Development of methodologies for fast analysis of ethanol in cyanobacteria cultures

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Abstract

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*

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 (1999) with *Synechococcus* sp. PCC 7942 and then by Pengcheng Fu (2009) 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.

Methods

Cyanobacteria culture

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, the result of each analysis has a specific meaning that needs to be optimised for the specific microalgae. Doing so, it generates the possibility of interpreting data more efficiently and expanding the understanding of the microalgae behaviour. In the case of Synechocystis 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 parameters that were correlated are: optical density at 730 nm, cell count and dry weight.

Optimization of Nutritive Media for Synechocystis cultivation

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. The tested nutritive media were:

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

The setting consisted of 6 bubble columns with duplicates of the 3 nutritive media at test. 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.

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 on 0.25 % (m/V) with an anti-septic and corrosive substance named cetrimide. 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.

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.

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.

Tested techniques

Near-Infrared Spectroscopy

There were two groups of tests done. The first was done by analysing samples of culture supernatant liquid with the following 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. 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: 1445, 2139, 2208, 2270 nm. The second test consisted in the preparation of a calibration curve based on the previous samples.

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 damage the measurements either. 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 1 - 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

Enzymatic method

The enzymatic method tests were done using a commercial ethanol test kit (UV method) supplied by NZYtech. The purpose was to determinate if the kit was fit to analyse the amount of ethanol in substances. Only a set of tests was executed and the following solutions were used:

Table 2 - Tested samples for 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 %.

Results

Optimisation of routine follow-up procedures for Synechocystis cultivation

The three methods that were correlated are: OD at 730 nm, cellular concentration and dry weight are summarized in Table 6.

Table 3 - Correlations for Synechocystis sp. PCC 6803 Kaplan summarized.

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

Optimization of Nutritive Media for Synechocystis cultivation

Nitrate consumption is relatively stable except for the periods corresponding to the peak microalgae concentration where it's naturally higher. Additionally, it can be observed that, in general, the nitrate consumption from Hubel 7 is relatively smaller than BG-11 and MMF. 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. The data indicates that the most productive medium to cultivate *Synechocystis* is the MMF industrial medium. However, the Hubel 7

proved to be consistent enough to be reliable as nutritive media – and considering it's possible to acquire it externally it becomes economically more advantageous. BG-11 as nutritive medium didn't show to be reliable for *Synechocystis* cultivation. *Synechocystis* culture should happen resorting either to MMF or Hubel 7.

Synechocystis tolerance to different types of ethanol

In this test the culture, the growth of contaminants (bacteria and fungi) was also observed – however mitigated by the renovations. Though during the daily renewal phase both conditions were identical, once 24h light photoperiod and aeration was established 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 – and as such they are probably dead.

Figure 1 - Culture evolution during the ethanol tolerance test.

Table 4 - Flux Citometry 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

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.

Pilot-scale production

The first test run lasted for 46 days (Figure 2). The test was interrupted due to the death of the culture due to micro-organism contamination. Once again, the main measure of productivity was the optical density and the adjusted correlation to dry weight.

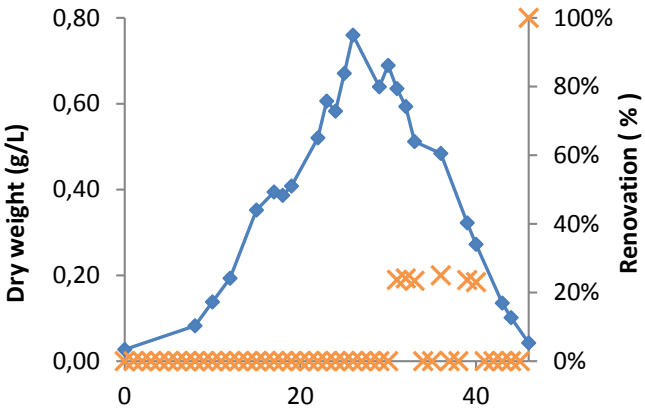

Figure 2 - Productivity of the PBR during the phase 1.

In the phase 2 (Figure 3), to respond to the sudden drop in productivity the nitrate set point was raised to 8 mM. The culture responded well to that raised and it allowed raising the renovation rate with success to 20%. However, with time, further contamination was noticed and by day 40 there was contamination

with fungi, bacteria, ciliates and other microalgae (specifically chlorella) – although not very intense.

