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Sónia Filipa Silva Ferreira

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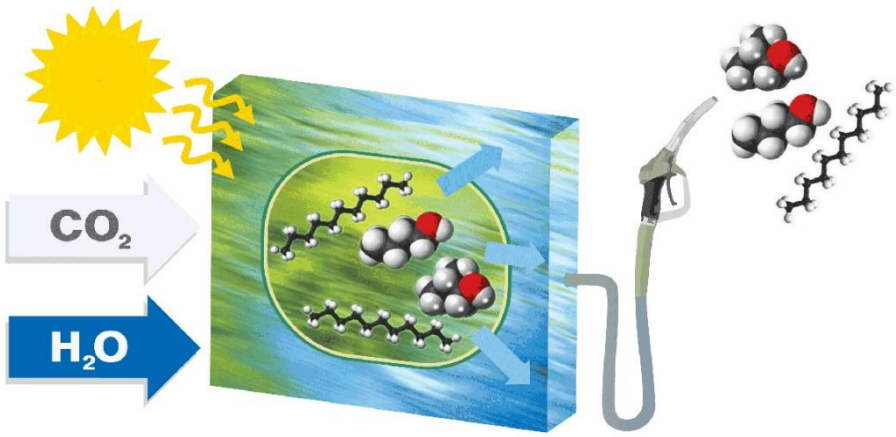

Pilot-scale cultivation of a genetically modified cyanobacterium producing 1-butanol

Sónia Filipa Silva Ferreira
Biologia Funcional e Biotecnologia de Plantas
Departamento de Biologia
2019

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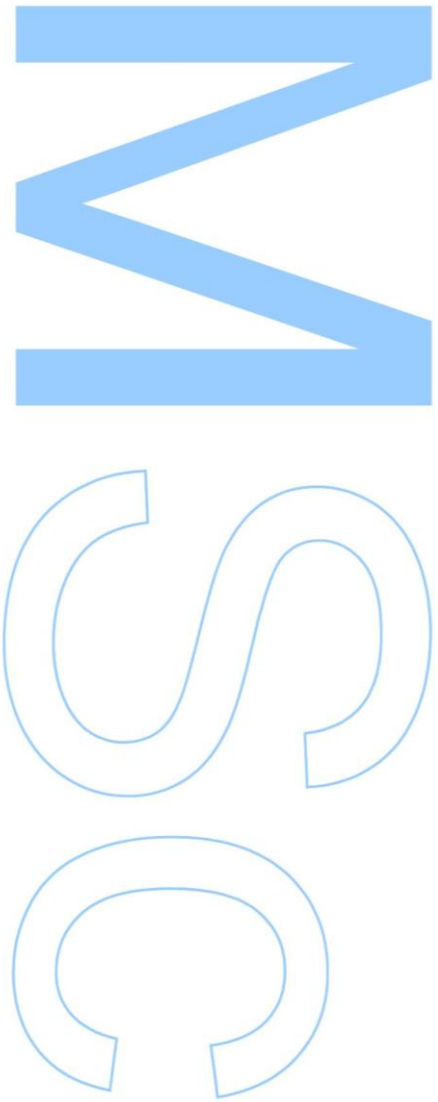

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Resumo

As alterações climáticas e a crise energética têm estimulado investigadores de todo o mundo à pesquisa por novas energias alternativas e sustentáveis. A produção de biocombustível a partir de fontes ecológicas é considerada uma das principais alternativas aos combustíveis fósseis, o que poderá contribuir para a redução da emissão de gases com efeito de estufa e da dependência de petróleo. A modificação genética de microalgas e cianobactérias para a produção e excreção de biocombustíveis revela ser uma alternativa promissora às tecnologias convencionais. O 1-butanol é um forte candidato a substituto da gasolina, cuja utilização não requer alterações nos atuais motores dos automóveis. *Synechocystis* sp. PCC 6803 e *Synechococcus elongatus* 7942 têm sido eficazmente modificadas a nível genético para a produção de 1-butanol.

O presente trabalho está integrado no projeto Photofuel, que objetiva desenvolver uma tecnologia de próxima geração para a produção biocatalítica de combustíveis líquidos de transporte. Este sistema requer apenas luz solar, CO_2 e água. Neste âmbito, *Synechocystis* sp. PCC 6803 foi geneticamente modificada para produzir e excretar 1-butanol para o meio de cultura, tendo sido designada por SynBuOH-44.

Este estágio em contexto empresarial centra-se na otimização das condições de cultivo de SynBuOH-44 de forma a maximizar a produção de 1-butanol, tendo sido realizados ensaios à escala laboratorial e à escala piloto. Ensaios laboratoriais indicaram que o uso do meio industrial desenvolvido e otimizado pela A4F – Algafuel S.A. (A4F-IM), e adotado para o cultivo de SynBuOH-44, não parece afetar negativamente a produtividade de 1-butanol quando comparado com o meio laboratorial comummente utilizado para o cultivo de cianobactérias (BG-11). Nesses mesmos ensaios, as concentrações de 1-butanol obtidas em cultivos a pH alcalino e a pH usualmente utilizado para cultivar *Synechocystis* sp. mostraram também não serem muito distintas. Estudos realizados à escala piloto evidenciaram perdas de 1-butanol por evaporação através do sistema de arejamento da cultura. Devido à volatilidade do 1-butanol, e para evitar perdas do produto de interesse, foi necessário conectar um condensador à saída de ar do reator de forma a recuperar o 1-butanol evaporado, verificando-se mesmo assim perdas substanciais de 1-butanol. O aparecimento de contaminantes nas culturas da unidade piloto mostrou ser um grande problema a enfrentar, dado que este evento parece conduzir a uma descida abrupta na concentração de 1-butanol acumulado na cultura para valores próximos de zero.

As máximas concentrações de 1-butanol medidas nas culturas do laboratório e da unidade piloto foram 165.01 mg L^{-1} e 53.36 mg L^{-1}, respetivamente. Otimizações futuras são cruciais para o aumento da produtividade de 1-butanol desta fábrica celular,

tornando este sistema de produção de biocombustível economicamente viável à escala industrial.

Palavras-chave: Sustentabilidade, engenharia metabólica, micro-organismo geneticamente modificado, *Synechocystis* PCC 6803, biocombustíveis, fotobioreato

Abstract

The climate change and energy crisis have stimulated worldwide researchers to search for new alternative and clean energies. Biofuel production from environmentally friendly sources is considered one of the main alternatives to petroleum-based fuels, which could help to reduce the greenhouse gases emission and the dependence on oil. Genetically engineered cyanobacteria and microalgae for the photoautotrophic production of excreted biofuels represent a promising alternative to conventional fuel technologies. 1-Butanol offers a great promise as a gasoline substitute, which can be directly used in existing internal combustion engines. *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* 7942 has been successfully engineered to produce 1-butanol.

The present work is integrated into the Photofuel project that aims to develop a biocatalytic production of liquid transportation fuels, which only requires sunlight, CO_2, and water. *Synechocystis* sp. PCC 6803 was genetically engineered to produce and directly excrete 1-butanol to the culture broth, having been called SynBuOH-44.

This internship in business context focuses on optimizing the cultivation conditions of SynBuOH-44 to maximize 1-butanol production, having been performed both laboratory and pilot-scale tests. Laboratory tests have shown that the use of the industrial medium developed and optimized by A4F – Algafuel S.A. (A4F-IM), adopted for the cultivation of SynBuOH-44, does not seem to negatively affect its 1-butanol yield when compared to the standard laboratory BG-11 medium as well as the cultivation at an alkaline pH comparing with the pH commonly used for *Synechocystis* sp. cultivation. In studies using a pilot-scale flat-panel photobioreactor, 1-butanol losses by evaporation through the aeration system of the culture were detected. Due to the volatility of 1-butanol and to avoid losses of the product of interest it was necessary to connect a condenser to the air outlet of the reactor in order to recover the 1-butanol evaporated, nonetheless substantial losses of 1-butanol were detected. The emergence of contaminating microorganisms in the cultures proved to be a huge problem to face at pilot-scale since this event leads to an abrupt drop in the 1-butanol titer accumulated in the culture for values near to zero.

The maximum 1-butanol content measured in the laboratory- and pilot-scale cultures were 165.01 mg L^{-1} and 53.36 mg L^{-1}, respectively. Future optimizations are crucial to increase the 1-butanol productivity of this cell-factory making this biofuel production system economically viable at industrial-scale.

Keywords: Sustainability, metabolic engineering, genetically modified microorganism, *Synechocystis* PCC 6803, biofuel, photobioreactor.

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List of Abbreviations and Acronyms

A4F	A4F – Algafuel, S.A.
A4F-IM	A4F Industrial Medium
ABE fermentation	Acetone Butanol Ethanol fermentation
Ach	Acetyl-CoA hydrolase
AckA	Acetate kinase
Acs	Acetyl-CoA synthetase
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BG-11	Standard laboratory medium for cyanobacteria
BuOH	1-Butanol
BuOH cond.	Condensed 1-butanol
BuOH cult.	1-Butanol in the culture
CBB cycle	Calvin-Benson-Bassham cycle
Ccr	Crotonyl-CoA reductase
DCM	Dichloromethane
Ddh	D-Lactate dehydrogenase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DW	Dry Weight
EIA	Energy Information Administration
F6P	Fructose 6-phosphate
FM	Final Mass
G3P | GA3P	Glyceraldehyde 3-phosphate
GC	Gas Chromatography
GC-FID	Gas Chromatography with a Flame Ionization Detector
GM	Genetically Modified
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
HGT	Horizontal Gene Transfer
hv	Photon energy
IEA	International Energy Agency
IM	Initial Mass
IPCC	Intergovernmental Panel on Climate Change

LED	Light-Emitting Diode
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NphT7	Acetoacetyl-CoA synthase
OD	Optical Density
PBR	Photobioreactor
PduP	CoA-acylating propionaldehyde dehydrogenase
PEG	Polyethylene glycol
PhaA	Acetyl-CoA acetyltransferase
PhaB	(R)-3-hydroxybutyryl-CoA dehydrogenase
PhaC	Poly(3-hydroxyalkanoate)
PhaE	Poly(3-hydroxyalkanoate)
PhaJ	(R)-specific enoyl-CoA hydratase
PHB	Polyhydroxybutyrate
Pk	Phosphokelotase
PPFD	Photosynthetic Photon Flux Density
Pta	Phosphate acetyltransferase
PTFE	Polytetrafluoroethylene
R&D	Research and Development
ROS	Reactive Oxygen Species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
Synechocystis	*Synechocystis* sp. PCC 6803
U.S.	United States
UV radiation	Ultraviolet radiation
Vol.	Volume
Xu5P	Xylulose 5-phosphate
3-PGA	3-Phosphoglycerate

Chapter 1.

Introduction

1. Introduction

1.1. Global energy concerns

For the last decades there has been a large world's population growth, coupled with an expectation of a higher standard of living, which has resulted in a substantial increase in global energy consumption – and it is expected the continuing increase of the world's population next years as well as the energy demand (United Nations, 2017; IEA, 2018; BP, 2019; EIA, 2019). Currently, fossil fuels such as oil, coal, and natural gas are the major energy sources although the consumption of renewable energy has been growing rapidly in recent years, tending to maintain strong growth by 2040 (BP, 2019).

The reserves of fossil fuel are limited and the tendency is that this non-renewable source of energy depletes in the coming years, no longer suppressing the needs of a growing global population that, according to "World Population Prospects: The 2017 Revision" of the official United Nations, is estimated to reach 8.6 billion in 2030, 9.8 billion in 2050 and 11.2 billion in 2100 (United Nations, 2017; Cheng, 2018; Ritchie and Roser, 2018). Moreover, burning fossil-based fuels causes devastating impacts on the environment due to greenhouse gas emissions, which is usually believed to be the major motive for global climate change (Wuebbles and Jain, 2001; Cheng, 2018). The Intergovernmental Panel on Climate Change (IPCC) reported that global warming is likely to reach 1.5 ºC between 2030 e 2052 if the global temperature continues to increase at the current rate (IPCC, 2018).

The rising energy dependency, the limited supply of fossil based-fuels, the increasing global concerns over energy security, environmental impacts of fossil fuel combustion such as increasing of greenhouse gases, acid rains, and urban smog, price fluctuations in the energy markets and rising costs of food have alerted governments to evolve new alternative and renewable energy strategies (Dwivedi *et al*., 2009; Mahmoud *et al*., 2009; Dillon and Barret, 2016; Cheng, 2018).

1.2. Renewable energy

Renewable energy is energy obtained from naturally repetitive and persistent flows of energy occurring in the environment – also referred to as green energy or sustainable energy. In contrast, non-renewable energy such as fossil fuels and nuclear energy is energy obtained from static stores of energy – finite supplies – that remain underground unless released by human interaction (Twidell and Weir, 2015).

Nowadays, there are several options of sustainable energy technologies in the market, which are more and more optimizing and efficient: hydropower, bioenergy,

geothermal, solar, and wind energy. The environmental pollution caused by these green energy technologies tend to be minimal (Demirbas and Demirbas, 2010; Twidell and Weir, 2015; Cheng, 2018). Bioenergy is becoming increasingly popular worldwide representing the biggest portion of the total renewable energy that is generated (IEA, 2018; EIA, 2019).

1.2.1. Bioenergy

The production of sustainable and environmentally friendly energy from biomass is so-called bioenergy and can be deployed as a solid, liquid, and gaseous fuels and used in different sectors (Demirbas and Demirbas, 2010; Creutzig *et al*., 2014). Currently, biomass provides 12 to 13 % of global primary energy (Kaltschmitt, 2018). Biomass is renewable organic matter derived from growing plants or from animal manure such as crops, plant wastes, forest wood by-products, algae, manure, and organic municipal wastes (Demirbas and Demirbas, 2010; Abdoli *et al*., 2018). Generally, biomass refers to any non-fossil biological materials, which are the direct or indirect products of photosynthesis which contains stored chemical energy (Abdoli *et al*., 2018).

Naturally, photosynthetic organisms such as higher plants, algae, and certain other microorganisms are capable of converting sunlight, water, and carbon dioxide, via photosynthesis, to produce a variety of organic molecules with the energy of sunlight stored in their chemical bonds, which can be used to generate biomass (Mckendry, 2002; Gilbert and Wilhelm, 2018). When these bonds are broken by digestion, combustion, or decomposition, there is released of their stored chemical energy that combined with oxygen produce CO_2, which is then available to produce new biomass, and water. Compared to burning fossil fuels, which take millions of years to be formed from biomass, burning new biomass reduces carbon emissions because during growth it captures and stores carbon that is released upon combustion, so no new carbon is released to the atmosphere (Mckendry, 2002).

Various types of organic origin feedstock as agricultural crops and wastes, industrial wastes, forest residues, or municipal solid waste are converted to biofuels – like ethanol, biodiesel, and biogas –, which are used as transportation fuels or directly to the production of electricity and heat (Cheng, 2018). In the last years, the transportation sector was the biggest consumer of petroleum and also the main responsible for the highest emissions of CO_2 (EIA, 2019). Transportation fuels, so-called biofuels, are an alternative to classical fuels produced from crude oil or from natural gas (Neuling and Kaltschmitt, 2018).

1.3. Different generations of biofuels

Biofuel is defined as a fuel with an 80 % minimum content by volume of materials derived from living organisms harvested within the ten years preceding its manufacture (Breeze *et al*., 2009). The paradigm in biofuel research and progress has substantially been shifted toward alternative and efficient biomass source (Dutta *et al*., 2014). Nowadays, biofuels are classified into four generations.

1.3.1. First-generation biofuels

The first-generation biofuels – biodiesel, bio-ethanol, and biogas – are made of sugar and vegetable oils from food crops like corn, wheat, barley, and sugarcane (Naik *et al*., 2010; Dutta *et al*., 2014). This generation biofuels is daily confronted by social and environmental challenges due to the use of feedstocks, which may have a negative impact on biodiversity and lead to competition with food crops for agricultural lands and water use pushing up food prices (Koh and Ghazoul, 2008; Naik *et al*., 2010; Sims *et al*., 2010; Dutta *et al*., 2014). Moreover, the sustainable production of first-generation biofuels is limited by the possibility of greenhouse gases increasing through emissions from land-use change when farmers plow up forests and grasslands to produce biofuels, which releases to the atmosphere much of the carbon previously stored on plants and soils (Searchinger *et al*., 2008).

1.3.2. Second-generation biofuels

Due to the impact of first-generation fuels on global food markets and on food security arose the second-generation fuels, which use low-cost non-food biomass like lignocellulosic feedstocks such as organic wastes, agricultural, and forestry residues, high yielding woody, grass, or other energy crops (Antizar-Ladislao and Turrion-Gomez, 2008; Sims *et al*., 2010). The use of these feedstocks for second-generation biofuels production – like bioethanol, biogas, biohydrogen, biobutanol, biodiesel – leads to decreasing of pressure on the use of agricultural land and all concerns related to food competition, and improving greenhouse gas emission reductions when compared to some first-generation biofuels (Antizar-Ladislao and Turrion-Gomez, 2008; IEA, 2009; Chye *et al*., 2018). However, biofuel production costs are likely to be relatively high and revenue low, thus more research, development, and processing technologies optimization are necessary in order to second-generation liquid transport biofuels become commercially competitive (Antizar-Ladislao and Turrion-Gomez, 2008; Sims *et al*., 2010).

