Pilot scale production of metabolites from cyanobacteria

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ABSTRACT

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

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

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

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

KEYWORDS:

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

1 INTRODUCTION

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

The need to unveil more sustainable energy sources in order to reduce dependence on fossil fuels has led to the development of a 3rd generation of biofuels, which are produced from microalgae (1). Microalgae possess the advantage of having a high growth rate, carbon dioxide (CO_2) fixation ability and high production capacity of lipids or polysaccharides. Furthermore, they do not compete for land use with food or feed crops and can be produced on non-arable land. Therefore, microalgae can represent an effective alternative to the substitution of conventional fuel produced from edible crops and lignocellulosic biomass from

dedicated non-edible crops like switchgrass and agricultural waste (2) (3) (4).

The first approach of ethanol production in a microorganism was made in 1999 by Deng and Coleman (5). They introduced heterologous genes encoding PDC (EC 4.1.1.1) and ADH (EC 1.1.1.1) from bacterium *Zymomonas mobilis* (*Z. mobilis*), a natural ethanol producer, in a *Synechococcus* sp. PCC 7942, under the control of cyanobacterial rbcLS promoter (6).

The EU has supported several R&D projects in order to fund biofuels technologies an alternative to fossil fuels. One of the main research activities of the European Union 7th Framework Program Project Direct Ethanol from MicroAlgae (DEMA), is the production of bioethanol as a secretion from cyanobacteria, *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis* 6803) (7).

Synechocystis 6803, a fresh-water, non-filamentous, non-nitrogen fixing microorganism capable of heterotrophic growth, was one of the first strains to be fully characterized in terms of physiology, biochemistry and genetics. The entire genome sequence, which includes four endogenous plasmids (pSYSX, pSYSM, pSYSA, pSYSG), was completely sequenced and over 3000 genes have been annotated which has allowed for the establishment of techniques for precise genome manipulation (4) (8) (9).

Considering their unique characteristics, cyanobacteria have been used as a model organism to produce a wide range of fuels. For instance, ethanol and hydrogen production attempts in three cyanobacteria strains (*Synechocystis* 6803, *Synechococcus* 7002 and *Synechococcus* 7942 - a parental strain) have already been reported (8).

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

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

2 MICROALGAE CHARACTERIZATON & SCALE-UP STRATEGIES

2.1 Strains

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

2.2 Nutritive media

The strains were maintained isolated in the microalgae culture collection of A4F with a nutritive media developed by A4F, in both liquid and solid media and also cryopreserved, which will be addressed below. The nutritive media was composed by macro-nutrients – nitrogen, phosphorous and iron; micro-nutrients – e.g. magnesium, zinc, etc., and is supplemented with vitamins and further sterilized by filtration (Ø = 0.2 µm, Whatman, USA).

2.3 Pre-culturing

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

2.4 Preservation

For long term conservation and genetic stability, cryopreservation was successfully tested in the studied cyanobacteria.

2.4.1 Procedure

Cyanobacteria were cultured in Erlenmeyer flasks (250 mL) with agitation, at 25 °C and under continuous illumination of 30 µmol $m^{-2}s^{-1}$.

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

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

2.4.2 Viability assay

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

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

2.5 Tolerance to ethanol

This experiment aimed to test different operating conditions, in terms of photoperiod and daily aeration period, in cultures growing in 1 L bubble column reactors in the presence of increasing ethanol concentrations. This ethanol tolerance test allows the evaluation of contaminant proliferation and the impact of ethanol toxicity in the cyanobacteria (loss of productivity, pigment leaching or agglomeration).

Experimental conditions and results

The test was carried out at 25 ºC in four 1000 mL glass bubble columns (designated by AL7, AL8, AL9 and AL10) containing 700 mL of culture, during 7 days. Turbulence was provided by bubbling air enriched with CO_2 through the gas distributor. Table 1 presents the growing conditions used during the test.

Table 1: Experimental conditions of the test.