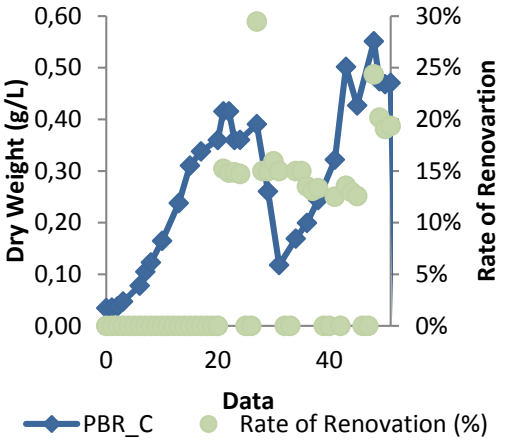

Figure 3 - Productivity of the PBR during the phase 2.

Near-Infrared Spectroscopy

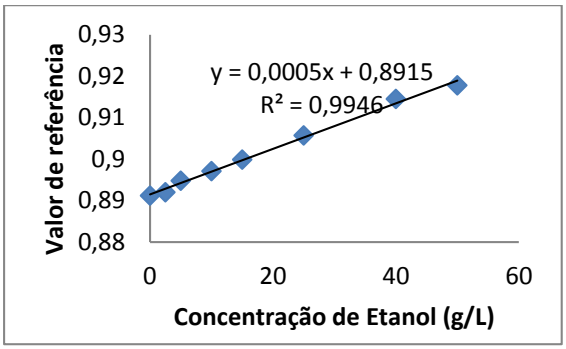

Figure 4 - Calibration curve at 2139 nm

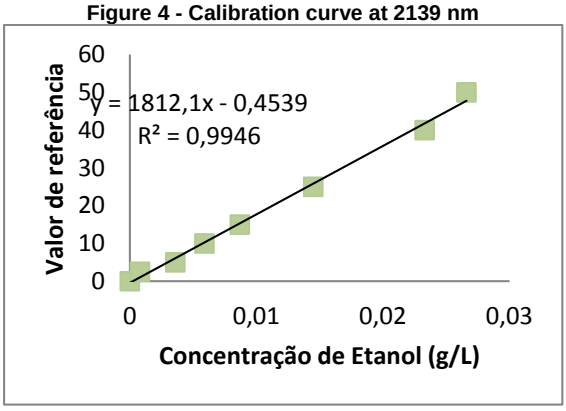

Figure 5 - Calibration curve at 2270 nm

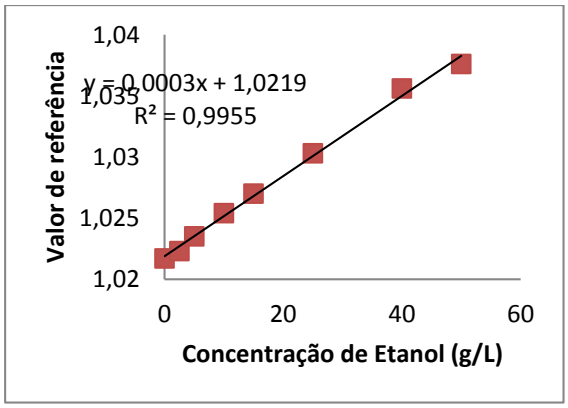

Figure 6 - Calibration curve at 2208 nm

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.

Refractometry

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

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

Equation 1- Correlation between Refractive Index and ethanol concentration (demineralized water).

However, since the smallest unit of the scale is 0,001, the smallest amount of ethanol the refractometer can measure is 2.5 g/L.

The test also allowed to conclude 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. Centrifuging samples should not be an issue when making measurements. There were no differences identified due to the presence of microalgae culture. 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.

Pycnometry

The results allowed concluding that it's possible to take in account both the effect of ethanol and OD in density:

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

Equation 2 - 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.

Enzymatic method

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.

Ethanol evaporation testing

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.

Pilot unit scale application

Similar to the conclusion from the previous test, the conclusion is that 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.

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. It was possible to conclude 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 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 – since culture productivity is also about the time put into preparing the medium and not just the material cost. *Synechocystis* sp. 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 to conclude 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* PC 6803 react to the process and to define methodologies to analyse the ethanol. Future work should incide over the testing of the methodologies with the genetically modified specie in order to achieve sustainable ethanol production. In the future, depending on the ethanol productivity, it might be advisable to use the NIR method as it allows detecting lower levels than refractometry.

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