1.3.3. Third-generation biofuels

Recently, algae have received a significant interest by researchers as alternative biofuel feedstock because of their higher photosynthesis and fast growth rate as compared to any territorial plant. This characteristic gives them the potential to produce considerably greater amounts of biomass and lipids per hectare than any kind of terrestrial biomass (Singh and Gu, 2010; Suali and Sarbatly, 2012; Dutta *et al*., 2014). The third-generation biofuels corresponds to fuels that are produced by processing algal biomass (Lu *et al*., 2011). The production of biofuels from algae frequently relies on the lipid content of the microorganisms (Lee and Lavoie, 2013). The oil yield of microalgal biofuel feedstocks exceeds that of the best oilseed crops, as illustrated in **Table 1** (Mata *et al*., 2010). An oil content of 20 to 50 % dry weight of biomass is quite common in microalgae (Chisti, 2007; Brennan and Owende, 2010).

Table 1 | Comparison of oil yield between microalgae and other biodiesel feedstocks. Adapted from Mata *et al*. (2010).

Feedstock	Oil yield (L ha^{-1} $year^{-1}$)
Microalgae **(oil content: 70 % in a dry weight basis)**	139900
Soybeans	636
Palm oil	5366
Rapeseed	974
Sunflower	1070
Corn/ Maize	172

In spite of algae grow in aqueous media, they need less water than terrestrial crops (Dismukes *et al*., 2008). No arable land and good water are required for algal production, so there is no competition for fertile lands or potable water. Algae uses CO_2 as a carbon source sequestering CO_2 permanently while growing, which contribute to reducing carbon footprint (Chisti, 2007; Rodolfi *et al*., 2009; Singh and Gu, 2010; Ullah *et al*., 2015). Wastewater can be the source of some of the other nutrients required for the growth of algae such as nitrogen, phosphorus, and potassium (Chisti, 2007; Cantrell *et al*., 2008). Algae cultivation does not require herbicides or pesticides application (Rodolfi *et al*., 2009). The biochemical composition of the algal biomass can be modulated by varying growth conditions and thus considerably enhance the oil yield (Sharma *et al*., 2012).

Several conversion technologies are available to carry out the transformation of algal biomass to different energy sources. From algal biomass, hydrogen gas, bioethanol,

acetone, butanol, methane, and hydrogen can be obtained by biochemical conversion. Thermochemical conversion is a process from which syngas, bio-oil, and charcoal are acquired. By chemical reaction algal biomass converts in biodiesel, and direct combustion of biomass generates power like heat and electricity (Behera *et al.*, 2015; Ndaba *et al*., 2015). However, the high cost of cultivation and processing of algal biomass for biofuel production as compared to conventional crops is the major obstacle to their worldwide implementation and commercialization (Ullah *et al*., 2015). For example, harvesting of algae requires a high energy input – approximately about 20 to 30 % of the total cost of production (Grima *et al*., 2003; Behera *et al*., 2015).

1.3.4. Fourth-generation biofuels

Recent research efforts have concentrated on applying metabolic engineering and genetic methods to algae and other photosynthetic microorganisms with the purpose of optimizing their productivity and energy value originating the fourth-generation biofuel production (Rosenberg *et al*., 2008; Mata *et al*., 2010; Lu *et al*., 2011). The cellular metabolism and properties of these organisms are directed modified through the introduction, deletion, and/or modification of photosynthetic metabolic pathways by using recombinant DNA and other molecular biological and bioengineering techniques (Lee and Papoutsakis, 1999). Thus, algae and other microorganisms can be genetically modified to continuously produce biofuels and excrete them to the medium while they are grown in photobioreactors (Lu *et al*., 2011).

1.4. 1-Butanol: overview

The most widespread biofuels are biodiesel, used in pure form or as a blend, and bioethanol, used as a blend (Durre, 2007). On the transportation sector, fuel ethanol such as E10 (a mixture of 10 % ethanol and 90 % motor gasoline) and E85 (a mixture of 85 % ethanol and 15 % motor gasoline) shows to be more used than biodiesel. In 2018, fuel ethanol was used almost 5 times more when compared to biodiesel (EIA, 2019). Currently, ethanol – the main gasoline additive in the fuel market – is normally made from biomass fermentation using starch- and sugar-based feedstocks such as corn grain (usual in the United States) and sugar cane (habitual in Brazil) (U.S. Department of Energy, 2018). In recent years, researchers have seen 1-butanol – also referred as butyl alcohol, n-butanol, and butanol – as an alternative biofuel to bioethanol due to many advantages over ethanol (Durre, 2007; Lee *et al*., 2008; Ndaba *et al*., 2015).

Butanol (C_4H_9OH) is one of the four-carbon alcohols that may be used as a liquid biofuel (Lu *et al*., 2011). It is a colorless, flammable liquid with strong alcoholic odor

whose vapor has an irritant effect on mucous membranes and a narcotic effect when inhaled in high concentrations. Irritation of the skin or eyes may be caused by direct contact of butanol. Butanol is completely miscible with organic solvents but only partly soluble in water (Durre, 2008; Lee *et al*, 2008). Compared to ethanol, 1-butanol is superior as a fuel additive in many regards (**Table 2**) (Lee *et al*, 2008). Butanol is safer to handle since it has a lower vapor pressure, thus making it less explosive. It is not hygroscopic allowing blending of gasoline at the refinery, not just shortly before use as occurs with ethanol. This feature of butanol will also prevent contamination of groundwater in case of spills. It is also less corrosive, which means that the complete existing infrastructure – such as pipelines, pumps, filling stations, and tanks– can be used. With its four carbons, 1-butanol has more energy content than ethanol increasing the mileage/gasoline blend ratio (Durre, 2007).

1-Butanol can be used as a direct gasoline substitute or as a fuel additive in existing car engines without the need for modification and/or substitution since it has sufficiently similar characteristics to gasoline, as evidenced in **Table 2** (Durre, 2007; Atsumi *et al*., 2008; Lee *et al*, 2008). 1-Butanol can be blended with gasoline in a higher ratio than ethanol, which can be blended only up to 85 % (Durre, 2007). In 2005, an old Buick fueled by pure 1-butanol was driven across the United States by David Ramey. The fuel consumption increased by 9 % compared to gasoline but emissions of greenhouse gases were substantially reduced (Durre, 2008).

Table 2 | Properties of 1-butanol compared to ethanol and gasoline. Adapted from Lee *et al*. (2008).

Properties	1-Butanol	Ethanol	Gasoline
Energy density (MJ L^{-1})	29.2	19.6	32
Air-fuel ratio	11.2	9.0	14.6
Heat of vaporization (MJ Kg^{-1})	0.43	0.92	0.36
Research octane number	96	129	91 to 99
Motor octane number	78	102	81 to 89

1-Butanol can be produced from biomass (bio-butanol) or from fossil fuels (petro-butanol) with the same chemical properties (Jin *et al*., 2011). Bio-butanol is usually generated from food crop biomass that is not a sustainable alternative (Ndaba *et al*., 2015). However, many efforts are doing in order to use sustainable, lower-cost substrates as corn stover (He and Chen, 2013), agricultural waste (Cheng *et al*., 2012), rice straw (Ranjan *et al*., 2013), barley straw (Qureshi *et al*., 2013), switchgrass (Jain *et*

al., 2014), lignocellulosic biomass (Kumar *et al*., 2009), and glycerol that is obtained as a byproduct during biodiesel production (Khanna *et al*., 2013). Also, algae have become one of the promising feedstock for 1-butanol production (Van der Wal *et al*., 2013; Castro *et al*., 2015; Cheng, 2015).

The acetone butanol ethanol (ABE) fermentation of sugar, glycerol or lignocellulose feedstock by different microorganisms from the *Clostridiaceae* bacterial family is the common process for bio-butanol production. However, as ABE fermentation process has a number of limitations – low final 1-butanol concentration and yield, and high costs of butanol recovery (Ndaba *et al*, 2015) – researchers are undertaking various studies in order to optimize ABE fermentation. Genetic manipulation of *Clostridium* (Schwarz *et al*., 2017; Qi *et al*., 2018) as well as other promising technologies such as aldol condensation has been discussed in the literature (Juben *et al*., 2007; Ndaba *et al*., 2015). More recently researchers have opted to genetically modify algae and other photosynthetic microorganisms to continuously produce biofuels while they are grown in closed photobioreactors. This approach, which has much fewer production steps than the previous processes that need to harvest and process biomass, avoids large capital and operational costs (Lu *et al*., 2011).

1.5. 1-Butanol production in engineered microorganisms

Many research groups have achieved 1-butanol production from metabolic engineering of several microorganisms (**Table 3**). These successful cases in producing 1-butanol from metabolic engineering microorganisms encouraged other researchers to move on to metabolic engineering of photosynthetic organisms for 1-butanol production (Lu *et al*., 2011).

Cyanobacteria, mainly *Synechocystis* sp. and *Synechococcus elongatus*, have been used as a favored target of metabolic engineering for producing desired biochemical products, as demonstrated in **Table 4**. So far, the amount of biofuel compounds that have been produced in cyanobacteria is much lower than the necessary to make possible to commercialize these products. A lot of effort is being made to increase the productivity of target compounds (Miao, 2018).

Table 3 | Examples of metabolically engineered microorganisms producing 1-butanol.

Microorganism	1-Butanol titer (mg L^{-1})	References
Escherichia coli	1200	Inui *et al*., 2008
Saccharomyces cerevisiae	2.5	Steen *et al*., 2008
Lactobacillus brevis	300	Berezina *et al*., 2010
Pseudomonas putida	120	Nielsen *et al*., 2009
Bacillus subtilis	24	Nielsen *et al*., 2009

Table 4 | Examples of biochemical substances produced by genetically modified cyanobacteria.

Biofuel	Titer (mg L^{-1})	References
Isobutanol	450	Atsumi *et al*., 2009
Ethanol	5500	Gao *et al*., 2012
1-Butanol	100	Josefine, 2016
2,3 - Butanediol	2400	Oliver *et al*., 2013
Ethylene	171	Ungerer *et al*., 2012
Fatty acids	197	Liu *et al*., 2011
Isobutyraldehyde	1100	Atsumi *et al*., 2009
2-Methyl-n-butanol	200	Shen and Liao, 2012
Acetone	36	Zhou *et al*., 2012
Isopropanol	146	Hirokawa *et al*., 2015
Isopreno	1260	Gao *et al*., 2016

1.6. Cyanobacteria

Cyanobacteria are a morphologically and developmentally diverse group of Gram-negative prokaryotes that can be found in most types of illuminated environment (Stanier and Cohen-Bazire, 1977; Whitton and Potts, 2012). They range from simple unicellular forms that reproduce by binary fission to complex filamentous forms capable of true branching or even truly multicellular that have vegetative cells for oxygenic photosynthesis and differentiated cells such as heterocysts for nitrogen fixation. The ability of some strains to fix nitrogen makes them independent of nitrogen and carbon sources. Cyanobacteria predominantly grow photoautotrophically, but some are capable of photoheterotrophy or chemoheterotrophy (Waterbury, 2006). These organisms shelter in their interior a photosynthetic apparatus – thylakoids – remarkably similar in functional,

structural, and molecular respects to that contained in the eukaryotic chloroplast presenting the capacity to perform oxygenic plantlike photosynthesis (Stanier and Cohen-Bazire, 1977; Waterbury, 2006). It is believed that cyanobacteria were the first organisms to carry out oxygenic photosynthesis leading to the accumulation of considerable amounts of oxygen in the atmosphere at around 2.45 billion years ago, which gave rise to the Great Oxidation Event (Olson, 2006; Flannery and Walter, 2012). According to the endosymbiotic theory, the chloroplasts of plants and algae descended from cyanobacteria (Martin and Kowallik, 1999).

Until 1960, these photosynthetic microbes were called blue-green algae due to their color given by chlorophyll *a* and phycobiliproteins primary photosynthetic pigments and were classified along with the green algae, the red algae, and the brown algae (Haselkorn, 2009; Whitton and Potts, 2012). In the 1960s, new biochemical evidence – sensitivity to antibiotics and lack of organelles such as chloroplasts and mitochondria – has emerged showing that the blue-green algae, unlike the other algae, are really bacteria. Therefore, these blue-green bacteria were removed from the realm of botany and integrated into the microbiology world with the new name cyanobacteria (Haselkorn, 2009). However, botanists and phycologists have had some difficulty in accepting this name change, so both denominations continue to be used depending on the interests and prejudices of each author (Wilmotte, 1994; Stanier and Cohen-Bazire, 1977).

Cyanobacteria is a very prominent group for biotechnological applications because it has a faster growth rate than plants and it can be more easily genetically engineered compared to algae, in addition to its capacity to use sunlight as an energy source and CO_2 as carbon source. Many molecular tools for genetic investigation and modification of cyanobacteria that have been developed over the last decades are currently available for genetic engineering of cyanobacteria (Heidorn *et al*., 2011). Much of these studies are performed with the two-model organisms *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* sp. PCC 7492. The introduction of optimized metabolic pathways into the metabolism of cyanobacteria allows the photosynthetic production of a wide range of valuable compounds such as biofuels from CO_2, light, and water, making them cyanobacterial cell factories (**Figure 1**) (Angermayr *et al*., 2009; Yu *et al*., 2013; Savakis and Hellingwerf, 2015).

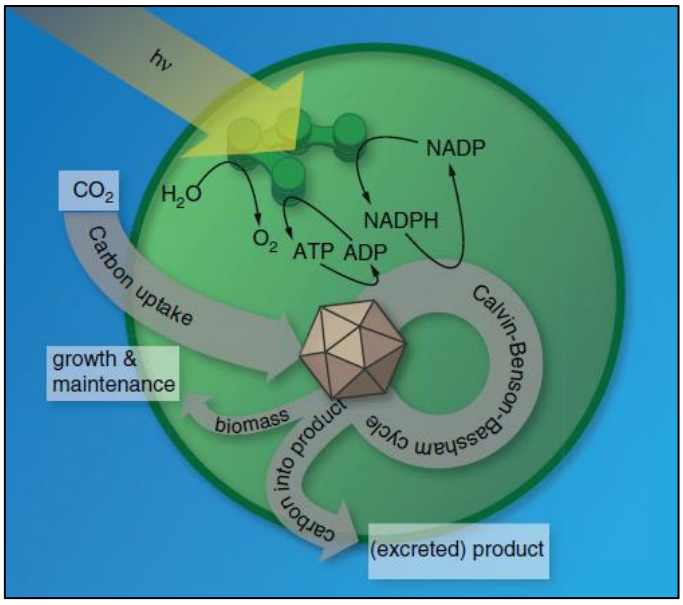

Figure 1 | Schematic representation of a cyanobacterial cell factory. In the thylakoids, photon energy drives water splitting, phosphorylation of ADP into ATP, and reduction of NADP into NADPH. Carbon dioxide gets assimilated in the Calvin-Benson-Bassham cycle and then the fixed carbon is reduced with the help of NADPH and ATP and channeled into biomass or into a production pathway. hv: photon energy, H_2O: water, O_2: oxygen, ATP: adenosine triphosphate, ADP: adenosine diphosphate, NADP: nicotinamide adenine dinucleotide phosphate, NADPH: nicotinamide adenine dinucleotide phosphate hydrogen, CO_2: carbon dioxide (Savakis and Hellingwerf, 2015).

1.6.1. Photosynthesis in cyanobacteria

The only process whereby photoautotrophs convert light energy to chemical energy in the form of organic matter is termed photosynthesis. The usual form is oxygenic photosynthesis in higher plants, algae, and cyanobacteria that can be expressed as a redox reaction driven by light energy in which carbon dioxide (CO_2) and water (H_2O) are converted to carbohydrates and oxygen (O_2) (Masojídek *et al*., 2013; Gilbert and Wilhelm, 2018). Furthermore, cyanobacteria have the additional capability of performing anoxygenic photosynthesis using hydrogen sulfide (H_2S) instead of H_2O as electron donor without releasing of O_2 (Shevela *et al*., 2013).

The oxygenic photosynthetic process comprises two major stages: the light-dependent reactions and light-independent reactions. The photosynthetic light-dependent reactions occur in the thylakoid membrane in most cyanobacteria. However, whereas in higher plants and algae the thylakoid membrane is located into the chloroplast, in cyanobacteria the membrane is within the cytoplasm. The first event in photosynthetic light-dependent reactions of cyanobacteria begins with the absorption of photons by phycobilisomes, which are attached to the cytoplasmatic surface of the photosynthetic membrane. Normally, the phycobilisomes system absorbs wavelengths range of 300 to 700 nm. Phycobiliproteins, which constitute the phycobilisomes, deliver the light energy absorbed to photosystems I and II. Photosystems are reaction centers

containing chlorophyll molecules that are integrated into the thylakoid membrane and work in series. The energy transfer from phycobiliproteins to photosystems I and II begins the conversion process of light energy into chemical energy. The photochemical acts within photosystem II involve fast, sequential electron transfers that result in the oxidation of H_2O and transfer of their electrons to nicotinamide adenine dinucleotide phosphate ($NADP^+$) leading to its reduction to NADPH and release of O_2. The light-driven electron transport from H_2O to $NADP^+$ catalyzed by both photosystems in addition to leading to reduction of $NADP^+$ to NADPH also leads to the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Shevela *et al*., 2013).

