since the smallest unit of the scale is 0.001, the smallest amount of ethanol that the refractometer can measure is 2.5 g/L. The test also allowed concluding that the nutrient concentration has an impact on the refractive index due to the dissolved salts. Multiple samples reinforce that for the approximately 6 mM the relative refractive index change is 0.0015.

The deviation between the values obtained in the second sample, based on the calibration curve defined before, is 0.0009 (20 g/L – 1.0061). Taking in account the effect of nutritive media and that the error of the scale is 0.0005, the value is acceptable. No differences between the analysis to the supernatant and culture were identified with or without ethanol. Also, (i) centrifuging samples

should not be an issue when making measurements; (ii) there were no differences identified due to the presence of microalgae culture and finally (iii) no significant differences were identified between media – as mentioned in the nutritive media analysis, the salt content is relatively similar.

Refractive Index is, therefore, a reliable method to measure the existence of ethanol. It provides the additional advantage that it can be measured “on spot” accounting for less mistakes due to ethanol evaporation.

4.1.3 Pycnometry

The pycnometry tests were done resorting to a 100 mL pycnometer with a purpose of establishing a correlation between the measured density and the concentration of ethanol in the sample – once again water and culture.

4.1.3.1 Experimental setting

Initially, tests were started with a 10 mL pycnometer. However, due to the small volume and to the nature of the operations involved, the errors were too widespread. In order to reduce them, the volume was increased through the use of a 100 mL pycnometer.

Table 28 - Pycnometer specifications

Pycnometer mass (g)	65,1316
Pycnometer volume (mL)	100

There were three sets of tests done. The first was aimed at establishing a calibration curve of ethanol in demineralized water, the second to create a similar calibration curve for ethanol in culture and a third to create a calibration curve that allowed correlating OD_{730} with density of the sample – as it is expected that different biomass contents will influence density in different ways. The tested concentrations were the same for the first two tests. The tested values were the following:

Table 29 - Concentration and OD experimental settings

Concentration (g/L)	OD_{730}
50	0,59
25	0,71
10	0,82
5	1,09
2,5	1,23
1	1,42
0	2,63

4.1.3.2 Results

The results obtained in the first test – demineralized water and ethanol – were the following:

Table 30 - First test measurements (ethanol + demineralized water)

m(g)	Density (g/dm^3)	$C_{ethanol}$ (g/L)
164,3121	991,805	50
164,6023	994,707	25
164,8516	997,2	10
164,8966	997,65	5
164,9506	998,19	2,5
164,9716	998,4	1
164,9616	998,3	0

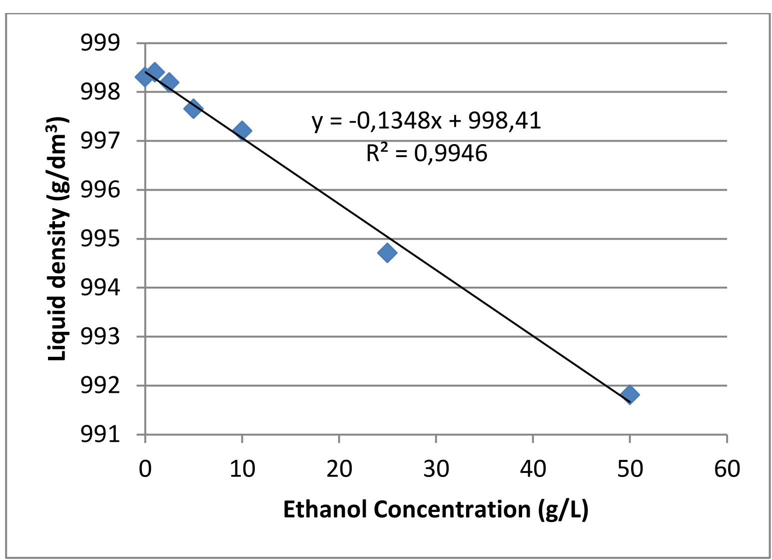


Figure 33 - First test calibration curve (ethanol + demineralized water).