System	[EtOH] g L^{-1}	Daily renewal	Air flow	Light intensity	Photoperiod
AL7 control	0	15 %	Day and night: 5 mL/min	170 µmol/m^2/s	10:14 h
AL8	~ 25				
AL9 control	0		Day: 5 mL/min Night: no air flow		
AL10	~25				

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

Regarding cell growth and, consequently, productivity, there were no significant differences between bubble columns with and without aeration during the first 4 days. There was an abrupt increase of OD at day 4 which is related to the stop of daily renewals, stronger aeration and changes in light availability (24h).

The average productivity of the different cell cultures was similar. The values showed that ethanol toxicity in the cyanobacteria does not have major impact in productivity, when cultures are submitted to daily renewals and photoperiod. However, the lower number of renewals and the short duration of the test may also influence this conclusion. Table 2 resumes the influence of aeration and ethanol in the average productivity.

Table 2: Average productivity of *Synechocystis* 6803 strains between the different conditions tested.

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

The nitrate ($NO3^-$) consumption of cultures with ethanol was 1.6 mM day^{-1} during the renewals and then, increased to about 1.8 mM day^{-1} when cultures are submitted to stronger aeration and 24 h of light whereas for those without ethanol, the nitrate consumption was around 0.1 mM day^{-1} until day 3 and then, when renewals stopped, increased to about 1.2 mM day^{-1}. This increase in consumption is due to the absence of renewals and increase in illumination period to 24 h which are better conditions to cellular growth and results in higher cellular concentrations. The higher values of nitrate consumption by cultures with ethanol may be due to the presence of bacteria which reduce the NO_3^- in the medium. Ethyl alcohol is a carbon source for bacteria that consumes nitrate, therefore, alcohol additions result in bacterial growth and proliferation. During this process, bacteria assimilate the nitrate leading to nutrient depletion in the culture medium. In the cultures without ethanol, there was an increase in consumption related to the increase in cell concentration.

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

According to the results obtained (Table 3), the progressive increase of ethanol concentration led to intense proliferation of bacteria on the culture, while the presence or absence of aeration appear to be unrelated to the occurrence of bacterial contamination. This indicates that the *Synechocystis* 6803 cell culture is much more sensitive to bacterial proliferation when ethanol is added, however, no relation was found between bacterial contamination and aeration conditions.

Table 3: Bacterial contamination quantitative evolution between the different conditions tested.

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

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

Flow cytometry measurements were also performed in order to assess the culture "well-being" in presence of ethanol and its implications to the cell enzymatic activity and membrane integrity. This showed a slight decrease in enzymatic activity (below 80 %) for *Synechocystis* 6803 cells under ethanol treatment, which may reveal that the cell culture was not in optimum conditions during the test. This may be associated with the increase of ethanol concentration in the medium, producing a harmful effect on the cyanobacteria. However, since there are no results for cultures without ethanol, no comparisons can be made. Nevertheless, results from previous tests with healthy cultures showed a percentage of 92 % viable cells growing without ethanol after 40 days (11). On the other hand, the presence of ethanol appeared not to affect the membrane integrity. In addition, the absence of aeration during the night had no negative effect on the culture health.

2.6 Characterization of WT and GMO: PCR

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

2.6.1 DNA preparation for culture collection sample analysis

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

2.6.2 DNA preparation for scale-up and production culture analysis

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

2.6.3 DNA Amplification

DNA amplification of the targeted sequence was carried out in accordance with the protocol provided with 2x Dream Taq Green Master Mix (Termo Scientific, US). Each amplification reaction required 2 µL of DNA, 1 µL of each primer (10 µM) and 12.5 µL of Master Mix up to a final volume of 25 µL. Primers DEMA9 f(5'GTCAGTTCCAATCTGAACATCGA) and DEMA13 r(5'CAATTTGCAGATTATTCAGTTGGCAT) were provided by the University of Limerick.

PCR amplification was run on a thermocycler (Termo Scientific, US) with the following program: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 49.9 °C for 30 seconds and extension at 72 °C for 6 minutes with a single final extension at 72 °C for 15 min.