The energy stored in NADPH and ATP obtained during light-dependent reactions is then used for the assimilation of CO_2 into carbohydrates during the light-independent reactions. This phase, which involves a cycle so-called Calvin-Benson cycle, takes place in the stroma (higher plants and algae) or cytoplasmic region (cyanobacteria) and do not depend directly on light (Najafpour *et al*., 2013; Gilbert and Wilhelm, 2018). These secondary reactions can be subdivided into three stages: carboxylation, reduction, and regeneration of ribulose-1,5-bisphosphate (RuBP) (**Figure 2**). Carboxylation corresponds to the first stage and consists of the binding of CO_2 by the enzyme complex ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to the sugar RuBP. The labile 6-carbon intermediate is hydrolyzed into two molecules 3-phosphoglycerate (3-PGA). In a second stage, 3-PGA is reduced to glyceraldehyde-3-phosphate (GA3P) with consume of ATP and NADPH. The third and last stage corresponds to the regeneration of RuBP through a complex arrangement of reactions with ATP consumption. Five from six GA3P molecules obtained when three CO_2 molecules enter the Calvin cycle are needed to regenerate three molecules of RuBP, being the remainder GA3P molecule used for the production of glucose. As a GA3P molecule contains three fixed carbon atoms, two GA3P are necessary to build a six-carbon glucose molecule that will be used to suppress the needs of carbon of the photosynthetic organisms (Gilbert and Wilhelm, 2018).

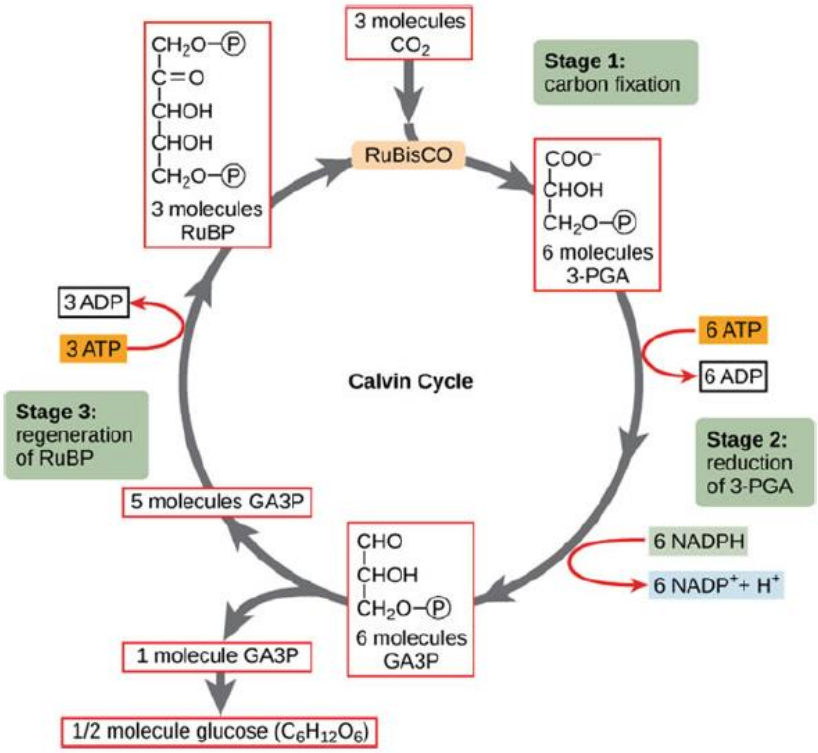

Figure 2 | Representative diagram of the Calvin cycle. CO_2: carbon dioxide, RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase, 3-PGA: 3-phosphoglycerate, ATP: adenosine triphosphate, ADP: adenosine diphosphate, NADPH: nicotinamide adenine dinucleotide phosphate hydrogen, NADP: nicotinamide adenine dinucleotide phosphate, H^+: hydron, GA3P: glyceraldehyde-3-phosphate, RuBP: ribulose-1,5-bisphosphate (Gilbert and Wilhelm, 2018).

1.6.2. 1-Butanol as a metabolite from cyanobacteria

1-Butanol can be produced by two different pathways (Lan and Liao, 2011): the CoA-dependent pathway (Atsumi *et al*., 2008) and the synthetic keto-acid pathway (Shen and Liao, 2008). The first route is used in nature by *Clostridium* sp. to produce 1-butanol along with ethanol and acetone and has been successfully transferred to non-native and facultative anaerobes organisms. Since the CoA-dependent pathway is derived from strict anaerobes, expressing this route into photoautotrophic organisms such as cyanobacteria, which produce oxygen during the photosynthesis, require some modifications because the enzymes involved may be negatively affected by the presence of oxygen (Lan and Liao, 2011). The 1-butanol production by CoA-dependent pathway in cyanobacteria is schematized in **Figure 3**. The synthetic keto-acid pathway that utilizes intermediates from amino acid biosynthesis routes is more commonly used for isobutanol production (Lan and Liao, 2011; Wong *et al*., 2017). In this pathway, 2-ketovalerate, a non-natural intermediate provided by the enzymes LeuABCD of leucine biosynthesis, is decarboxylated and then reduced to 1-butanol (Shen and Liao, 2008; Lan and Liao, 2011).

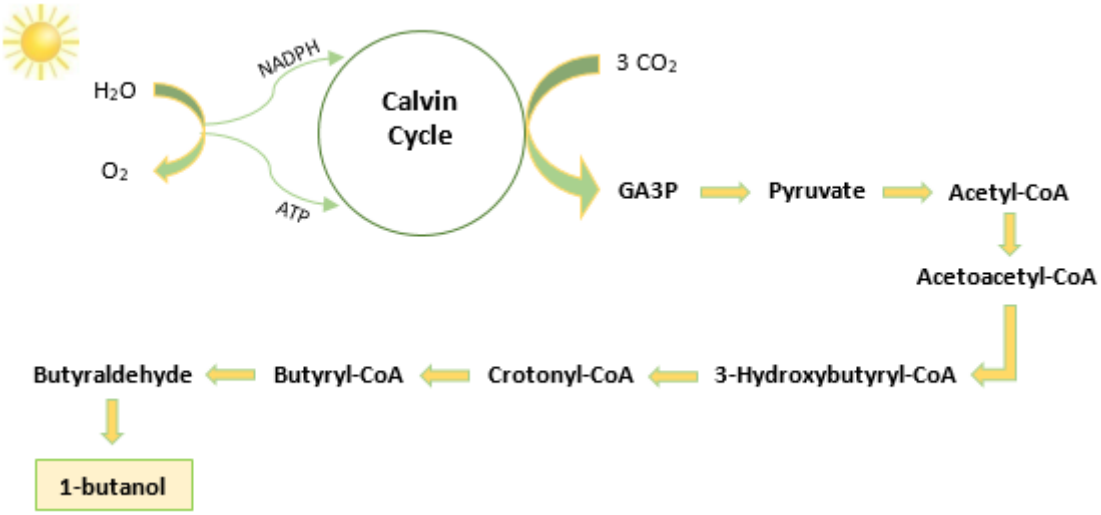

Figure 3 | Schematic of 1-butanol production from CO_2 for cyanobacteria metabolism. H_2O: water, O_2: oxygen, NADPH: nicotinamide adenine dinucleotide phosphate hydrogen, ATP: adenosine triphosphate, CO_2: carbon dioxide, GA3P, glyceraldehyde-3-phosphate. Adapted from Lan and Liao (2011) and Lan *et al*. (2013).

The first photoautotrophic production of 1-butanol in a genetically modified *Synechococcus elongatus* 7942 expressing the modified CoA-dependent 1-butanol synthesis pathway was demonstrated by Lan and Liao (2011) with a titer of 14.5 mg L^{-1} obtained under darkness and anoxic conditions. Further manipulations of the strain used by this group helped to increase 1-butanol titers to 29.9 mg L^{-1} (Lan and Liao, 2012) and further to 317 mg L^{-1} under photosynthetic conditions (Lan *et al*., 2013). 1-Butanol production has also been demonstrated in metabolically engineered *Synechocystis* sp. PCC 6803 reaching maximum butanol concentrations in the flask of 37 mg L^{-1} (Anfelt *et al*., 2015) and 100 mg L^{-1} (Josefine, 2016), for example.

Synechocystis sp. PCC 6803 is one of the most popular organisms for genetic and physiological studies of photosynthetic processes because its genome is completely sequenced and biochemistry and physiological information available, and is able to be transformable by exogenous DNA (Grigorieva and Shestakov, 1982; Ikeuchi, 1996; Lu *et al*., 2011). The cyanobacterium strain used in this master's project was a genetically modified *Synechocystis* sp. PCC 6803 for 1-butanol production. The genetic modification of this strain, referred to hereafter as SynBuOH-44, is presented on the **Materials and Methods** section. The maximum in-flask titer observed with this optimized metabolically engineered *Synechocystis* sp. strain for photosynthetic production of 1-butanol was 2.13 g L^{-1}. The cumulative titer obtained was 4.71 g L^{-1} (Miao, 2018). A4F – Algafuel, S.A., the company where this internship was developed, has been focused on the optimization of the cultivation of this strain at pilot-scale for maximum production of 1-butanol. So far, the maximum 1-butanol concentration achieved at A4F's pilot-scale was 117 mg L^{-1}.

Although this value is very far from the value we know to be the potential of the strain under laboratory conditions (2.13 g L^{-1}), this concentration obtained at pilot scale cultivations (42 L) is higher than most of the values obtained at laboratory-scale found in the literature mentioned above (Lan and Liao, 2011, 2012; Anfelt *et al*., 2015; Josefine, 2016).

1.6.3. *Synechocystis* sp. PCC 6803

The cyanobacterium *Synechocystis* sp. PCC 6803 is one of the most extensively studied species since it was isolated from a freshwater lake in 1968 in California (Yu *et al*., 2013). It was the first phototrophic organism to have its entire genomic sequence (Kaneko *et al*., 1996), including four large endogenous plasmids (Kaneko *et al*., 2003), fully determined allowing its genetic manipulation (Vermaas, 1996). The genome database for this cyanobacterial strain is publicly available at CyanoBase (Nakamura, 2000). *Synechocystis* sp. PCC 6803 is naturally transformable once it incorporates external isolated DNA without pre-treatment of the cells, and it evidence efficient homologous recombination between the foreign DNA inserted in the cell and regions of identical sequence in the cyanobacterial genome (Grigorieva and Shestakov, 1982; Vermaas, 1996). This cyanobacterium has been genetically engineered as a cell factory for the production of valuable compounds (Luan *et al*., 2015; Zhu *et al*., 2015; Miao, 2018).

Synechocystis sp. PCC 6803 can grow photoautotrophically, heterotrophically, mixotrophically, and anaerobically. Photoautotrophic growth, which requires CO_2, light, and the functional presence of both photosystems (I and II), has doubling times of 10 to 15 hours depending on light intensity and CO_2 concentration (Vermaas, 1996). The activities of the photosystems I and II and the Calvin cycle enzymes are influenced by environmental factors such as light intensity (Aurora *et al*., 2007), radiation wavelength (Hübschmann *et al*., 2005), light and dark cycles (Gill *et al*., 2002), and nutrient availability (Imamura *et al*., 2006). The specific growth rate of any photoautotrophic organism depends essentially on light irradiance, pH, temperature, and concentrations of CO_2, nitrogen, and phosphorus (Kim *et al*., 2011). *Synechocystis* sp. PCC 6803 is usually cultivated at 30 ºC using BG-11 culture medium at a pH between 7 and 8, however, the cells can grow in alkaline culture medium (Yu *et al*., 2013). The concentration of inorganic carbon (C_i) in the culture is a crucial factor since carbon is the nutrient consumed in the largest quantity constituting about 50 % of the cyanobacterial biomass dry weight (Kim *et al*., 2011). The cyanobacterium *Synechocystis* sp. PCC 6803 has systems for carbon acquisition with transporters for CO_2 and HCO_3^- input (Badger *et*

al., 2002). The concentrations of CO_2 and HCO_3^- depend strongly on the ambient pH whereby a very high pH may indicate that the concentrations of these inorganic carbon are low, affecting the growth rate. Other crucial macronutrients that can be limiting for growth of the culture are inorganic nitrogen (Ni), phosphorus (Pi), and sulfur (Si), which constitute around 12 %, 1.5 %, and 0.6 % of the biomass dry weight, respectively (Kim *et al*., 2011).

Although there are optimal conditions for the cultivation of the *Synechocystis* sp. PCC 6803, it supports a wide range of environmental variations, therefore, it is an excellent candidate for large-scale biomass production (Kim *et al*., 2011).

1.7. Pilot-scale cultivation: photobioreactors

Phototrophic organisms are grown in photobioreactors that allows photobiological reactions (Tredici, 2004). Photobioreactors can be defined as culture systems for phototrophic organisms in which photons, the main source of energy for growth, do not impact directly on the culture surface but need to pass through the transparent walls of the reactor before reaching the cells (Zittelli *et al*., 2013). In closed photobioreactors the exchange of liquids and particles between the culture and the atmosphere is strongly limited, thus maintaining controlled culture conditions and protecting the culture from contamination by competing microorganisms (Tredici, 2004; Zittelli *et al*., 2013). The amount of energy that reaches the surface of the culture should be maximized through the design and orientation of the photobioreactor since the distribution of the intercepted radiation on the culture surface depends on the geometry of the reactor (Acién *et al*., 2013).

In terms of design, photobioreactors can be classified into three categories: 1) flat or tubular, 2) horizontal, inclined, spiral, or vertical, and 3) manifold or serpentine (Tredici, 2004). Tubular photobioreactors and flat panels are the main systems belonging to the category of closed reactors. Flat panels consist of two linked parallel transparent panels where the culture is stored, on which the culture is illuminated from one or both sides and stirred by aeration (**Figure 4**). This type of culture system used for mass production of photoautotrophic microorganisms can be made of glass or plastic, and can be rigid or flexible (Tredici, 2004; Acién *et al*., 2013). Tubular photobioreactors (**Figure 5**) are the most usual design of closed systems for commercial phototrophs cultivation. These reactors are habitually made of plastic or glass tubes in which the culture is circulated through pumps or preferably by air streams (airlift system) (Zittelli *et al*., 2013; Acién *et al*., 2017).

Figure 4 | Pilot facility of flat panel photobioreactors installed inside a thermoelectric power station located at Tocopilla (Chile) (Acién, 2017).

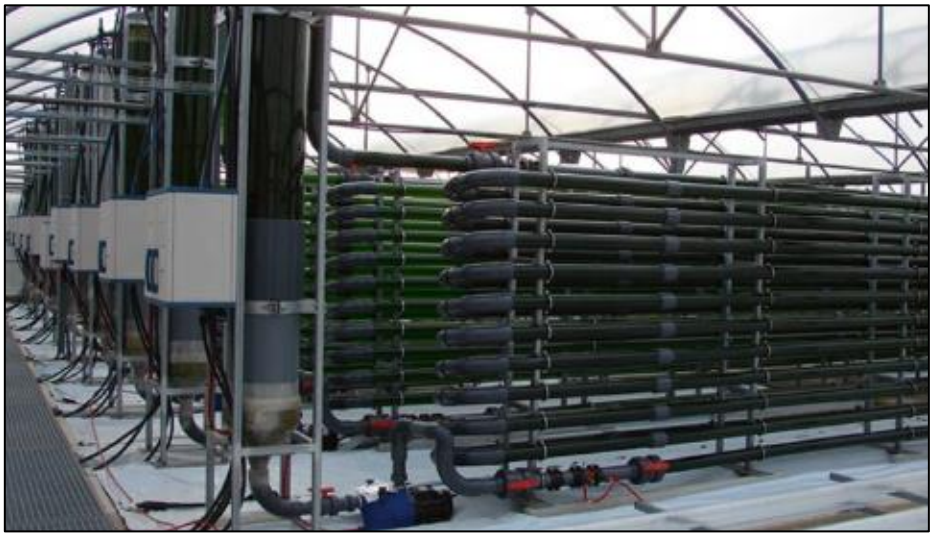

Figure 5 | Serpentine photobioreactors operated in a 30 m^3 plant installed at the Estación Experimental de Cajamar "Las Palmerillas" (Almeria) (Acién, 2017).

Phototrophs can also be cultivated in open systems that include lakes, natural ponds, and raceway ponds. These systems are easier and less expensive to build and operate than most of the closed reactors in addition to being more durable. However, the risk of contamination of the culture by fungi, bacteria, protozoa, or other microorganisms, and their competition with the original species used as inoculum is quite large so that not all microorganisms can be grown in open reactors (Tredici, 2004).

Although there is a wide range of systems available for the cultivation of photosynthetic microorganisms, in all of them it is necessary the satisfaction of requirements for the growth of these organisms such as light availability, nutrients

supply, pH and temperature control, agitation and mixing of the culture (Acién *et al*., 2017; Yen *et al*., 2019).

The cultivation of genetically modified microorganisms (GMM) requires specific containment measures to limit their contact with the general population and the environment (European Parliament and Council of the European Union, 2009). The main concern with large-scale cultivation of GMM seems to be related either to the potential escape of viable GM microorganisms or the relevant transgene(s) into the natural ecosystem. The latter can occur in cyanobacteria by horizontal gene transfer (HGT), which is the transfer of genetic material between organisms in a manner other than by the transmission of DNA from parent to offspring (Kumar, 2015). In order to prevent contact between the genetically modified cells and the environment, closed photobioreactors seem to be the most suitable culture system since it can be considered contained when placed inside a building (Wijffels, 2015).