The results allow establishing a correlation between density and ethanol concentration in water. As for the second test, the results obtained – culture and ethanol – were the following:

Table 31 - Second test measurements (ethanol + culture).

m(g)	Density (g/dm^3)	$C_{ethanol}$ (g/L)
163,6196	984,886	50
163,9826	988,516	25
164,2464	991,154	10
164,4307	992,997	5
164,4712	993,402	2,5
164,4907	993,597	1
164,5343	994,033	0

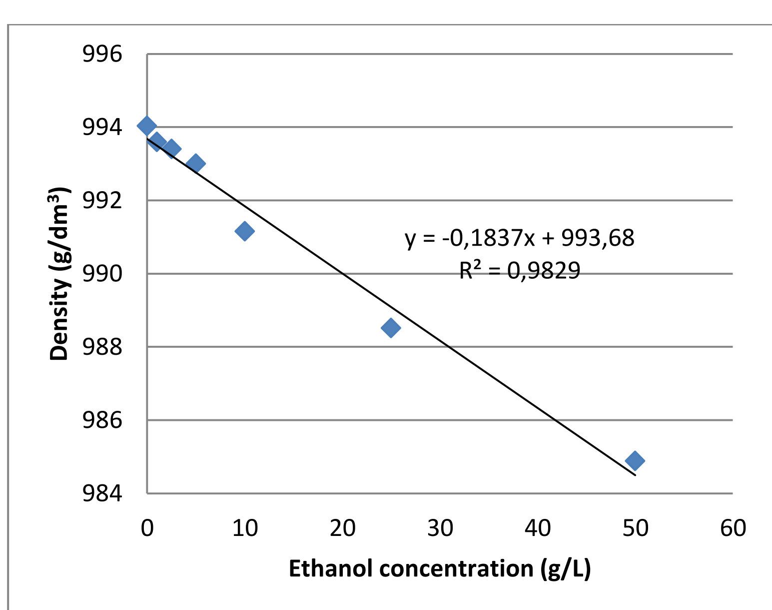


Figure 34 - Second test calibration curve (ethanol + culture).

While not as adequate as the first, the results also allow the establishment of a correlation. Finally, the results of the third test were the following:

Table 32 - Third test measurements (culture).

m(g)	Density (g/dm^3)	$C_{ethanol}$ (g/L)
164,5544	994,173	2,63
164,5267	993,896	1,42
164,5124	993,753	1,23
164,5021	993,65	1,09
164,5056	993,685	0,82
164,491	993,539	0,71
164,485	993,479	0,59

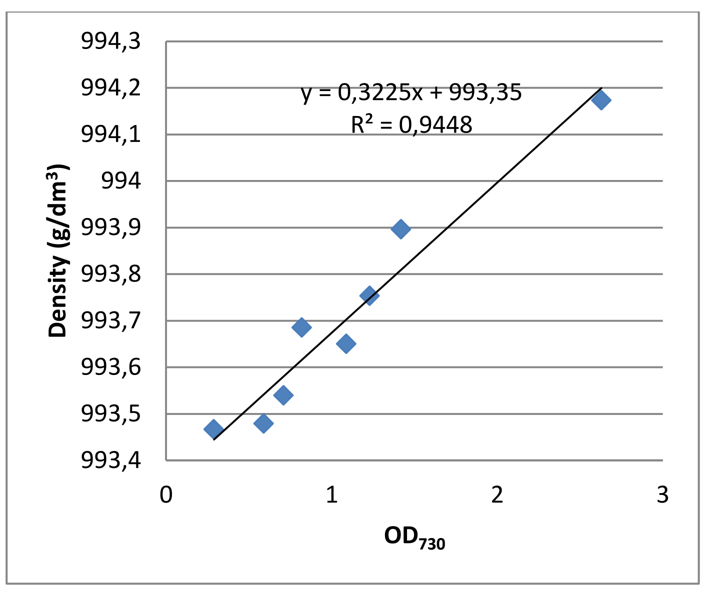


Figure 35 - Third test calibration curve (culture).