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

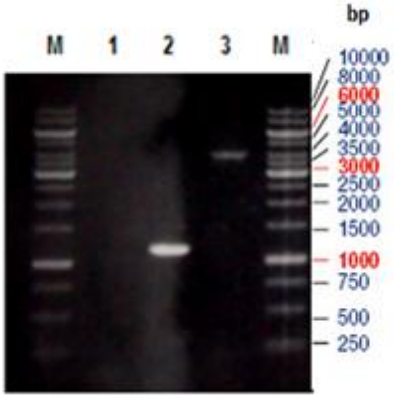


Figure 1. Agarose gel electrophoresis of PCR-amplified DNA fragments of GMO *Synechocytis* 6803.M: PCR markers, 1: NTC, 2: Positive control- Syn WT (plates), 3: PCR product of GMO Synechocytis 6803 (plates).

2.7 Scale-up strategy

At pilot scale, antibiotic treatment is not cost effective and therefore it is important to analyze the genetic stability of GMO strains when cultivated in absence of kanamycin. For this purpose, specific growth rates of the GMO strains with and without kanamycin were established and compared to the WT.

2.7.1 Specific growth rate determination

The specific growth rate (µ) was determined by the natural logarithm (Ln) of the cell concentration versus time. The slope of the linear regression of the early exponential growth phase corresponds to the µ (Equation 1), where N_1 and N_2 are the concentration of cells at the beginning (t_1) and at the end (t_2) of the exponential growth phase, respectively. Generation time (G) was calculated according Equation 2.

$$\mu = \frac{Ln\,(N_2 - N_1)}{t_2 - t_1} \qquad \text{Equation 1}$$

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

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

Experimental conditions and results

The specific growth rate (Table 4) was calculated during the exponential growth phase, which occurred during the initial four days of the test. Moreover, the end of the exponential growth phase was determined with a half log plot.

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

Table 4. Specific growth rate and generation time of WT and GMO strains. Each value represents the average of three cultures. Correlation coefficient (R^2) = 1.000 (WT); 0.9857 (GMO without kanamycin); 0.9121 (GMO with Kanamycin).

Species	Specific growth rate (h^{-1})	Doubling time (h)
WT	0.03	23.62
GMO without kanamycin	0.02	32.75
GMO with kanamycin	0.01	66.25

2.7.2 Use of fixed carbon by the microalgae: biomass vs etanol

The quantification of the fixed carbon that is converted into biomass or into the production of ethanol may be a parameter to determine the genetic stability of the culture.

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

The wt% of carbon in the biomass was found to be 51.4 % and was determined using the molecular formula of the biomass ($C\ H_{1.62}O_{0.4}N_{0.22}P_{0.01}$) (10).

The amount of carbon (in mmol) used to synthesized ethanol was determined with the ethanol concentration (g L^{-1}) measurement and then converted to mmol of C (C_{EtOH}) considering the volume used (Equation 3).

$$C_{EtOH}(mmol) = \frac{[EtOH](g\ L^{-1}) \times \%\ C\ \times Volume\ (L)}{MM_{EtOH}} \quad \text{Equation 3}$$

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

Experimental conditions and results

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

As it was expected, and it is observed, GMO cultures perform better – towards ethanol production – in the presence of kanamycin (Table 5). This should be adopted in for the scale-up strategy.

Table 5. Results from the reference test.

Strain	Inoculum reference	C -> Ethanol (%)	C -> Biomass (%)
WT	2.1.1.1.1.1 (6L) scale-up	-	100
GMO without kanamycin	4 (1L) scale-up	15	85
		16	84
GMO with kanamycin	3.1 (1L) scale-up	30	70
		33	67

2.8 Disinfection and sterilization methods

GMO *Synechocystis* 6803 culture was treated with 0.005 % (50 ppm) of sodium hypochlorite in order to inactivate/destroy the microalgae before wastewater disposal and different exposure times were tested (Table 6).