1.8. Aim

The project developed in this internship is integrated into a Horizon 2020 European project named Photofuel, which aims to develop a next-generation technology for the biocatalytic production of solar fuels, only requiring sunlight, CO_2, and water. The Photofuel project is working on a technology of microalgae cultivation in closed photobioreactors for the production of phototrophic cyanobacteria or algae microorganisms, which are genetically modified to produce engine-ready fuels or precursor substances and excrete them to the culture broth. The product of interest is directly separated without the need to harvest biomass, which makes this process more sustainable and economically viable.

The work's main goal alongside A4F – Algafuel, S.A. in this project is optimizing the culture conditions at pilot-scale of a genetically modified cyanobacterium *Synechocystis* sp. PCC 6803 strain for 1-butanol production in order to enhance alcohol productivity. It is expected that the optimizations carried out make viable the commercialization of 1-butanol as an innovative, cost-competitive, and liquid-transportation fuel from a non-food feedstock.

Chapter 2.

Materials and Methods

2. Materials and Methods

This project was fully performed within the R&D group of A4F – Algafuel, S.A. located at the Lisbon laboratory, which is a GMO Class II certified laboratory, and at the Lisbon experimental unit, which has a GMO compliant area. Biosafety procedures on the contained use of genetically modified microorganisms required by the European Union were guaranteed in order to provide a high level of safety for the general population and the environment (European Parliament and Council of the European Union, 2009).

2.1. Biological material

The genetically modified cyanobacterium *Synechocystis* sp. PCC 6803 strain capable of photosynthetic production and excretion of 1-butanol – SynBuOH-44 (Miao, 2018) – used in this study was provided by the University of Uppsala located in Sweden, a partner in the Photofuel project. The engineered *Synechocystis* was maintained cryopreserved (Day, 2007), what will be addressed below.

Synechocystis sp. PCC 6803 was genetically altered to produce 1-butanol via an acetyl-CoA dependent optimized pathway with six reaction steps to synthesize the biofuel precursor, as shown in **Figure 6**. Resistance genes to kanamycin, erythromycin, and spectinomycin were inserted into the genome of this strain to act as a selective pressure on transformed cells.

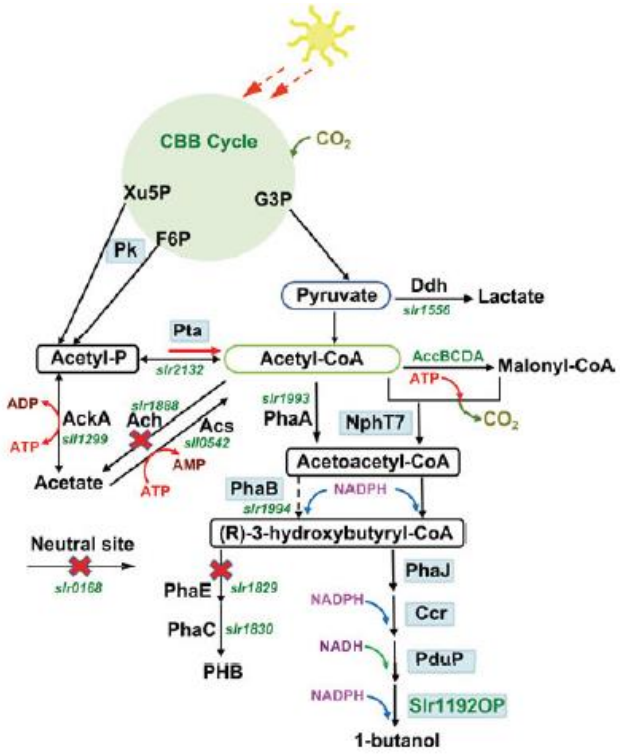

Figure 6 | Schematic overview of 1-butanol biosynthesis pathway introduced into *Synechocystis* sp. PCC 6803. The endogenous genes are in green and the introduced genes are in blue boxes. The red crosses indicate the knockout *loci* in *Synechocystis* genome. NphT7: acetoacetyl-CoA synthase from *Streptomyces* strain CL190, PhaB (T173S): mutated version of acetoacetyl-CoA reductase from *R. eutropha*, PhaJ: (R)-specific enoyl-CoA hydratase from *A. caviae*, Ccr: crotonyl-CoA reductase from *S. collimus*, PduP: CoA-acylating propionaldehyde dehydrogenase from *S. enterica*, Slr1192OP: codon re-optimized version of Slr1192 (ADH) from *Synechocystis*, Pk: phosphoketolase from *P. aeruginosa*, Pta: phosphate acetyltransferase from *B. subtilis,* Xu5P: xylulose 5-phosphate, F6P: fructose 6-phosphate, G3P: glyceraldehyde 3-phosphate, PHB: Polyhydroxybutyrate, Ddh: D-lactate dehydrogenase, PhaA: acetyl-CoA acetyltransferase, PhaE and PhaC: poly(3-hydroxyalkanoate), AckA: acetate kinase, Ach: acetyl-CoA hydrolase, Acs: acetyl-CoA synthetase, CBB cycle: Calvin-Benson-Bassham cycle, CO_2: carbon dioxide, ATP: adenosine triphosphate, ADP: adenosine diphosphate, NADH: nicotinamide adenine dinucleotide, NADPH: nicotinamide adenine dinucleotide phosphate hydrogen. Miao (2018).

2.1.1. Cryopreservation

SynBuOH-44 cells in exponential growth phase were cryopreserved by a method based on Day (2007) by freezing with dimethylsulfoxide (DMSO; Me_2SO) (*Carlo Erba*) as a cryoprotectant. The 100 % (V V^{-1}) DMSO was sterilized by filtration with 0.2 µm polytetrafluoroethylene (PTFE) membranes (*Whatman*®) and added to 10x concentrated cultures for a final concentration of 8 % (V V^{-1}). After mixing with DMSO, the cyanobacterial cultures were frozen at -70 ºC in autoclaved cryopreservation vials.

To revive, the cryopreservation vials were quickly thawed, centrifuged (*MiniSpin*® *5453, Eppendorf*) at 14100 x *g* for a few seconds, and the pellet was resuspended with an industrial culture medium developed and optimized by A4F – Algafuel S.A. (A4F-IM), described in **2.2.**. The genetically modified *Synechocystis* cells were transferred to a

culture system and diluted with fresh culture medium until the desired concentration for recovery. The procedure described was performed into a class II laminar flow chamber (*Bio II Advance*, *Telstar*), previously disinfected with 70 % (V V^{-1}) ethanol and decontaminated with ultraviolet (UV) radiation.

The cultures were incubated at a pH 7 to 8 in a culture chamber with a temperature of 25 ºC, under a photoperiod of 24 h with continuous aeration of air supplemented with 0.5 % CO_2.

2.2. Scale-up strategy

The production of inoculum in the laboratory from cryopreserved culture was necessary whenever a new pilot- or laboratory-scale assay was initiated, ensuring genetic stability of the strain and lower risk of loss or contamination (Saks, 1978). All the manipulations in the cultures grown in the laboratory were performed in a laminar flow chamber (*Bio II Advance*, *Telstar*), as mentioned in **2.1.1.**, to maintain axenic cultures. At pilot-unit, aseptic conditions were more complicated to achieve although there has been a great effort in the use of a flame and 10 % (V V^{-1}) bleach and/or 70 % (V V^{-1}) ethanol (never simultaneously with the use of the flame) during all the handling of the culture.

SynBuOH-44 cultures were initiated with a few milliliters – usually 4 mL – of cryopreserved culture from the cyanobacteria culture collection of A4F – Algafuel. S.A. in Lisbon, which was used to inoculate 60 to 80 mL of liquid A4F industrial medium (A4F-IM) in a small aerated bottle (*SCHOTT Duran®*) with inlet and outlet air filters with 0.2 µm membranes (*Sartorius, Midisart® 2000*) to maintain aseptic conditions and prevent cross-contamination. The A4F-IM is based on an algal culture medium (Fábregas *et al*., 2000) and was optimized by A4F – Algafuel, S.A. for laboratory-scale cultivation of freshwater cyanobacteria. It comprises water with a low concentration of salt supplemented with a nutritive medium constituted by nitrogen, phosphorous, iron, sodium, magnesium, zinc, cobalt, molybdenum, copper, manganese, vitamins, etc. In order to protect the intellectual property of A4F – Algafuel, S.A., the recipe of the A4F-IM is not presented. When the cultures are scaled-up also proper sterilized antibiotics – 25 mg L^{-1} kanamycin sulfate (*BioChemica*), 25 mg L^{-1} spectinomycin dihydrochloride pentahydrate (*TCI Chemicals*), and 50 mg L^{-1} erythromycin (*Farmaquímica Sur*) – were added to the cultures to keep the selective pressure of the genetic modifications. The antibiotics were sterilized by filtration with 0.2 µm cellulose acetate membranes (*Neoreax, Lda*) (kanamycin and spectinomycin) and with 0.2 µm polytetrafluoroethylene (PTFE)

membranes (*Whatman*®) (erythromycin). Antibiotics were not added to the cultures after their reanimation since afterward this process the cells may be fragile.

During the inoculum production in the laboratory, the cultures were cultivated according to the conditions described in **2.1.1.**. Under these conditions, the cultures concentrated rapidly allowing the sequential scale-up process with A4F-IM into more and bigger aerated bottles (*SCHOTT Duran*®) with inlet and outlet air filters with 0.2 µm membranes (*Sartorius, Midisart*® *2000*), as represented in **Figure 7**. In order to avoid very diluted cultures and, consequently, possible photoinhibition each scale-up step was limited to a 2 to 5 fold volume increase from the previous step.

The pilot-scale cultivation of 0.05 m^3 of SynBuOH-44 requires the production of at least 18 L of laboratory inoculum with an average concentration of 0.5 g L^{-1} to inoculate the 55 L outdoor photobioreactor with an initial target concentration of around 0.15 g L^{-1}. Using 4 mL of cryopreserved culture it took around three weeks to produce a sufficient volume of inoculum for inoculation of the photobioreactor.

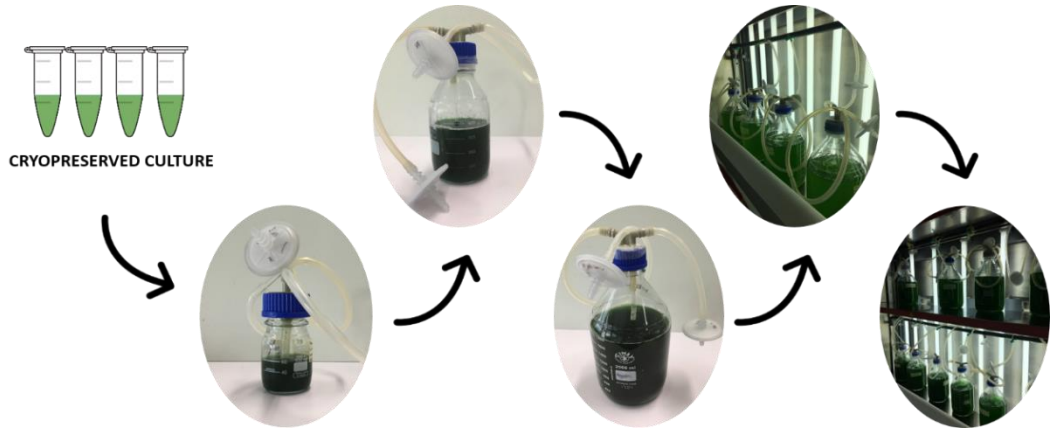

Figure 7 | Diagram of the scale-up procedure in the laboratory for inoculum production.

2.3. Cultivation of SynBuOH-44

2.3.1. Laboratory-scale

The cultivation of the *Synechocystis* sp. PCC 6803 strain that was genetically altered to produce and excrete 1-butanol was carried out at laboratory and pilot unit. Parallel to the pilot-scale tests, laboratory assays were performed in order to assess the influence of certain conditions on the accumulation of 1-butanol in the culture to function as a preliminary test for pilot-scale assays, or simply to corroborate in controlled conditions results obtained from pilot-scale tests.

Two culture mediums were used to cultivate SynBuOH-44 at laboratory: a modified version of a standard laboratory medium for cyanobacteria (BG-11) originality formulated

by Hughes *et al.* (1958), and the A4F industrial medium (A4F-IM) developed and optimized by A4F – Algafuel, S.A. for freshwater cyanobacteria, as mentioned in the previous section. The modified recipe of the BG-11 is disclosed in **Table 5**. 25 mM HEPES buffer was further added to the prepared BG-11.

Table 5 | Composition of the modified version of a standard laboratory medium for cyanobacteria (BG-11).

Compound	Concentration in BG-11
$NaNO_3$	8.80 mM
$MgSO_4 \cdot 7H_2O$	0.15 mM
$CaCl_2 \cdot 2H_2O$	0.12 mM
$C_6H_8O_7 \cdot H_2O$	16.90 µM
$Na_2EDTA \cdot 2H_2O$	1.30 µM
H_3BO_3	46.30 µM
$MnCl_2 \cdot 4H_2O$	9.15 µM
$ZnSO_4 \cdot 7H_2O$	0.77 µM
$Na_2MoO_4 \cdot 2H_2O$	1.62 µM
$CuSO_4 \cdot 5H_2O$	0.32 µM
$Co(Cl_2) \cdot 6H_2O$	0.17 µM
$C_6H_5FeO_7$	21.43 µM
Na_2CO_3	18.87 µM
K_2HPO_4	17.51 µM
$C_6H_{15}NO_6S$	0.02 M

The cultivation systems used for the laboratory tests were 25 cm^3 cell culture flasks (*Sarstedt*). Their filter cap with 0.22 µm pore size hydrophobic membrane for gas exchange were capped with insulating tape in order to avoid possible losses of 1-butanol. The closed cell culture flasks were inoculated with culture to an optical density at a wavelength of 730 nm (OD_{730nm}) of 0.5 in 25 mL of culture medium supplemented with 50 mM $NaHCO_3$, which ensure the carbon supply for the cells. The proper antibiotics – 25 mg L^{-1} kanamycin sulfate (*BioChemica*), 25 mg L^{-1} spectinomycin dihydrochloride pentahydrate (*TCI Chemicals*), and 50 mg L^{-1} erythromycin (*Farmaquímica Sur*) –, previously sterilized as described in **2.2.**, were also added to the cultures. The cultures prepared in duplicates were placed in the orbital incubator (*INCU-Line*®, *VWR*), positioned as shown in **Figure 8**, under continuous shaking (120 rpm) and 24 h

photoperiod with a light intensity of 30 μmol m^{-2} s^{-1}. The laboratory-scale assays were performed in a semi-continuous regime by removing 10 % (V V^{-1}) of the culture and adding the same volume removed of fresh culture medium supplemented with 500 mM $NaHCO_3$ and the proper antibiotics three times a week. The pH was measured with pH test strips and controlled for values of 7 to 8 or around 10 by the addition of 10 M HCl and 8 M NaOH to decrease or increase the pH, respectively.

Figure 8 | Arrangement of the cell culture flasks in test in the orbital incubator.

2.3.2. Pilot-scale

The culture medium used to cultivate SynBuOH-44 at pilot-scale was the A4F industrial medium (A4F-IM), as stated above.

For the pilot-scale assays, a vertical flat-panel photobioreactor with a capacity of 60 L, also named green wall panel, developed by A4F – Algafuel, S.A. and installed in a greenhouse located at the Lisbon Experimental Unit of A4F – Algafuel, S.A., was used. The photobioreactor is capable to sustain temperatures over 100 ºC in order to allow decontamination after genetically modified microorganisms cultivation and reactor sterilization prior inoculation, which is crucial for the production of a culture free of contaminants. Before inoculations, the photobioreactor, made of glass and stainless steel, was disinfected with 50 ppm sodium hypochlorite and sterilized with two cycles of hot steam at 100 ºC for one hour. Additionally, all the operations in the green wall panel were performed using a flame and 10 % (V V^{-1}) bleach and/or 70 % (V V^{-1}) ethanol.

The inoculations of the photobioreactor were made with culture from the laboratory transported into an autoclavable jerrican under aseptic conditions to the Lisbon experimental unit and added to the culture system by gravity. The cultures grown on the photobioreactor with an air outlet and inlet were homogenized by bubbling CO_2 enriched air. The temperature set-point was 30 $\pm$ 1 ºC controlled by a temperature controller connected to a chiller allowing the temperature to be maintained to the predefined values. The cultures were cultivated under a 24 h photoperiod, as result of natural light supplemented with 24 h light-emitting diode (LED) radiation (161 μmol m^{-2} s^{-1}), at a pH

set-point of 7 to 8 or 10.5, regulated by the manually addition of CO_2 and $NaHCO_3$ to decrease or increase the pH, respectively, and to simultaneously provide a carbon source. Air filters with 0.2 µm hydrophobic PTFE membrane (*PolyVENT*™, *Whatman*®) were used at the outlet of the culture aeration preventing the escape of genetically modified organisms to the environment. Depending on the conditions to be tested, the photobioreactor set-up, as illustrated in **Figure 9**, was adapted in order to reach the desired conditions for the assay.