4.1.3.3 Conclusions

The three tests were successful in the sense they allowed to establish effective correlations that allow calculating the concentration of ethanol based on the density. The obtained correlations were:

$$C_{ethanol/water}(g.l^{-1}) = \frac{998{,}41 - d\ (g.dm^{-3})}{0{,}1348}$$

Equation 10 - Correlation between ethanol concentration in water and density.

$$C_{ethanol/culture}(g.l^{-1}) = \frac{993{,}68 - d\ (g.dm^{-3})}{0{,}1837}$$

Equation 11 - Correlation between ethanol concentration in a culture and density.

$$d_{\ culture}(g.dm^{-3}) = 993{,}35 + 0{,}3225\ \times OD_{730}$$

Equation 12 - Correlation between density and OD.

The last two equations (culture and ethanol and culture) can be combined in order to take into account the effect of OD in density:

$$C_{ethanol}(g.l^{-1}) = \frac{993{,}35 + 0{,}3225\ \times OD_{730} - d\ (g.dm^{-3})}{0{,}1837}$$

Equation 13 - Correlation between ethanol concentration in a culture, OD and density

The general conclusion, however, is that pycnometry is not the ideal method for ethanol testing for the following reasons:

- The test is rather sensitive and one drop can remove the accuracy of it;
- The combined equation multiplies the errors;
- The test requires specific equipment – a precision scale which can read up to 200 g;
- It cannot be done *in situ* – and that may cause ethanol evaporation during transportation and compromise the accuracy of the test;
- For it to work properly it requires large volumes of samples – which can only function in a PBR and not at laboratory scale.

4.1.4 Enzymatic method

The enzymatic method tests were done using a commercial ethanol testing kit (UV method) supplied by NZYtech. The purpose was to determinate if the kit was fit to analyse the amount of ethanol in substances.

4.1.4.1 Experimental setting

Only a set of tests was executed and the following solutions were used:

Table 33 - Tested samples using the ethanol kit.

Blank
5 g/L of ethanol
20 g/L of ethanol
Assay control solution (5 g/L) ethanol

All the solutions were prepared according to kit manual but the 5 g/L and 20 g/L required further preparation as it also implied 100 and 1000 times dilution, respectively; so the spectroscopy would fit into the linearity limits. Solutions were prepared with ethanol 99.6 %.

4.1.4.2 Results

The results obtained were the following:

Table 34 - Results of ethanol kit tested samples.

Sample	A_1	A_2	$\Delta A_{2\text{-}1}$	ΔA	$C_{ethanol}$ (g/L)
Blank	0,072	0,184	0,112	-	-
Control (5 g/L)	0,083	0,699	0,616	0,504	4,7
5 g/L	0,077	0,695	0,618	0,506	4,7
20 g/L	0,089	0,368	0,279	0,167	19,2

4.1.3.3 Conclusions

The results of the test indicate that the kit is reasonably accurate at determining ethanol concentration. While it has a limited application in the field due to the need of sample transportation, it is a good method to validate results as it’s simple and easy to apply.

4.2 Ethanol evaporation testing

The ethanol produced by the microalgae is excreted into the culture medium and the evaporation in the PBR tank might not be negligible. Indeed, through preliminary study of the vapour-liquid equilibrium, it has been concluded that the fraction of ethanol present in the gas phase can be up to 0.185 (for a maximum concentration of 50 g/L of ethanol) which, by itself and taking into account the concentrations used, can be considered an appreciable amount. Moreover, given that aeration (which will disrupt the vapour-liquid equilibrium) will be used, the amount of ethanol that will evaporate is likely to be higher.

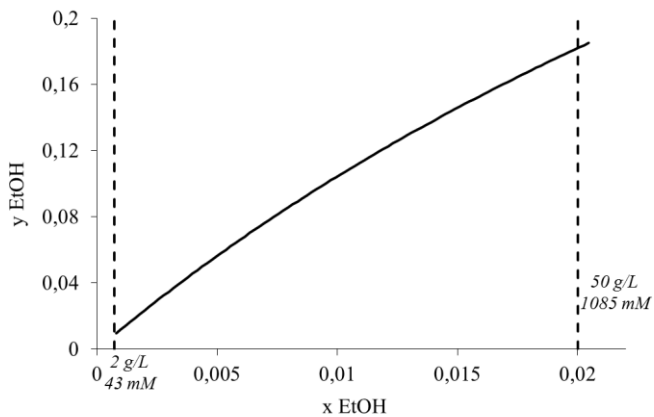


Figure 36 - Ethanol/water liquid-vapour equilibrium at 25 ºC.