Table 6. Experimental conditions to determine the effectiveness of the disinfection method.

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

The culture pigmentation decreases with the time of exposure. Moreover, for the lowest biomass concentration tested, it was possible to observe the complete destruction of microalgae after 1 h treatment with a concentration of 50 ppm. The absence of cell growth was assessed by plating aliquots of treated culture in agar plates and liquid growth media. Tests demonstrated that after 7 days of incubation none GMO cyanobacteria had grown (data not shown).

3 Pilot scale Cultivation of *Synechocystis* 6803

The A4F experimental pilot plant, located at Lisbon, is a compliant GMO pilot unit with a total capacity of 4.3 m^3. This existing infrastructure accommodates a 1.1 m^3 PBR – a DEMA project dedicated production line – where *Synechocystis* 6803 WT and GMO strains were cultivated.

Cyanobacteria production in the PBR was carried out at the optimum growth temperatures (30 ± 1 °C, pH at 8.3 ± 0.5) (10), while the radiation supplied was natural light inside the greenhouse.

Overall Performance

The biomass concentration evolution for both strains is represented in Figure 2. The curves exhibited 3 distinct phases: a lag phase, which

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

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

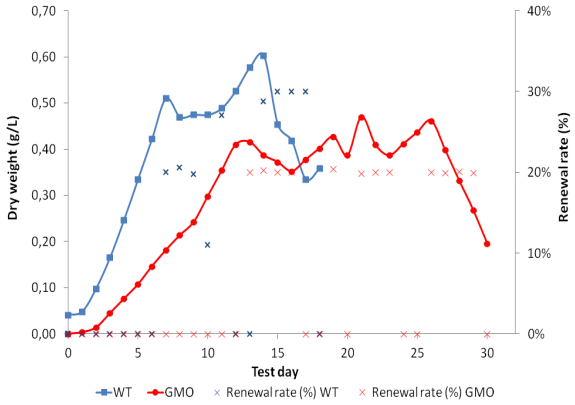


Figure 2: Evolution of biomass concentration of *Synechocystis* 6803 strains expressed in g L^{-1}.

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

Although the GMO culture was growing favorably, there was a pronounced decrease of biomass concentration in the final week of the trial (days 26 to 30). This was due to poor radiation with high renewal rate and a contamination that led to culture degradation and decrease in the culture productivity – biomass production rate did not match the biomass removed from the PBR. The poor condition of the culture after contamination led to the proliferation of more contaminants (bacteria, fungi, cists, ciliates and other microalgae (specifically *Chlorella*)).

The average biomass concentration and the productivity of each operating conditions is presented in Table 7. A loss of daily volumetric productivity of about 50 % is verified between the GMO and WT strains, at 20 % daily renewal.

Table 7: Average productivity and biomass concentration of *Synechocystis* 6803 strains.

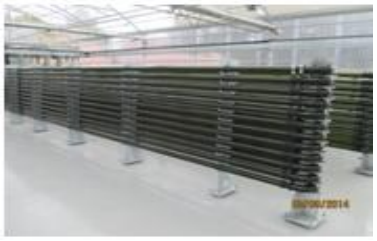
Species	Renewal rate (%)	Average Biomass Concentration (g L^{-1})	Average Productivity (g L^{-1} day^{-1})
WT *Synechocystis*	20	0.47 ± 0.003	0.09 ± 0.02
	30	0.42 ± 0.08	0.07 ± 0.04
GMO *Synechocystis*	20	0.36 ± 0.07	0.04 ± 0.03

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

Macro- and Microscopic observation

Figure 3 illustrates the macroscopic evolution of the reactor where WT and GMO *Synechocystis* 6803 strains were cultured, respectively. In general, both *Synechocystis* 6803 strains had similar appearance, showing an intense blue-green color, characteristic of cyanobacteria. At inoculation, cultures showed a yellowish aspect due to the nutrients solution which has a typical brownish color. Once the PBR was concentrated the blue-green color was dominant. At the end of the test the GMO strain showed a brownish color due to the poor state of the culture and contaminants present, soon after the trial was terminated.