The culture growth rate was determined using **Equation 1**.

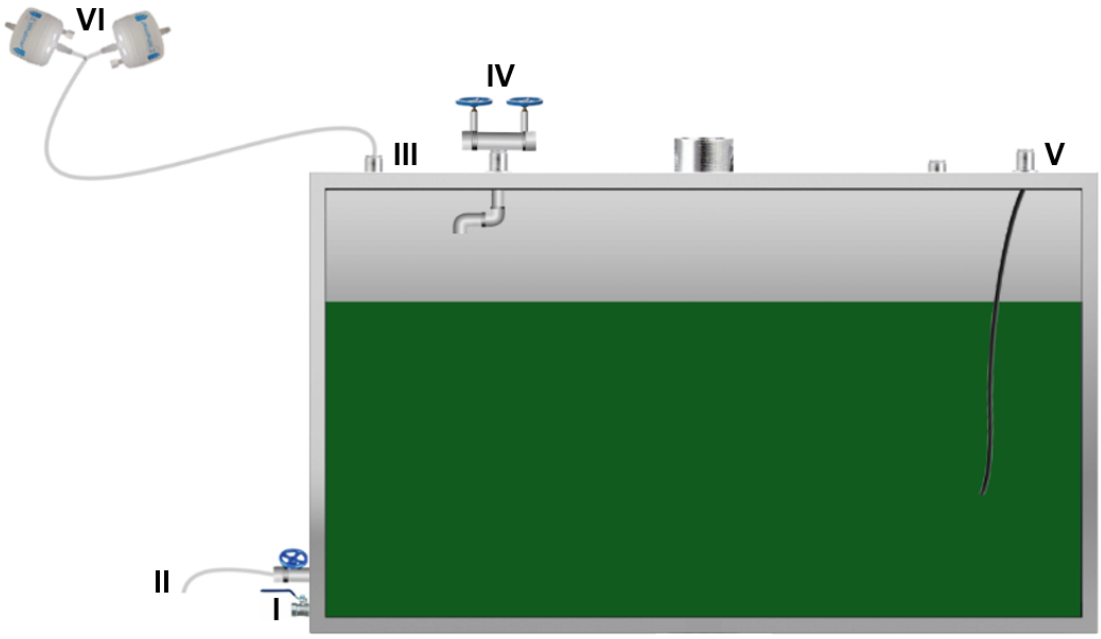

Figure 9 | Set-up of flat-panel photobioreactor. I: air and CO_2 inflow, II: sampling site, III: air outlet, IV: entry of inoculum, nutrients, or other solution to be added to the culture, V: temperature probe, VI: outlet air filters.

$$\boldsymbol{Culture\ growth\ rate\ \left(g\ L^{-1}\ day^{-1}\right) = \frac{\Delta DW}{\Delta t}}$$

Equation 1 | Determination of the culture growth rate in a certain period of time (g L^{-1} day^{-1}). *ΔDW* corresponds to the change in biomass dry weight (g L^{-1}) in the stipulated time interval, *Δt* is the time interval selected for the determination of the culture growth rate, in days.

2.3.2.1. Cultivation regime

Two cultivation regimes were used: fed-batch and continuous regime (chemostat). For the fed-batch regime, the photobioreactor was not changed in relation to the illustration presented in **Figure 9**, it was only necessary the addition of the nutritive medium developed by A4F to the culture using compressed air through a 0.2 µm air filter (*Sartorius, Midisart® 2000)*. The need to add the nutritive medium was determined by the

nitrate concentration available in the culture, measured as described in **2.4.4.**, in order to avoid nitrate deficiency in cells.

For the chemostat cultivation regime, PR7 peristaltic pumps (*Seko*) were used to provide continuous input of fresh culture medium (A4F-IM) into the photobioreactor while simultaneously removing the same culture volume from the growing system. The fresh culture medium was stored in an autoclavable jerrican and the culture removed from the system was collected to another jerrican, as represented in **Figure 10**. Each of these jerricans was connected to one peristaltic pump. Initially, peristaltic pumps were regulated to a constant flow rate of 9 L day^{-1} during the week, based on an assay accomplished with SynBuOH-44 by a project partner (data not published), and 5 L day^{-1} at the weekend due to the limitations of the capacity of the available jerricans (20 L), which are unable to sustain the operation of the chemostat at a flow rate of 9 L day^{-1} during the whole weekend time. The data obtained by using these two flow rates led to the choice of only a flow rate of 5 L day^{-1} to perform a second assay.

The biomass dilution rate was calculated according to **Equation 2**.

$$\boldsymbol{Biomass\ dilution\ rate\ \left(g\ L^{-1}\ day^{-1}\right) = DW\ x\ \frac{Flow\ rate}{Vol.}}$$

Equation 2 | Determination of the biomass dilution rate in g L^{-1} day^{-1}. *DW* corresponds to the biomass dry weight present in the culture (g L^{-1}), *Flow rate* corresponds to the flow rate at which the peristaltic pumps operate (L day^{-1}), *Vol.* is the volume of the culture (L).

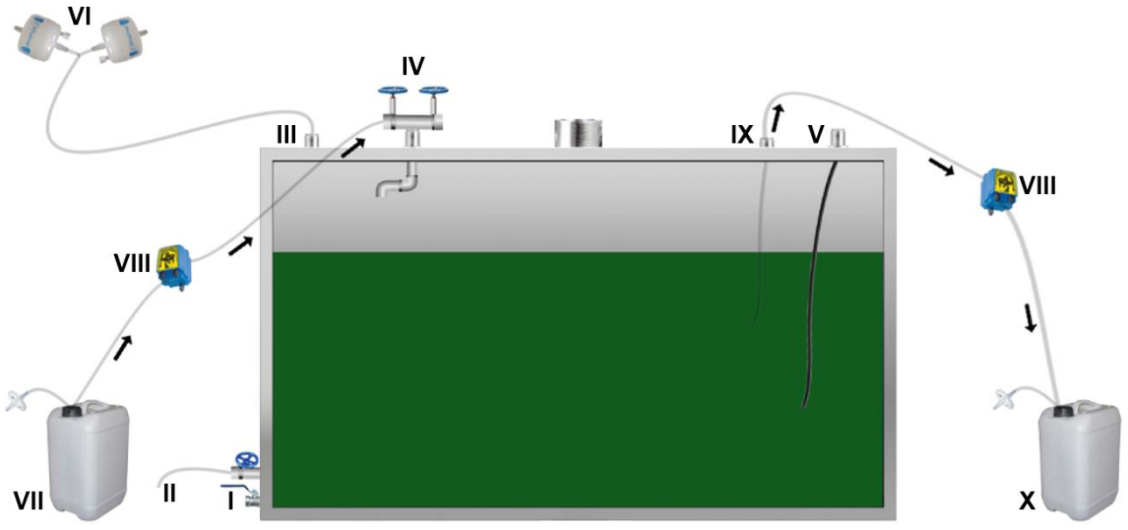

Figure 10 | Set-up of flat-panel photobioreactor in chemostat regime. I: air and CO_2 inflow, II: sampling site, III: air outlet, IV: entry of inoculum, fresh culture medium, or other solution to be added to the culture, V: temperature probe, VI: outlet air filters, VII: jerrican with fresh culture medium inside, VIII: peristaltic pump, IX: culture outlet, X: jerrican with culture removed from the photobioreactor inside. The arrows (→) indicate the flow direction of the fresh culture medium and the culture.

2.3.2.2. 1-Butanol lost by evaporation

A coil condenser was attached to the air outlet of the vertical flat-panel photobioreactor in order to condense the air that was lost from the culture system to a collection bottle, as illustrated in **Figure 11**. A recirculating chiller was connected to the condenser to circulate cooling liquid at about -14 ºC inside the condenser coil in order to condense the air entering to the condenser. The collection bottle was changed three times per week.

The percentage of 1-butanol lost by evaporation and condensed in the collection bottle through the condensing system was calculated based on the concentration of 1-butanol present in the culture and in the condensate, the culture and the condensate volumes, and the elapsed time, following **Equations 3** and **4**.

$$\boldsymbol{BuOH\ (mg) = [BuOH]\ x\ Vol.}$$

Equation 3 | Determination of the amount of 1-butanol (mg) presents in the sample. [*BuOH*] is the concentration of 1-butanol (mg L^{-1}) obtained for the sample, *Vol.* corresponds to the volume of culture or condensate.

$$\boldsymbol{BuOH\ cond.}\left(\%\ \boldsymbol{day^{-1}}\right) = \frac{\dfrac{\boldsymbol{BuOH\ cond.}}{\boldsymbol{BuOH\ cult.}}}{\boldsymbol{\Delta t}}\ \boldsymbol{x\ 100}$$

Equation 4 | Calculation of the percentage of condensed 1-butanol relative to the amount of 1-butanol obtained in the culture at t0, per day (% day^{-1}). *BuOH cond.* corresponds to the amount of condensed 1-butanol (mg) in the ***Δt*** considered, *BuOH cult.* corresponds to the amount of 1-butanol (mg) presents in the culture at t0 considered for the calculation, ***Δt*** corresponds to the time interval between samplings, in days.

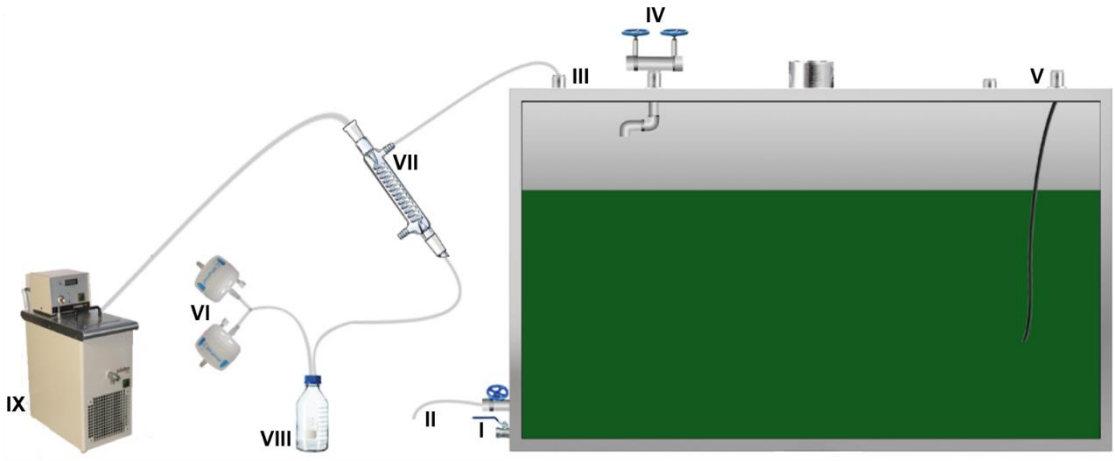

Figure 11 | Set-up of flat-panel photobioreactor with the condensing system attached to it. I: air and CO_2 inflow, II: sampling site, III: air outlet, IV: entry of inoculum, fresh culture medium, or other solution to be added to the culture, V: temperature probe, VI: outlet air filters, VII: coil condenser, VIII: collection bottle, IX: recirculating chiller.

2.3.2.2.1. Control test: 1-butanol lost by evaporation

In the course of assays carried out using a coil condenser to evaluate possible losses of 1-butanol by evaporation, a control test was necessary in order to understand if the condensing system to be used had the capacity to condense all the air that passed through it. To do so, the flat-panel photobioreactor with a coil condenser attached, as represented in **Figure 11**, was inoculated with water supplemented with a theoretical concentration of 1 g L^{-1} 1-butanol (*Chem-Lab*).

The photobioreactor was disinfected as described in **2.3.2.**.The volume of water added to the photobioreactor was 50 L since it is the volume with which it is usually inoculated with culture. The temperature set-point was 30 $\pm$ 1 ºC, which was the temperature used to cultivate SynBuOH-44. At the beginning of the test, the pH of the solution was adjusted to 12 with 8 M NaOH in order to have a better control of contaminants since high pH seems to be incompatible with the growth and survival of some contaminants, according to the literature (Touloupakis *et al*., 2016). Three times per week samples were collected from the photobioreactor and the condensate for 1-butanol analysis and microscopic observation to control the appearance of contaminants. The bottle where the condensate was stored was changed three times per week.

Knowing the concentration of 1-butanol inside the photobioreactor and in the condensate, it is possible to determine the percentage of 1-butanol lost to the environmental through **Equations 3**, **4**, **5** and **6**.

$$\boldsymbol{BuOH\ lost\ from\ PBR}\left(\%\ \boldsymbol{day^{-1}}\right) = \frac{\dfrac{\boldsymbol{\Delta BuOH}}{\boldsymbol{BuOH\ cult.}}}{\boldsymbol{\Delta t}}\ \boldsymbol{x}\ \mathbf{100}$$

Equation 5 | Determination of the percentage of 1-butanol lost from the photobioreactor (PBR), per day (% day^{-1}). Δ*BuOH* corresponds to the variation of the 1-butanol in the photobioreactor between samplings (mg), *BuOH cult.* corresponds to the amount of 1-butanol (mg) present in the culture at t0 considered for the calculation, *Δt* corresponds to the time interval between samplings, in days.

$$\boldsymbol{BuOH\ lost\ to\ the\ environment}\ (\%\ \boldsymbol{day^{-1}}) = \ \boldsymbol{BuOH\ lost\ from\ PBR - BuOH\ cond.}$$

Equation 6 | Calculation of the 1-butanol lost from the photobioreactor (PBR) to the environment (% day^{-1}). *BuOH lost from PBR* corresponds to the percentage of 1-butanol lost from the photobioreactor (% day^{-1}), *BuOH cond.* Corresponds to the percentage of condensed 1-butanol per day (% day^{-1}).

2.4. Routine analyses

In order to monitor cultures from the laboratory and outdoor cultivations, analyses were carried out frequently throughout the assays, which enabled the detection of anomalies that could influence the culture. The sampling of the culture of the flat-panel photobioreactor was made through the opening of a valve located in the lower zone of the reactor using 70 % (V V^{-1}) ethanol to keep the sample zone sterile. In the laboratory, the samplings were collected into a laminar flow chamber (*Bio II Advance*, *Telstar*), previously disinfected with 70 % (V V^{-1}) ethanol and decontaminated with UV radiation, using a sterile pipette.

2.4.1. Optical density

The optical density (OD) was measured at a wavelength of 730 nm, using a spectrophotometer (*Genesys™ 10S UV-Vis, Thermo Scientific™*) in order to evaluate the culture growth. Each sample was read in duplicate against distilled water using plastic cuvettes of 1 cm path. For samples of smaller volumes, the OD_{730nm} was measured in quadruplicate using a microplate reader (*SPECTROstar Nano, BMG LABTECH*). For concentrated samples, dilutions were performed in order to be in the linearity range of the Beer-Lambert law. The optical density measurement was performed three times per week, either in cultures being tested in the laboratory or in the pilot unit.

2.4.2. Biomass dry weight

The determination of biomass dry weight (DW) was used as a method to follow the culture growth. The dry cell weight was obtained by filtration of a known volume of culture through glass microfiber filters of 0.7 µm (*Glass microfibers filter 698, VWR*). An isotonic solution of 0.5 M ammonium formate (*ACROS organics™*) was used in order to remove salts. After filtration, the filters prepared in triplicates were weighted on a moisture analyzer (*MS-70, AnD*) at 180 °C. The biomass dry weight was obtained through **Equation 7**.

$$\boldsymbol{DW\ (g\ L^{-1}) = \frac{FM - IM}{Vol.}}$$

Equation 7 | Determination of dry weight in g L^{-1}. *FM* corresponds to the dry mass of the filters and biomass after filtration (g), *IM* corresponds to the mass of the filter before filtration (g), *Vol.* is the volume of the culture sample passed through the filters (mL).

2.4.2.1. Correlation between OD_{730nm} and DW

A correlation coefficient between optical density at a wavelength of 730 nm and dry weight (g L^{-1}) for SynBuOH-44 was determined. Through the use of the correlation coefficient, the determination of a theoretical dry weight value was possible using only the optical density value avoiding measure the dry weight, which is more laborious and takes much more time than measuring the optical density.

Using laboratory cultures, several measurements of DW and OD_{730nm} were performed following the procedures already described above.

2.4.3. Microscopic observation

Culture samples were analyzed under the microscope (*Olympus BX53*) to determine cell color and morphology, cell division, the presence of cellular debris, cell agglomerates, and contaminants, among others. The cultures tested were microscopically evaluated three times per week.

2.4.4. Nitrate concentration

The nitrate concentration in the culture was regularly measured and controlled for a set-point of 8 mM. Nitrate measurements were done with a method based on Carvalho *et al*. (1998). Specifically, 15 mL of culture sample was centrifuged at 1789 x *g* for 10 minutes (*Nahita 2655*). The concentrated supernatants obtained were diluted using distilled water in order to be in the linearity range of the Beer-Lambert law. 1 M HCl was added to the supernatants for a final concentration of 3 % (V V^{-1}) in order to prevent interferences from other absorbing compounds such as carbonate or hydroxide anions.

Samples were then analyzed for the nitrate ion content using a spectrophotometer (*Genesys™ 10S UV-Vis, Thermo Scientific™*). The absorbance value was then converted to nitrate concentration using the calibration curve, previously determined, obtained with the nitrate standard solutions (KNO_3). Every sample was read in duplicate utilizing quartz cuvettes of 1 cm path. Nitrate concentration of the cultures in test was estimated three times per week.