4.2.1 Laboratorial scale application

To simulate the environment in a PBR, a laboratory scale bubble columns reactor was used to determine the amount of ethanol lost in the gas phase. For that, measurements of the liquid phase were made on a timescale with the purpose of identifying the loss of ethanol from the bubble columns.

4.2.1.1 Experimental setting

The experimental setting consisted in a bubble column with a volume of 700 mL and an air flow of 5 mL/s of air. The measurement was made with the refractometer and the ethanol used was the 96% (v/v). The samples were extracted from within the bubble column without stopping the air flow through a silicone tube with a syringe. A drop of that sample was then immediately analysed in the refractometer. Experiment time was registered through the use of a chronometer.

Table 35 - Experimental setting for Laboratory scale evaporation.

AL	Mass concentration of ethanol (g/L)	Molar concentration of ethanol (mM)	Mass fraction of ethanol (wt)	Volume fraction of ethanol (v/v)
AL1	25	542,6	0,0253	0,0301

4.2.1.2 Results

The results achieved from the successive measurements were the following:

Table 36 - Results of ethanol evaporation at laboratory scale.

Time (minutes)	IR	$C_{ethanol}$ (g/L)
0	1,0065	21,67
5	1,0065	21,67
10	1,0065	21,67
15	1,0065	21,67
20	1,0065	21,67
25	1,0065	21,67
31	1,0060	20,00
37	1,0060	20,00
46	1,0060	20,00

56	1,0060	20,00
62	1,0060	20,00
71	1,0060	20,00
77	1,0060	20,00
88	1,0060	20,00
97	1,0060	20,00
120	1,0060	20,00
166	1,0060	20,00

4.2.1.3 Conclusion

The experiment leads us to conclude that, with such a low air flow, the rate of evaporation of ethanol is not significantly altered and the losses of ethanol are relatively small. Taking into account that in the real case the ethanol concentration would be much lower, that also means the ethanol loss would be much reduced – much more than it is already – in laboratory conditions.

4.2.2 Pilot Unit scale application

After gathering the data of the previous experience it became necessary to test ethanol evaporation in the PBR. This became necessary because, as mentioned before, the PBR is installed in a greenhouse and subject to daily sun irradiation which might impact the temperature and the evaporation of the ethanol. For that, a large amount of ethanol was added to the PBR and measurements taken throughout the day. The result of this experiment gave us the conclusion on the amount of ethanol lost in the reactor.

4.2.2.1 Experimental setting

The experience was conducted in the PBR with the following features:

Table 37 - PBR features.

Pump type	Centrifugal pump – ITT Lowara 500/30/P
Power	3 kW
Frequency	38 Hz
PBR total culture volume	1100 L
Tubes material	Glass
Tank material	Stainless steel 316

Since the primary objective of the test is to evaluate ethanol evaporation rate, it was necessary to measure the temperature inside and outside of the greenhouse and of the water/ethanol mixture in the PBR. Ethanol concentration was measured on site using the Refractometer FG 201-211 and at the Laboratory using an enzymatic test kit. Data was recorded on an hourly basis. The samples for the ethanol determination using the enzyme test were stored in the freezer (-20ºC) and analysed afterwards.

4.2.2.2 Results

The results of the measurements, both on site and in the laboratory are the following:

Table 38 - Ethanol test results of PBR evaporation.