(a)



(b)

Figure 3. Photographic record of WT (a) and GMO (b) cultures growing in PBR: (a) day 16; (b) day 15.

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

Ethanol production

Ethanol culture concentration and respective OD were measured twice a day, at 9 am and 5 pm. During GMO cultivation no significant ethanol concentrations were detected in the PBR. Ethanol measurement in the first two days of the test was of 4.09 and 10.40 mg L^{-1}, respectively, and only residual values were measured for the remaining days (see Figure 4).

Prior to the inoculation of the PBRs, scale-up cultures growing in 6 L round flasks were examined for the presence of contaminants and ethanol concentration was measured (ca. 25 to 50 mg L^{-1}). The most likely explanation for the non-detection of ethanol on the PBRs is related with ethanol consumption by contaminants such as bacteria (alcohol is a viable carbon source for bacteria growth).

The ethanol evaporation from the PBR may also be a hypothesis; however, this is unlikely to occur since the PBR is a closed system.

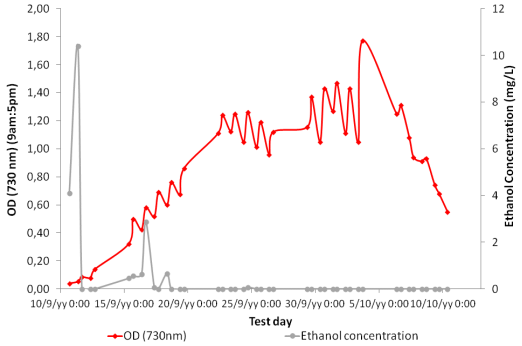


Figure 4: Optical density (730 nm) and ethanol concentration over time.

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

The ethanol construct was amplified by PCR with specific primers developed in the scope of the project.

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

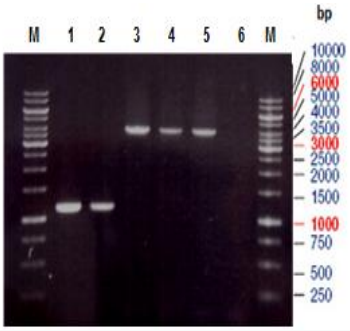


Figure 5: Agarose gel electrophoresis of PCR-amplified DNA fragments of GMO *Synechocytis*- M: PCR markers; 1: Positive control- Syn WT (plates); 2: Positive control- Syn WT (liquid); 3: PCR product of GMO Synechocytis (plates); 4: PCR product of GMO Synechocytis (scale-up); 5: PCR product of GMO Synechocytis (PBR) and 6: NTC.

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

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

5. Conclusions and Future work

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

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

Although the use of long-term preservation is widespread in laboratory and clinical settings, few data is available for algae cryopreservation. In this study, a standardized methodology for freezing, thawing and assessment of viability was tested in WT and GMO *Synechocystis* 6803. It was clear that both strains had tolerance to freezing stress resulting in the development of a satisfactory and acceptable cryopreservation protocol valid for the cyanobacteria in study.

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

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

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

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

No phenotype of ethanol stress was observed in the PBR, namely a more yellowish pigmentation characteristic of the ethanol producer compared to the rather blue-green of the reference strain. This change in cellular pigment composition was observed in the seven-day ethanol exposure test, where WT

culture started with an ethanol concentration of 2 g L^{-1} and reached 25 g L^{-1} after 7 days.

The differences registered in terms of bacterial contamination for both strains were not significant when compared to the ethanol tolerance test, where the increase of ethanol concentration led to intense propagation of bacteria on the culture.

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

Future work

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

- Improve disinfection/sterilization procedures.
- PBR inoculation should be carried out with kanamycin in order to maintain the selective pressure of the cells;
- Perform tests in order to understand if there might be ethanol evaporation from the PBR;
- Evaluate the effect of the photoperiod in ethanol production

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

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

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

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