2.4.5. pH monitoring

The pH of the outdoor cultures was controlled three times a day on every working day using a field pH meter (*HI-98130*, *Hanna Instruments*). The pH of the laboratory cultures was controlled three times per week using pH test strips 1 to 14.

2.4.6. 1-Butanol quantification

Samples for 1-butanol analysis were sent to an external laboratory. A project partner carried out the 1-butanol extraction and analysis, as described below, and the results were provided to A4F – Algafuel, S.A..

1-Butanol was quantified from the culture medium of the cultivation samples. For 1-butanol extraction, 2.3 mL of cell culture was centrifuged at 14100 x *g* for 10 minutes (*MiniSpin®*, *Eppendorf*) and 2.07 mL of supernatant was collected in a 10 mL screw cap tube (*VWR*). Then, the supernatant was mixed with 30 µL 7000 mg L^{-1} isobutanol (*Sigma-Aldrich*), as an internal standard, and 700 µL (1:3 volume) 100 % (V V^{-1}) dichloromethane (DCM) (*Sigma-Aldrich*), which is an extraction solvent. The isobutanol standard was obtained by dilution with fresh BG-11 medium for a final concentration of 7000 mg L^{-1}. The mixture was shaken on multi-tube vortexer (*VX-2500*, *VWR*) for 5 minutes at maximum speed and afterwards centrifuged at 14100 x *g*, 4 °C, for 10 minutes, to get a faster and clearer separation of the two phases. The DCM phase (bottom phase) was transferred into 1.5 mL clear glass gas chromatography vials (*VWR*).

The extracted samples were analyzed on a gas chromatography (*Clarus® 580 GC*, *PerkinElmer*) system equipped with a flame ionization detector (GC-FID) and a polyethylene glycol (PEG) series capillary column 30 m x 0.25 mm x 0.25 µm (*Elite-WAX*, *PerkinElmer*). The carrier gas was nitrogen with a 10 mL min^{-1} flow rate. The injector and detector were working at temperatures of 220 ºC and 240 ºC, respectively. The initial oven temperature was 50 ºC and then it raised to 100 ºC with a rate of 10 ºC min^{-1} followed by a rise to 180 ºC with a rate of 20 ºC min^{-1}.

The retention time was determined using 100 mg L^{-1} isobutanol standard (*Sigma-Aldrich*) and 1-butanol standard (*VWR*). Chromatographic peaks of 1-butanol and isobutanol were indicated at the retention time 4.55 and 4.00 minutes, respectively. The presence of 1-butanol was identified by retention time and comparison with 1-butanol standard. All samples were spiked with the same amounts of isobutanol (100 mg L^{-1}), which was used as the internal standard for quantitative determination. Thus, a standard curve was made by measuring extractants (extracted in the same way as the culture samples of this work) from liquid BG-11 medium with nine different concentrations of 1-butanol standard (10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 75 mg L^{-1}, 100 mg L^{-1}, 250 mg L^{-1}, 500 mg L^{-1}, 750 mg L^{-1}, and 1000 mg L^{-1}) and 100 mg L^{-1} of isobutanol as internal standard. The amount of 1-butanol in the culture (in-flask titer of 1-butanol) was calculated based on the ratio of its signal peak area and that of the internal standard. The cumulative titer of 1-butanol takes into account the dilutions made to the cyanobacterial culture broth by feeding. The GC results were analyzed using *TotalChrom Navigator* version 6.3.2.

Culture samples were taken three times per week for 1-butanol content analysis and the most informative selection of those were sent to analysis.

2.5. Statistical analysis

The growth of the cultures developed in the laboratory, indicated by OD_{730nm}, herein presented resulted from the calculated mean ± standard deviation of two replicated assays. Although laboratory assays were performed in biological duplicates, only one replicate was analyzed over a time course for 1-butanol content. However, in certain cases where the 1-butanol content data did not follow the expected pattern, both replicates were analyzed and that results were expressed as mean ± standard deviation. The mean ± standard deviation was obtained using *Microsoft Excel* software. The statistical analysis of the laboratory cultures growth data was carried out using an unpaired *Student's T-Test* via *GraphPad Prism 8* software. Significance was determined at a *p-value* inferior to 0.05.

Each assay performed at pilot-scale was carried out only once since it was a work of culture conditions sequential optimization.

Chapter 3.

Results and Discussion

3. Results and Discussion

A4F – Algafuel, S.A., the company that welcomed me to develop my master's internship, is part of the Photofuel project consortium contributing to the upscaling of biocatalytic systems and the outdoor fuel production. The tests carried out during this work were sometimes based on unpublished results obtained throughout the project by the consortium partners or other companies. Since this project started in May 2015, also results obtained by the A4F – Algafuel, S.A. before my integration into the team were crucial for the assays performed and herein presented.

With the aim of enhancing 1-butanol production and accumulation in SynBuOH-44 cultures at pilot-scale making viable its commercialization, several assays in an outdoor photobioreactor were performed as well as laboratory experiments allowing to test certain conditions in a more controlled environment.

3.1. Laboratory-scale

Although the focus of this work is the pilot-unit, laboratory assays were crucial to obtaining insight into the impact of certain conditions, such as culture medium and pH, on 1-butanol production and accumulation in the culture. This prior knowledge, besides being directly applicable in pilot-scale tests, also allows a better interpretation of the results achieved there.

3.1.1. Correlation between OD_{730nm} and DW

A correlation coefficient between OD_{730nm} and DW (g L^{-1}) for SynBuOH-44 was determined in order to monetize the time dedicated to these routine analyses in business context. This correlation would avoid regular measurements of biomass dry weight that are much more laborious and time-consuming than the measurement of optical density values.

The data of OD_{730nm} and DW obtained were plotted (**Figure 12**) and the correlation reached is **Equation 8** with a $R^2 = 0.9945$. The correlation coefficient can be applied to all assays that were performed.

$$\boldsymbol{DW\ (g\ L^{-1}) = 0.3032\ x\ OD_{730nm}}$$

Equation 8 | Correlation between OD_{730nm} and DW, in g L^{-1}, for SynBuOH-44 cultures. The correlation coefficient is 0.3032.

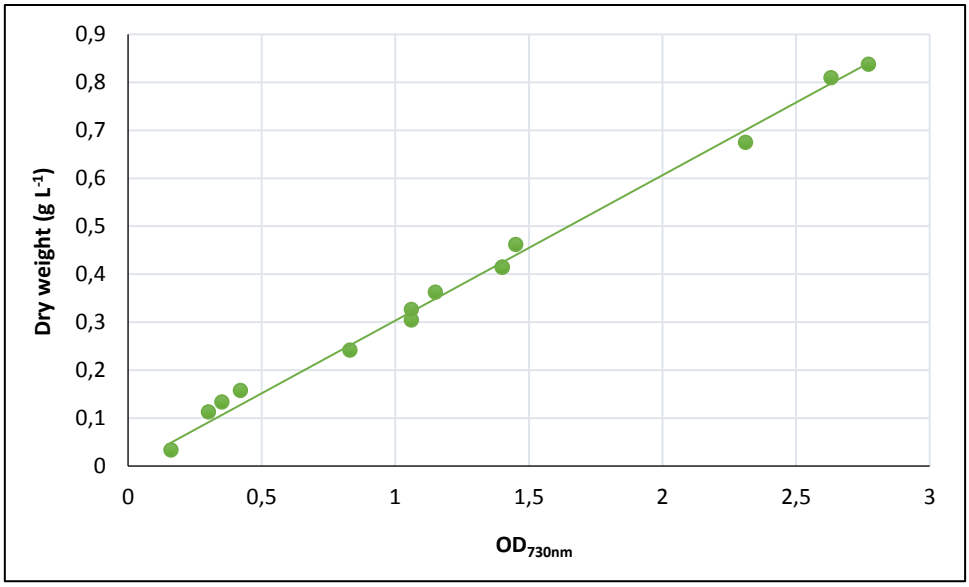

Figure 12 | Correlation curve between DW, in g L^{-1}, and OD_{730nm} for SynBuOH-44 cultures. The correlation coefficient is 0.3032 with a R^2 = 0.9945.

3.1.2. Impact of the culture medium on 1-butanol production

In order to understand the possible influence of the culture medium used for cultivating SynBuOH-44 on 1-butanol production and accumulation, a laboratory assay was carried out. For this purpose, it was used an industrial culture medium developed and optimized by A4F – Algafuel, S.A. for freshwater microalgae and cyanobacteria (A4F-IM) and a modified version of a standard laboratory medium for cyanobacteria (BG-11). The culture conditions are shown in **Table 6**.

Figure 13 presents the growth curves of the cultures as well as the 1-butanol in-flask titer obtained during the assay.

Table 6 | Experimental conditions of the assay aimed to determine the impact of the culture medium on 1-butanol production and accumulation.

System	Culture regime	Culture medium	Volume	pH	Photoperiod	Light intensity	Temperature set-point	Carbon source
Cell culture flasks	Semi-continuous	BG-11 and A4F-IM	25 mL	7 to 8	24 h	30 µmol m^{-2} s^{-1}	30 ºC	$NaHCO_3$

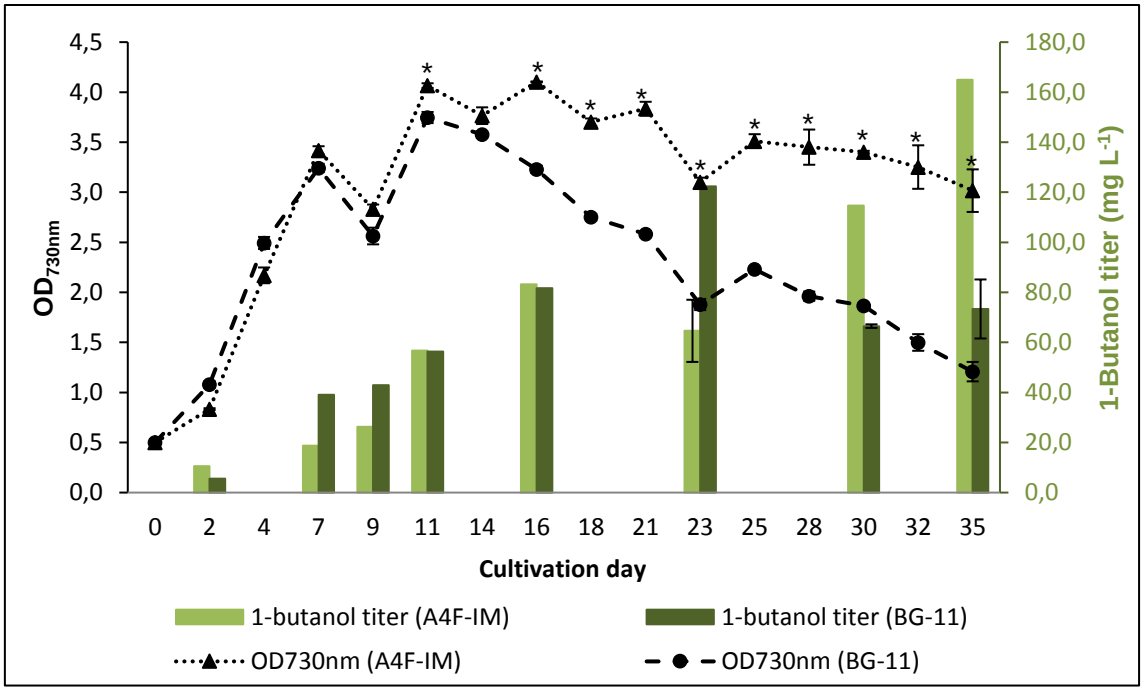

Figure 13 | Growth curves of SynBuOH-44 indicated by OD_{730nm} and 1-butanol in-flask titer (mg L^{-1}) during 35 days of cultivation in both A4F-IM and BG-11 culture medium at pH commonly used for *Synechocystis* sp. cultivation (pH 7 to 8). The values of OD_{730nm} and three values of 1-butanol titer (23rd (A4F-IM), 30th (BG-11) and 35th (BG-11) day of cultivation) are expressed as mean ± standard deviation. Error bars indicate standard deviation of biological duplicates. * indicates statistically significant differences in the growth of the cells developed in the two culture media tested for each cultivation day (unpaired *T-test*, $p < 0.05$).

Comparing both growth curves (**Figure 13**), from day 0 until the 11th day of cultivation (exponential growth phase), there are no statistically significant differences between the cultures cultivated in A4F-IM or BG-11 culture medium. However, from the 11th day of cultivation, the cell concentration of the cultures grown in BG-11 decreased more sharply than those cultivated in A4F-IM revealing statistically significant differences between both conditions in this period. These differences in cell concentration between cultures grown in two different culture media at the intermediate to late stages of the assay were also evident at the macroscopic level by changes in the color intensity of the cultures (**Figure 14**).

Of all samples analyzed, the maximum 1-butanol in-flask titer was obtained in cultures grown in A4F-IM (165.01 mg L^{-1}) on the 35th day of cultivation (**Figure 13**). A trend analysis of 1-butanol content results shows that, until the 16th day of cultivation, do not appear to be major differences between cultures grown in A4F-IM and BG-11 medium. However, during the intermediate to late death phase of the cultures grown in BG-11, the differences between 1-butanol titer obtained with both culture media became more pronounced since there was a much smaller number of living cells producing 1-butanol in BG-11 medium compared to A4F-IM.

Figure 14 | Cultivation of SynBuOH-44 in two different culture media at pH 7 to 8: A4F-IM (flasks on left) and BG-11 (flasks on right). This image corresponds to the 35th day of cultivation and depicts a faded green color in cultures grown in BG-11 (flasks on right).

The maximum 1-butanol in-flask titer obtained in this test (165.01 mg L^{-1}) is below what is known to be the potential of this strain under laboratory conditions (2.13 g L^{-1}) (Miao, 2018). However, the maximum 1-butanol value achieved is higher than most of the values found in the literature obtained at laboratory-scale with other genetically modified cyanobacteria strains (**Table 7**).

Table 7 | Photoautotrophic production of 1-butanol in genetically modified cyanobacteria.

Microorganism	1-Butanol titer (mg L^{-1})	References
***Synechococcus elongatus* 7942**	14.5	Lan and Liao, 2011
***Synechococcus elongatus* 7942**	29.9	Lan and Liao, 2012
***Synechocystis* sp. PCC 6803**	37.0	Anfelt *et al*., 2015
***Synechocystis* sp. PCC 6803**	100.0	Josefine, 2016

The results obtained in this assay indicate that the use of A4F-IM, which consists of water with a percentage of salt and supplemented with a nutritive medium, as a culture medium for SynBuOH-44 does not seem to harm its growth. Although cultures growth was similar in both culture media during growth phase, thereafter statistically significant differences between cultures grown in different culture media were evident. Cultures grown in the standard medium used to grow *Synechocystis* sp. (BG-11) experienced a marked decrease in their cell concentration. Although the reason for this event is unknown, it is suspected that the limitation of some nutrient may have caused that. According to the literature, sulfate is the limiting nutrient in BG-11 and its limitation leads to ROS formation followed by bleaching and death of the *Synechocystis* sp. culture (Alphen *et al*., 2018).

In addition to being infeasible to use a culture medium formulated for laboratory practice for large-scale cultivations, the data obtained in this assay revealed that the cultivation of SynBuOH-44 in A4F-IM, in general, does not appear to impair the 1-butanol production and accumulation in the culture as well as its growth. These reasons make the industrial medium developed by A4F – Algafuel, S.A. a promising medium for the cultivation of this genetically engineered cyanobacterium for 1-butanol production. However, these positive indications about the use of A4F-IM for cultivations of SynBuOH-44 should be confirmed by repeating this assay, ideally in triplicates, and analyzing the 1-butanol content for all replicates.

3.1.3. Impact of the pH on 1-butanol production

The need to evaluate in a laboratory environment the effect that the cultivation of SynBuOH-44 at alkaline pH may have on cell growth and 1-butanol production and accumulation in the culture led to the performance of this assay. For this purpose, the genetically modified strain for 1-butanol production was cultivated with A4F industrial medium developed and optimized by A4F – Algafuel, S.A. (A4F-IM) at pH normally used for *Synechocystis* sp. cultivation (between 7 and 8) and alkaline pH (about 10). The experimental conditions are presented in **Table 8**.

Figure 15 presents the cell growth curves and the 1-butanol in-flask titer obtained during this assay.

Table 8 | Experimental conditions of the assay aimed to determine the impact of the pH on 1-butanol production and accumulation.