Hour	T_o GH (ºC)	T_i GH (ºC)	T PBR (ºC)	Radiation (kW/m^2)	RI	Ethanol (g/L)	$Ethanol_e$ (g/L)
10:20	20,5	29,2	25,3		1,0095	31,67	26,28
11:25	21,1	29,3	25,7	529	1,01	33,33	
12:17	21,3	28	26,2	187	1,0095	31,67	
13:24	22,9	26,9	26	343	1,0095	31,67	
14:34	22,3	30,4	26,9	249	1,009	30,00	31,85
15:30	23,9	33,2	28,1	286	1,009	30,00	
16:24	22,6	30,2	28,6	225	1,009	30,00	
17:20		31	28,6	244	1,009	30,00	
18:22		26,9	28,5	155	1,009	30,00	28,14
09:17		22,4	19,7	74	1,009	30,00	30,74

4.2.2.3 Conclusion

Similar to the conclusion from the previous test, the rate of ethanol evaporation is not significant to imply major evaporation losses. The temperature inside of the PBR was not revealed to have a direct impact in the evaporation rate. However, the temperature inside the greenhouse was higher than inside the PBR and that might have an impact in the evaporation rate – as the exhaustion tube of the PBR would not be removing air from the deposit and the equilibrium would be preserved there. Additionally, the use of the enzymatic kit validated the use of the refractometer as a tool to analyse the ethanol concentration in a medium.

5. Conclusion

In the present study of methodologies for the determination of ethanol in microalgae culture it was possible to conclude that the two most reliable methods for ethanol determination are refractometry and near-Infrared Spectrometry. Also, it was concluded that, for fast and *in situ* determinations, it's feasible to quantify down to 2 g/L of ethanol through the use of refractometry – without the need of sample treatment. The method showed itself to be vulnerable to the nutritive media however in a predictable way. The advantages of the method are its portability and the flexibility towards samples. It is also relatively inexpensive to acquire the equipment.

Regarding the NIR, the method proved to have a greater range to determined ethanol but it requires sample processing and cannot be executed quickly. Additionally, it's a rather expensive process if there are no other alternate uses for it. Neither the pycnometry nor the enzymatic method revealed themselves to be useful for fast measurements. The first requires several measurements that risk the integrity of the sample as well as treatment operations. The second was efficient to validate other methods but, besides similar sample problems to the pycnometry, it has an additional sensitivity to possible contents in microalgae culture.

This dissertation also approached the topic of the study of the cultivation of *Synechocystis* sp. PCC 6803 where two nutritive media were identified as acceptable: MMF and Hubel 7. MMF demonstrated to be the most adequate for culture development and growth. Hubel 7, while less efficient at generating culture productivity, showed to be consistent and capable of providing sustained growth. MMF requires preparation while Hubel 7 can be acquired already prepared which may make the Hubel 7 more advantageous.

Synechocystis PCC 6803 was observed to be relatively vulnerable to contaminations while in pilot-scale PBR. This allowed concluding preventive measures should be taken to avoid the development of fungi, bacteria and ciliates. On the topic of contaminations, it was observed that the ethanol presence in *Synechocystis* sp. PCC 6803 culture supports the development of bacterial and fungi contaminations due to additional carbon sources. These issues can be prevented by applying adequate anti-contamination treatment.

Finally, this thesis allowed concluding that ethanol evaporation from both the laboratory scale unit and the pilot-scale unit is relatively reduced and therefore does not require special procedures to recover or prevent loss of ethanol.

The work accomplished had a primary character related to understanding how would a culture of *Synechocystis* sp. PCC 6803 react to the process and to define methodologies to analyse the ethanol. Future work should focus on the testing of the methodologies with the genetically modified specie in order to achieve sustainable ethanol production. Another possible field of work is to develop a strategy to prevent culture contamination as it revealed itself to be a possible issue.

In the future, depending on the ethanol productivity, it might be advisable to use the NIR or the enzymatic test kit method as it allows detecting lower levels than refractometry.

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7. Annexes

7.1 Annex 1

7.1.1 Correlation of optical density (microplate reader) with dry weight

This procedure consisted in preparing a set of samples of both diluted and concentrated culture obtained from Scale-up culture. The optical densities measured in microplate reader and the respective Dry Weights were:

Table 39 - Optical Density$_{730}$ (MPR) vs Dry weight.

Optical Density$_{730}$ (MPR)	Dry Weight (g/L)
0,4294	0,108
0,2625	0,049
0,6156	0,178
1,0415	0,295
1,485	0,42
1,397	0,431
2,19	0,571

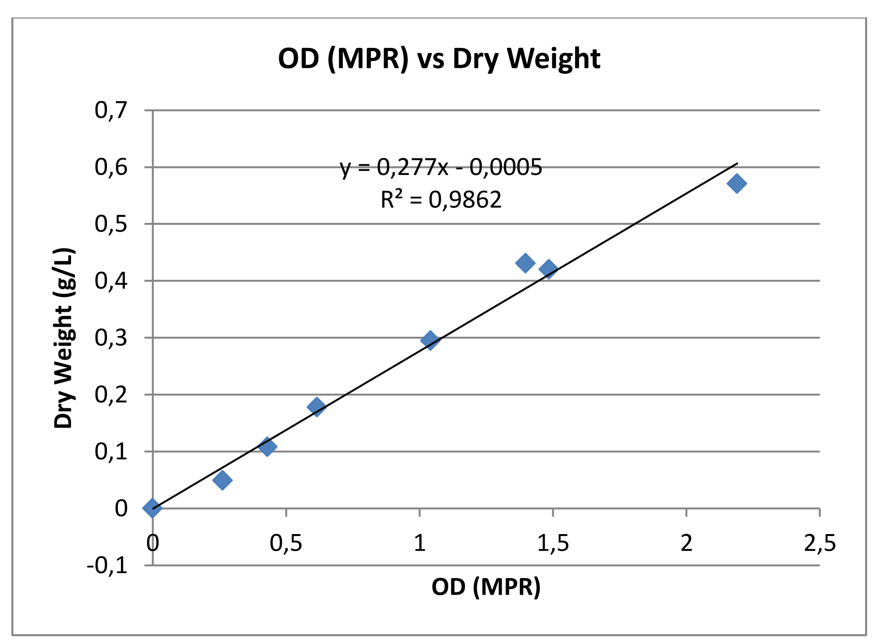

Figure 37 - Optical Density (MPR) vs Dry Weight.

It can be concluded that Dry Weight is satisfactorily correlated with Optical Density by the following proportion:

$$DW = 0{,}277 \times OD_{730}\ (g/L)$$

Equation 14 - Dry weight correlation to OD.

7.1.2 Correlation of optical density (microplate reader) with cellular density (Neubauer method)

The procedure was similar to the previous and the same data with the difference that, instead of using the dry weight, the correlation is done with the the cell count measured for different Optical Densities:

Table 40 - Optical Density730 (MPR) vs Cell Count (Neubauer method).

Optical Density$_{730}$ (MPR)	Cell Count (cel/mL) / 10^{-7}
0,4294	3,28
0,2625	1,75
0,6156	3,68
1,0415	5,03
1,485	7,44
1,397	7,89
2,19	13,6

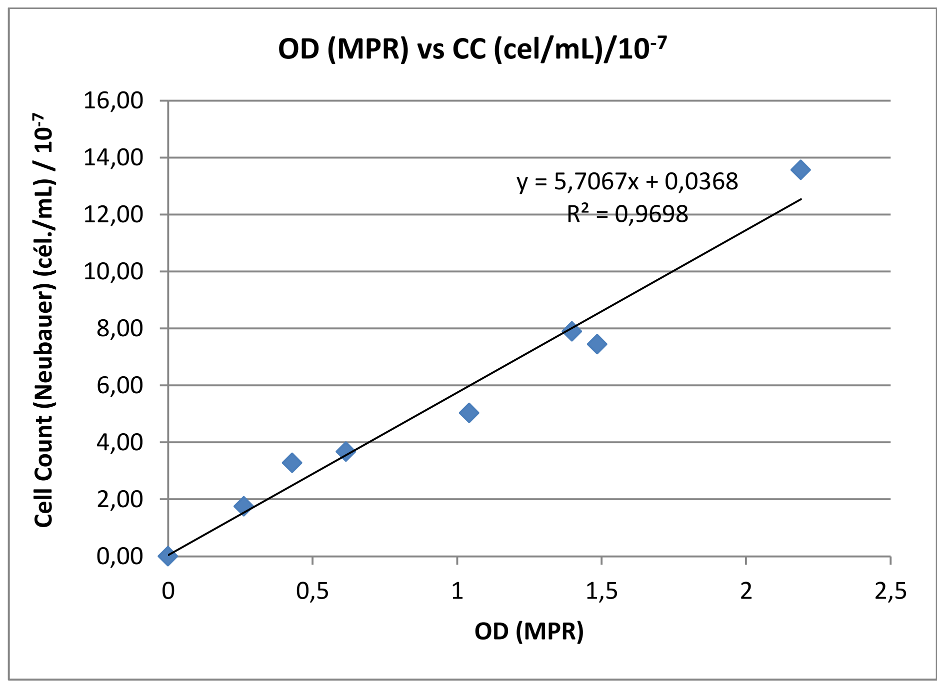

Figure 38 - Optical Density (MPR) vs Cell Count (Neubauer method)