System	Culture regime	Culture medium	Volume	pH	Photoperiod	Light intensity	Temperature set-point	Carbon source
Cell culture flasks	Semi-continuous	A4F-IM	25 mL	7 to 8 and around 10	24 h	30 µmol m^{-2} s^{-1}	30 ºC	$NaHCO_3$

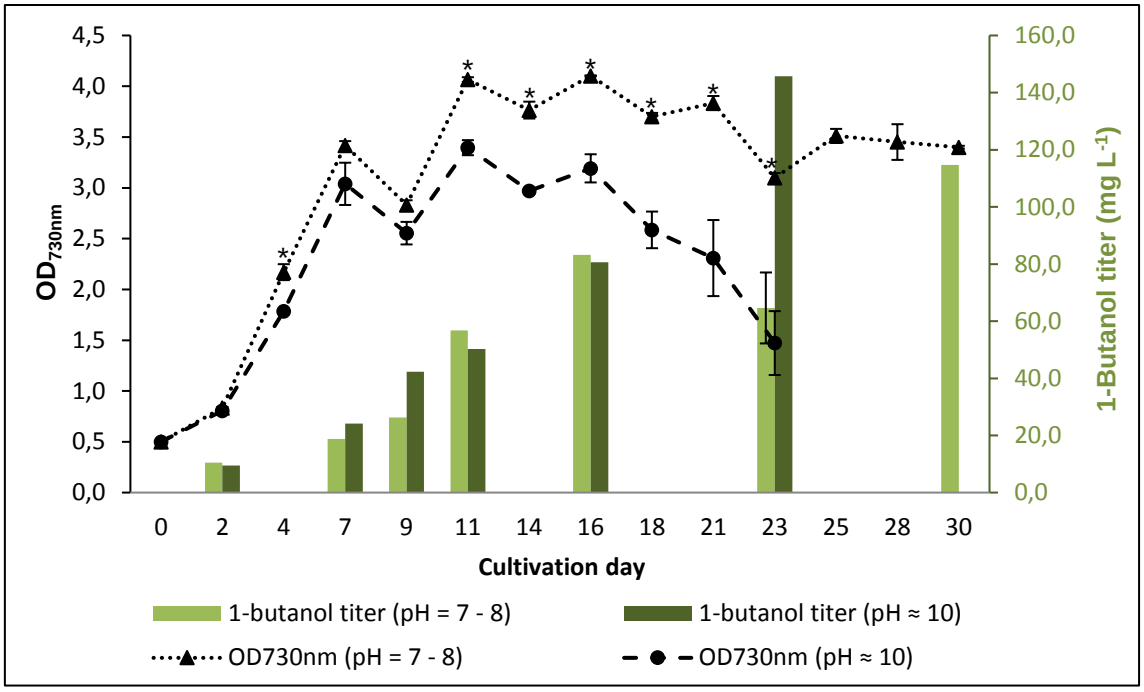

Figure 15 | Growth of SynBuOH-44 indicated by optical density at a wavelength of 730 nm (OD_{730nm}) and 1-butanol in-flask titer (mg L^{-1}) during the 30 days of cultivation in A4F-IM culture medium at two different pH: between 7 and 8 and about 10. The values of OD_{730nm} and one value of 1-butanol titer (23rd (A4F-IM) day of cultivation) are expressed as mean ± standard deviation. Error bars indicate standard deviation of biological duplicates. * indicates statistically significant differences in the growth of the cells developed in the two pH tested for each cultivation day (unpaired *T-test*, $p < 0.05$).

Through the statistical analysis of the growth curves obtained in this assay (**Figure 15**) it is concluded that, in general, significant differences exist between the cultures grown at pH frequently used for *Synechocystis* sp. cultivation (between 7 and 8) and alkaline pH (around 10). The cell growth of the cultures grown at alkaline pH is lower than that observed in cultures grown at pH 7 to 8. Moreover, from the 11th day of cultivation, the cell density observed in the cultures grown at alkaline pH started to decrease sharply culminating in the bleaching and death of the culture. The yellowish color of the cultures is visible in **Figure 16**.

Taking into account only the samples selected and analyzed for determination of the 1-butanol content, the maximum concentration of 1-butanol obtained was 145.75 mg L^{-1} corresponding to the last cultivation day of the culture grown at pH around 10 (**Figure 15**). The trend of the graph in **Figure 15** indicates that cultures cultivated at alkaline pH (around 10) do not appear to negatively influence the 1-butanol production and accumulation in the culture when compared to cultures developed at pH 7 to 8.

Figure 16 | Cultivations of SynBuOH-44 in A4F-IM culture medium at two different pH values: between 7 and 8 (flasks on left) and around 10 (flasks on right). This image corresponds to the 23rd day of cultivation.

Once again, the maximum 1-butanol in-flask titer obtained in this assay (145.75 mg L^{-1}) did not reach a value close to what is the known potential of this strain (2.13 g L^{-1}) (Miao, 2018). Nonetheless, it surpassed most of the values found in the literature for 1-butanol producing cyanobacteria strains (**Table 7**). Interestingly, the maximum 1-butanol titer was achieved during the death phase of the culture grown at alkaline pH. Cell death will lead to cell lysis and release of dissolved organic carbon (Franklin, 2013), such as 1-butanol, promoting an increase in the accumulated product of interest in the culture.

While the tested pH does not appear to negatively influence the 1-butanol production and accumulation in the culture, the biomass productivity was significantly affected in cultures grown at alkaline pH compared to those grown at pH between 7 and 8. These results were not surprising since the pH of 7.5 has been reported to be the pH value that leads to optimal biomass productivity in *Synechocystis* sp. cultures (Zhang *et al*., 2009; Touloupakis *et al*., 2016). The pH values between 7.5 and 10 lead to a slight reduction in productivity of about 5 %. However, an increase of the pH from 10 to 11 shows a sharper reduction in productivity of more than 30 % (Touloupakis *et al*., 2016). Several studies found in the literature have shown that cultivation of *Synechocystis* sp. at pH from 7.5 up to 11 is viable (Panda *et al*., 2006; Zhang *et al*., 2009; Touloupakis *et al*., 2016). In this experiment, it was possible to grow SynBuOH-44 at pH around 10, however, the cultures reached a faster cell death in which the culture bleached and the cell concentration decreased sharply. The cause for this event is not clear, however, it is believed that it may have occurred due to the unavailability of carbon in the chemical forms that can be incorporated into the cells. *Synechocystis* sp. PCC 6803 owns systems for inorganic carbon acquisition, which consists of CO_2 uptake mechanisms and HCO_3^- transporters (Badger *et al*., 2002). The concentrations of HCO_3^- and CO_2 in solution depend strongly on the ambient pH. These chemical species tend to drop at high pH increasing the concentration of CO_3^{2-}, which becomes the major form of inorganic carbon

in solution. However, CO_3^{2-} is not taken up by *Synechocystis* sp. PCC 6803 whereby the growth rate could be influenced (Kim *et al*., 2011).

Although 1-butanol production and accumulation do not seem to be impaired with SynBuOH-44 cultivation at high pH (around 10), under the conditions in which this assay was performed, there is a high risk of premature death of the culture since pH plays an important role in carbon supply. Thus, unless there is a considerable reason to do an alkaline pH cultivation at a pilot-scale, cells should ideally be cultivated at a pH regularly adjusted to a value between 7 and 8. Nonetheless, as in the previous assay, the indicative results obtained should be corroborated by repeating this test in triplicates and performing the 1-butanol content analysis for all replicates.

3.2. Pilot-scale

This master's project focuses on optimizing SynBuOH-44 cultivation in the company's pilot-unit. The following presented assays were performed in the same flat-panel photobioreactor at different times, although with different engineered adaptations. The optimization of the culture conditions is fundamental to improve 1-butanol production in the culture to become a viable system for the sustainable production of this biofuel at industrial-scale.

3.2.1. 1-Butanol lost by evaporation

Due to the low productivity of 1-butanol obtained in previous assays performed by the A4F – Algafuel, S.A. team (unpublished results) when compared to what it is known to be the potential of this strain, 2.13 g L^{-1} (Miao, 2018), it was suspected that 1-butanol might be lost through aeration of the culture. In order to understand if there were losses of 1-butanol by evaporation due to aeration during cultivation at pilot-scale, a coil condenser was connected to the air outlet of the flat-panel photobioreactor, as illustrated in **Figure 11** (Baez *et al*., 2011). The air leaving the production system was condensed and accumulated in a collection bottle. The culture conditions are presented in **Table 9**.

The photobioreactor inoculated with SynBuOH-44 culture and operated in a fed-batch culture regime is shown in **Figure 17** and the condensing system attached to it, essentially constituted by the coil condenser, the cooling system, the collection bottle, and the air outlet filters, is presented in **Figure 18**.

Table 9 | Experimental conditions of the assay aimed to determine possible losses of 1-butanol by evaporation due to aeration during cultivation at pilot-scale.

System	Culture regime	Culture medium	Initial volume	pH set-point	Light	Temperature set-point	Carbon source
Flat-panel PBR with condensing system attached to it	Fed-batch	A4F-IM	50 L	7 to 8	Natural and natural + LED system	30 ºC	CO_2 + $NaHCO_3$

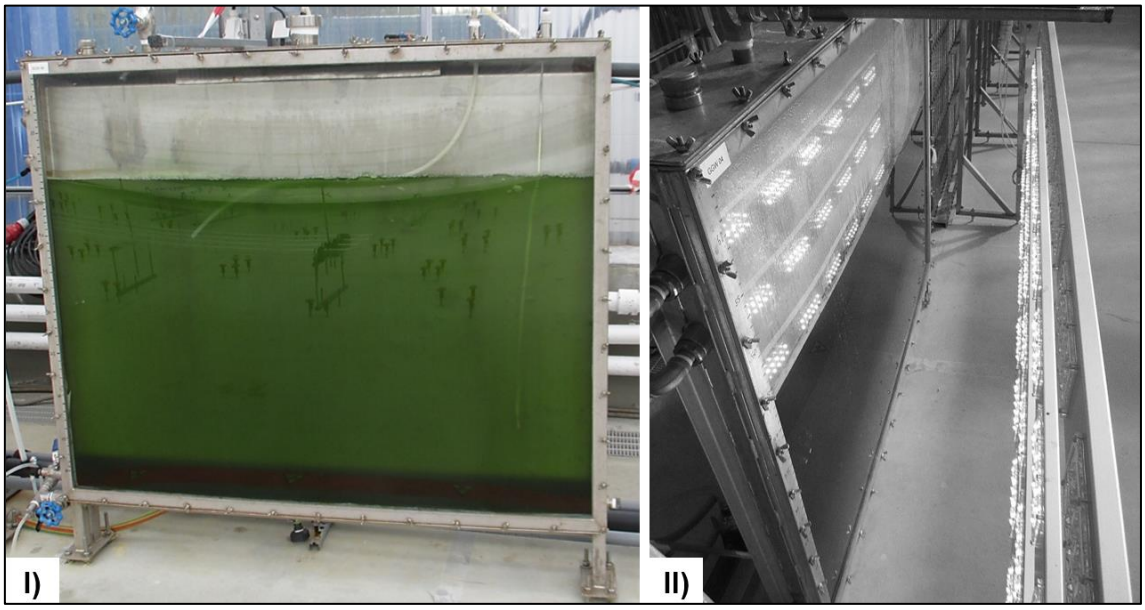

Figure 17 | Flat-panel photobioreactor inoculated with culture of SynBuOH-44. I) Photobioreactor without a LED artificial lighting system. II) Photobioreactor with a LED system (colorless image for confidentiality reasons).

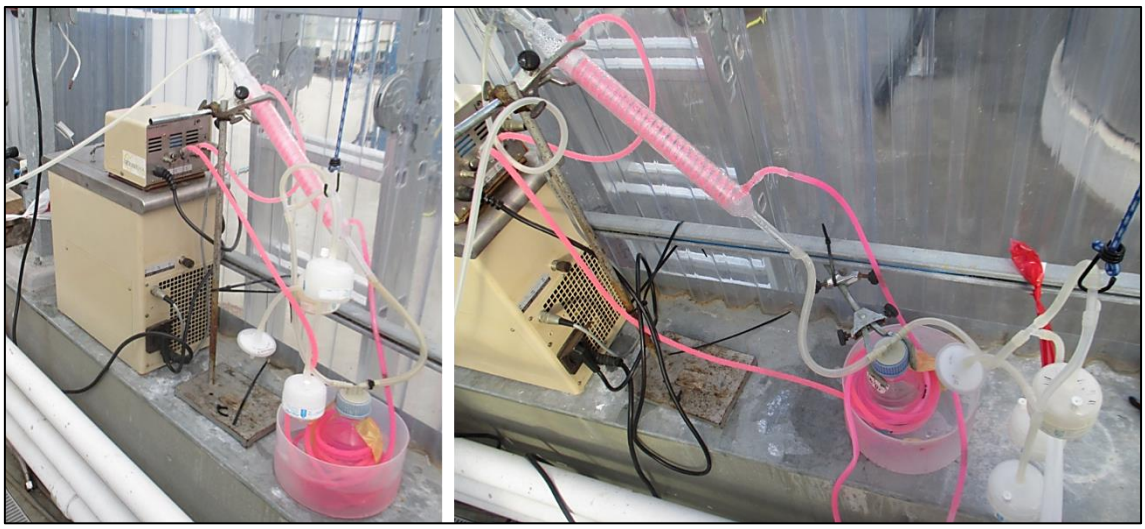

Figure 18 | Condensing system attached to the flat-panel photobioreactor.

The growth of the culture and the 1-butanol titer in the culture and in the condensate accumulated into a collection bottle are presented in **Figure 19**.

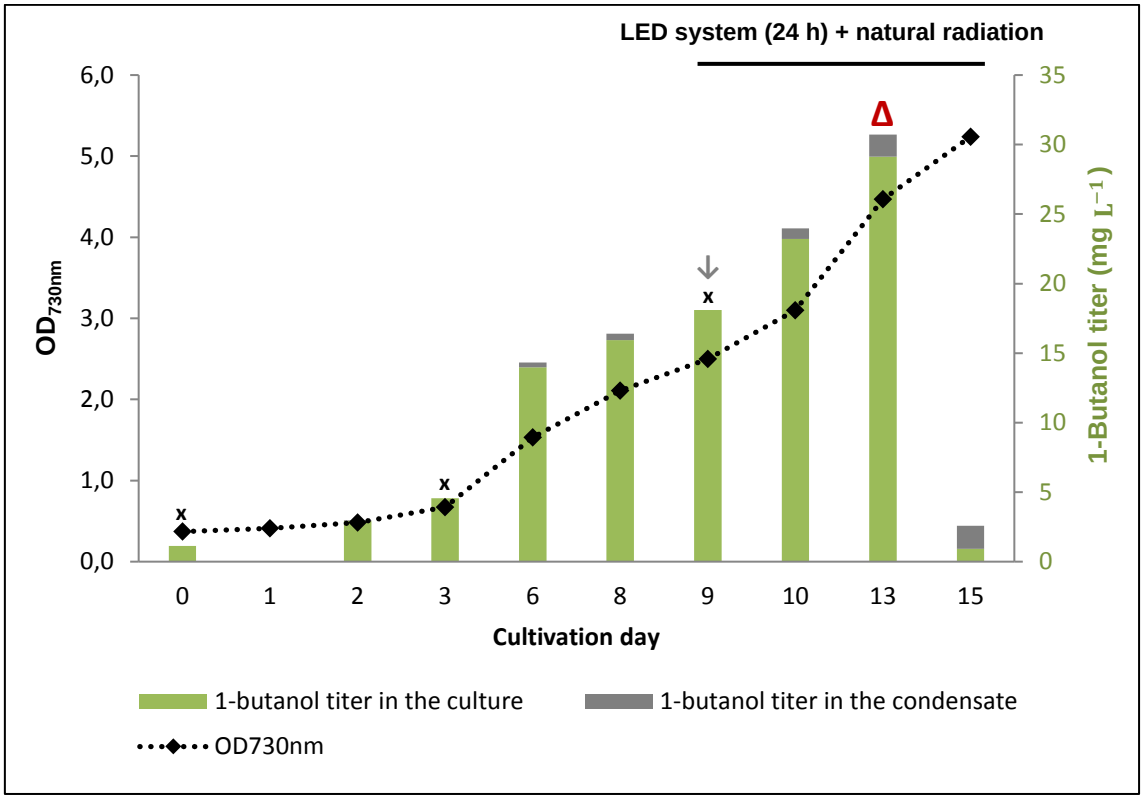

Figure 19 | Growth of SynBuOH-44 indicated by OD_{730nm} and 1-butanol titer (mg L^{-1}) presented in the culture and in the condensate accumulated into a collection bottle during 15 days of cultivation in a flat-panel photobioreactor at pilot-scale. The cumulative values of 1-butanol titer in the condensate are calculated in relation to the volume of culture in the photobioreactor at each time point. LED artificial lighting system was attached to the photobioreactor as a supplement to the natural radiation from the 9th day of cultivation (↓). At 13th day of cultivation, fungi were observed in the culture (**Δ**). **X** indicates that no condensate sample was collected and analyzed.

Figure 19 evidences an exponential growth of the culture that, as expected, seems to be slightly more pronounced from the moment in which LED artificial lighting system was installed and turned on 24 hours per day as a supplement to the natural radiation, once the radiation received per each cell is increased. Accumulation of 1-butanol in the culture was observed until the 13th day of cultivation. At day 13, fungi were detected in the culture by microscopic observation (**Figure 20**) and 1-butanol titer drop to values near to zero. The maximum 1-butanol titer obtained in the samples analyzed was 29.12 mg L^{-1}, which is quite low for the maximum 1-butanol in-flask value already achieved by this genetically modified *Synechocystis* sp. PCC 6803 strain (2.13 g L^{-1}) (Miao, 2018). In one of the laboratory assays performed in this work and already herein presented, the maximum 1-butanol concentration attained with a culture also grown on A4F-IM at pH between 7 and 8 was almost six times higher than that obtained in this pilot-scale assay. 1-Butanol titer in the condensate, presented in **Figure 19**, evidences that the culture

system used to cultivate SynBuOH-44 cells caused the loss of 1-butanol from the culture by evaporation.