Similarly to the previous case, it can be concluded that cell count is satisfactorily correlated with Optical Density by the following proportion:

$$CC = 5{,}7067 \times 10^{7} \times OD_{730}\ (cel/mL)$$

Equation 15 - Cell Count correlation with OD

7.1.3 Correlation of dry weight with cell count (Neubauer method)

This last correlation is a combination of both previous data – the samples used were the same – and it allows to establish the relationship of Synechocystis cells per amount of Dry Weight:

Table 41 - Dry Weight (g/L) vs Cell Count (Neubauer method) (cel/mL) / 10^{-7} .

Dry Weight (g/L)	Cell Count (cel/mL) / 10^{-7}
0,108	3,28
0,049	1,75
0,178	3,68
0,295	5,03
0,42	7,44
0,431	7,89
0,571	13,6

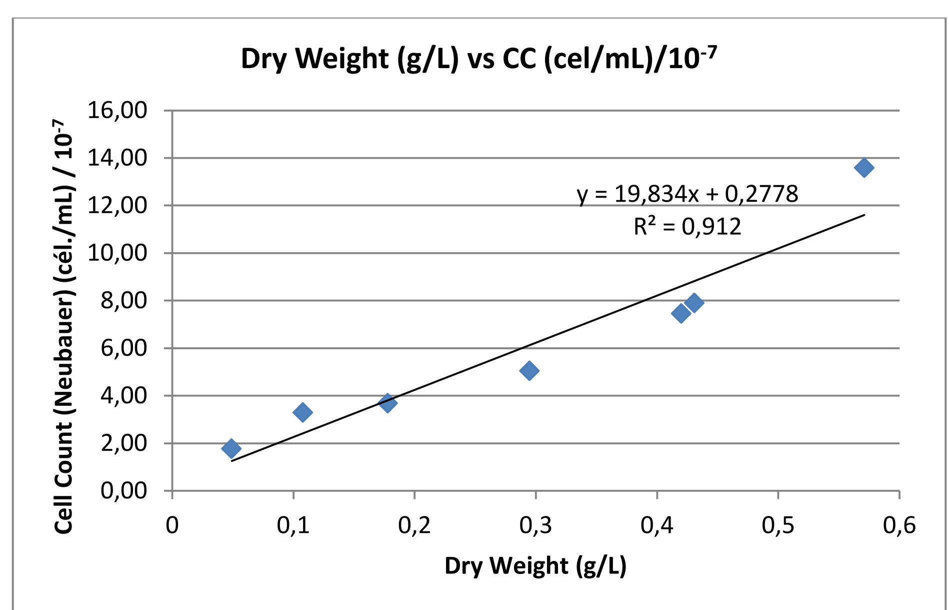

Figure 39 - Dry Weight (g/L) vs Cell Count (Neubauer method)

In this case the correlation is worse than the previous but it still provides an acceptable approximation to the amount of cells per mass of Synechocistis:

$$CC = 19{,}834 \times 10^{7} \times [DW\ (g/L)]\ (cel/mL)$$

Equation 16 - Cell Count Correlation with Dry Weight

This correlation can be simplified to:

$$Cells = 19{,}834 \times 10^{10} \times Dry\ Mass\ (g)$$

Equation 17 - Cell number correlation with Dry mass

7.2 Annex 2

7.2.1 MMF industrial recipe

Table 42 - MMF industrial recipe composition

Element	Concentração no meio nutritivo (mM)
N	2000
P	100
Mg	2
Zn	1
Mn	1
Mo	1
Cu	0,1
Co	0,1
Fe	20