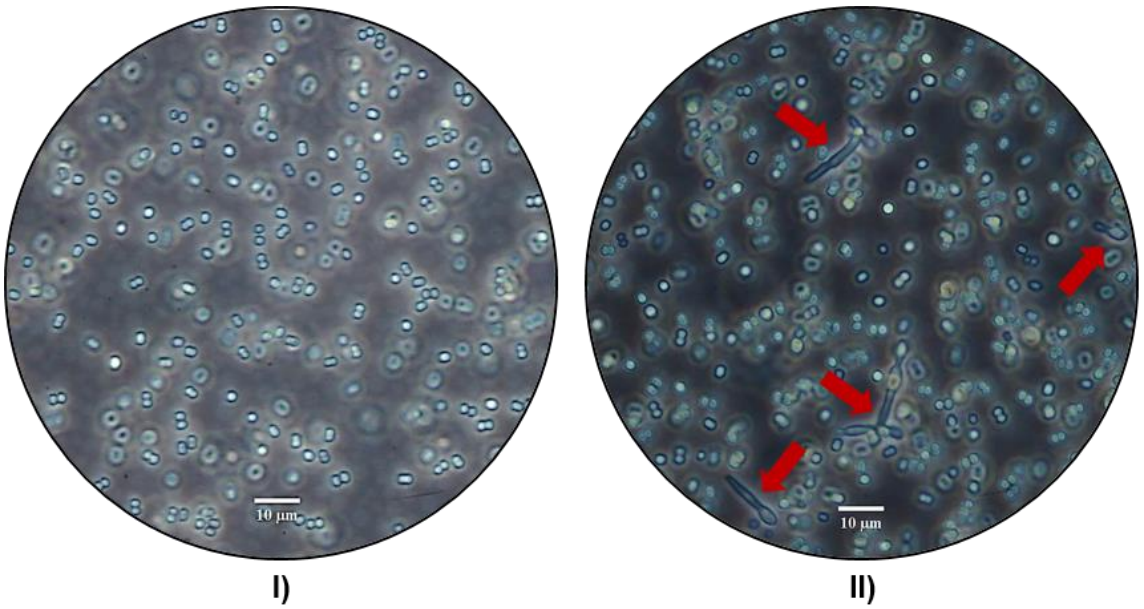

Figure 20 | Microscopic observation of SynBuOH-44 culture cultivated at pilot-scale (phase contrast). I) Non-contaminated culture. II) Culture contaminated with fungi. Red arrows indicate fungi.

The Henry's law constant for 1-butanol is 8.7634 x 10^{-6} atm mol^{-1} m^{-3} at 25 ºC (Yaws *et al*., 1998), which indicates that 1-butanol is expected to volatilize from water surfaces (NCBI, 2019). The high risk of 1-butanol evaporation through aeration during cultivation could be the reason for the low values of 1-butanol titer obtained. However, according to the results attained in this assay, on average, only 0.84 % (**Equation 4**) of the 1-butanol accumulated in the culture was lost and condensed through the condensing system coupled to the photobioreactor, per day. In order to corroborate these results, the condensing system was again attached to the photobioreactor in another assay with a different study purpose that will be presented later. In that confirmation test, related to the 1-butanol titer in the culture, the average of 1-butanol condensed per day was 0.41 % (**Equation 4**). The percentage of 1-butanol daily lost from the culture and condensed by the condensing system (0.84 % and 0.41%) does not seem to be the reason for the low 1-butanol concentrations that have been obtained by A4F – Algafuel, S.A. at pilot-scale. The need arose for a control test in order to verify if all the 1-butanol that was being lost from the photobioreactor was being condensed or if there was also a part of 1-butanol to be released into the environment. This experiment will be presented below.

The appearance of fungi in the culture led to the end of the assay after 15 days. The presence of fungi appears to have led to a drastic drop in 1-butanol titer in the culture. Alcohols are usually considered to be disinfectants because of their diverse toxic effects

on vegetative forms of bacteria, viruses, and fungi. An aqueous solution of 1-butanol with a concentration of 80 to 96 % (V V^{-1}) was reported to be the most useful alcohol solution for use as a disinfectant in the form of vapors against fungi (Bacílková, 2006). However, fungi can use a variety of carbon sources, included some alcohols in a non-toxic concentration such as ethanol and methanol (Walker and White, 2018). The experience in the cultivation of SynBuOH-44 that A4F – Algafuel, S.A. has achieved over the course of several assays indicate that 1-butanol may be consumed by contaminating microorganisms, namely bacteria and fungi, since there is a marked decrease in the concentration of 1-butanol accumulated in the culture medium immediately after the appearance of these contaminants (unpublished results). According to the literature, some bacteria are capable of assimilating 1-butanol as carbon source (Hao, 2003; Veeranagouda *et al*., 2006) as well as some fungi, although 1-butanol alone as a carbon source does not support a great fungal growth (Ewaze *et al*., 2007).

The results achieved in this test confirmed the suspicions that there should be 1-butanol losses from the culture by evaporation through the aeration system of the photobioreactor used for the cultivation of SynBuOH-44. Thus, in future assays it will be necessary to attach the condensing system to the photobioreactor if there is an interest in recovering the lost and condensed 1-butanol.

3.2.1.1. Control test: 1-butanol lost by evaporation

The performance of this control test intended to understand if the condensing system that was being used to condense 1-butanol lost by evaporation due to the aeration of the culture growing in photobioreactors at pilot-scale would be sufficient to condense all the 1-butanol lost or if there was any part to be lost to the environment. For this, the condensing system (Baez *et al*., 2011) was attached to the air outlet of the flat-panel photobioreactor having been inoculated with water containing a known concentration of 1-butanol under sterile conditions. In order to mimic the culture conditions under which SynBuOH-44 have been grown in the same culture system, the volume, the temperature set-point, and the aeration intensity inside the photobioreactor were the same as that practiced with culture. The pH of the water was set to a value of 12 to have better control of contaminants (Touloupakis *et al*., 2016). Since the 1-butanol pKa is 16.1 (NCBI, 2019) there will be no chemical dissociation problems of the compound at a pH of 12.

The flat-panel photobioreactor inoculated with water supplemented with 1-butanol and the condensing system is shown in **Figure 21**.

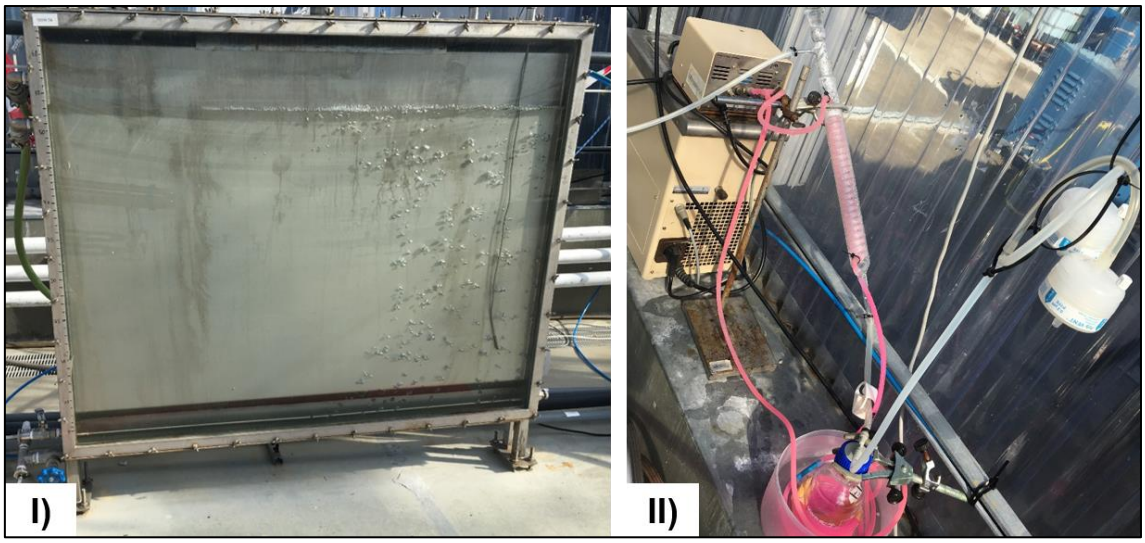

Figure 21 | Control test for 1-butanol lost by evaporation through aeration at pilot-scale. I) Flat-panel photobioreactor inoculated with water supplemented with 1-butanol. II) Condensing system attached to the photobioreactor.

1-Butanol concentrations obtained by analyzing some of the samples collected during this test are presented in **Figure 22**. Although it was planned to start the test with a theoretical 1-butanol concentration of 1 g L^{-1}, due to the possible 1-butanol evaporation, retention of 1-butanol in the reactor structures, and inaccuracy in pilot-scale measurements, the actual 1-butanol concentration measured at the start of the test was 433.83 mg L^{-1} (**Figure 22**).

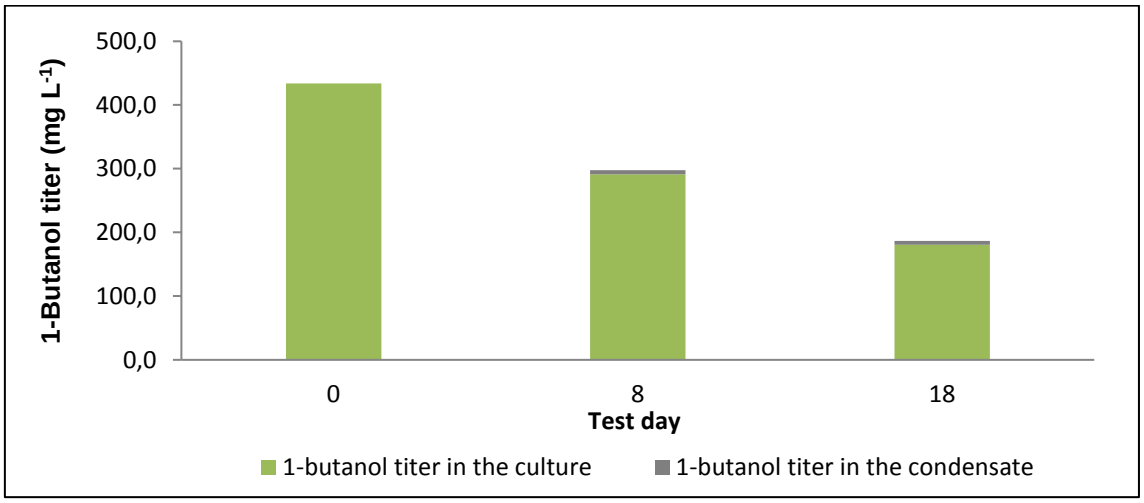

Figure 22 | 1-Butanol titer (mg L^{-1}) presented in the water supplemented with 1-butanol and in the condensate accumulated into a collection bottle just in the 2 days preceding the test day shown in the chart (8 and 18). The values (not cumulative) of 1-butanol titer in the condensate are calculated in relation to the volume of the water present in the photobioreactor. On the day 0 of the test, there was no condensate to be sampled.

A clear tendency for the decrease of 1-butanol titer present in the water supplemented with 1-butanol contained in the photobioreactor during the test is

evidenced in **Figure 22**. At the time of sample collection, on day zero of the test, there was no condensate in the collection bottle whereby the concentration of 1-butanol condensed on that day was considered to be zero. On the remaining days analyzed for 1-butanol content, 1-butanol condensed through the condensing system attached to the photobioreactor was evident showing loss of 1-butanol by evaporation through the air outlet of the photobioreactor. The average daily percentages of condensed 1-butanol, 1-butanol lost from the photobioreactor, and 1-butanol lost to the atmosphere were calculated using the **Equations 4**, **5** and **6**, respectively. As all the points in which the collection bottle was changed and sampled were not analyzed for 1-butanol content, a theoretical value had to be calculated for the amount of 1-butanol in the photobioreactor for the points not analyzed. For this purpose, the amount of 1-butanol lost from the photobioreactor per day was calculated assuming that the same amount is lost every day in two-time intervals: from day 0 to 8 and day 8 to 18. These theoretical values were important for **Equations 4** and **5** where it was necessary to know the amount of 1-butanol present in the photobioreactor at time zero considered. Daily, on average, 4.18 % of the 1-butanol contained in the photobioreactor was lost with only 0.77 % being condensed through the condensing system. In this way, 1-butanol was lost to the environment by evaporation with a daily mean loss of 3.41 %. Taking the overall mean balance, at the end of 18 days of the control test, 75 % of the 1-butanol was lost from the photobioreactor. Of this 75 %, 14 % was condensed through the condensing system and 61 % was released into the environment.

Depending on the application and process scale, different types of condensing system can be chosen (Butterworth D, 1991; Papari and Hawboldt, 2018). The condensing system used in this assay does not appear to have sufficient capacity to condense all the evaporated 1-butanol from the photobioreactor. The use of a condensing system capable of condensing all evaporated 1-butanol from the culture would allow its recovery minimizing losses of 1-butanol productivity due to evaporation. The acquisition of a larger capacity condenser or even the use of several smaller condensers in series seems to be a solution for the 1-butanol losses by evaporation through the aeration of the culture. In addition to the outside collecting system, there are organic traps such as dodecane that allow the volatile compounds in the headspace to be trapped in the hydrophobic solvent overlay inside the photobioreactor (Newman *et al*., 2006; Lauersen *et al*., 2016).

3.2.2. Continuous cultivation regime

Intending to understand if a continuous cultivation regime leads to a higher 1-butanol yield in SynBuOH-44 cultures compared to fed-batch cultivation regime (Miao *et al*., 2018), a chemostat regime assay was performed at pilot-scale. The 1-butanol productivity data obtained in this assay would be compared with the experiment of the 1-butanol lost by evaporation, mentioned in point **3.2.1.**, since they had been carried out under the same culture conditions (**Table 9** and **10**), to realize which culture regime leads to a higher 1-butanol titer accumulated in the culture medium. In order to operate the flat-panel photobioreactor in a chemostat regime (Monod, 1950; Novick and Szilard, 1950), two peristaltic pumps regulated for the same flow rate were connected to the photobioreactor. Thus, the photobioreactor had a continuous inflow of fresh culture medium and outflow of culture at identical rates. The fresh culture medium was stored in an autoclavable jerrican and the culture removed from the system was collected to another jerrican. Each of these jerricans were connected to the peristaltic pumps, as illustrated in **Figure 10**. Once again, the condensing system was coupled to the air outlet of the photobioreactor to have more confidence in the results obtained in the tests in which the condensing system was also used. All the engineered constituents coupled to the photobioreactor (**Figure 17**) are shown in **Figure 23**.

Table 10 | Experimental conditions of the assay aimed to determine whether the continuous cultivation regime (chemostat) enhances 1-butanol production.

System	Culture regime	Flow rate	Culture medium	Initial volume	pH set-point	Light	Temperature set-point	Carbon source
Flat-panel PBR with condensing system attached to it	Che-mostat	9 and 5 L day^{-1}	A4F-IM	60 L	7 to 8	Natural and natural + LED system	30 ºC	CO_2 + $NaHCO_3$

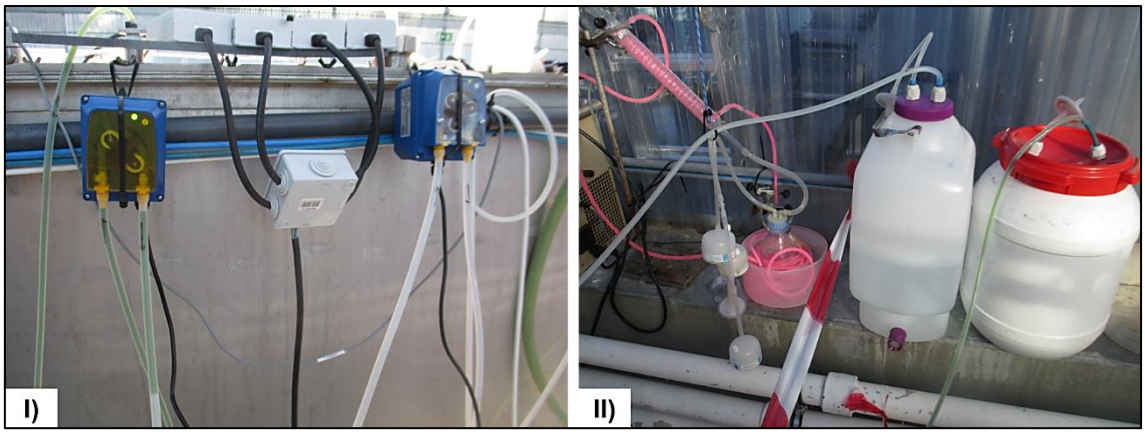

Figure 23 | Constituents of the continuous culture system attached to the flat-panel photobioreactor. I) Two peristaltic pumps for fresh culture medium inlet into the photobioreactor and culture outlet from the culture system. II) Condensing system and two jerricans: one of them contains fresh culture medium and the other contains culture removed from the photobioreactor.

The growth curve and the 1-butanol titer obtained over 26 days of cultivation are presented in **Figure 24**.

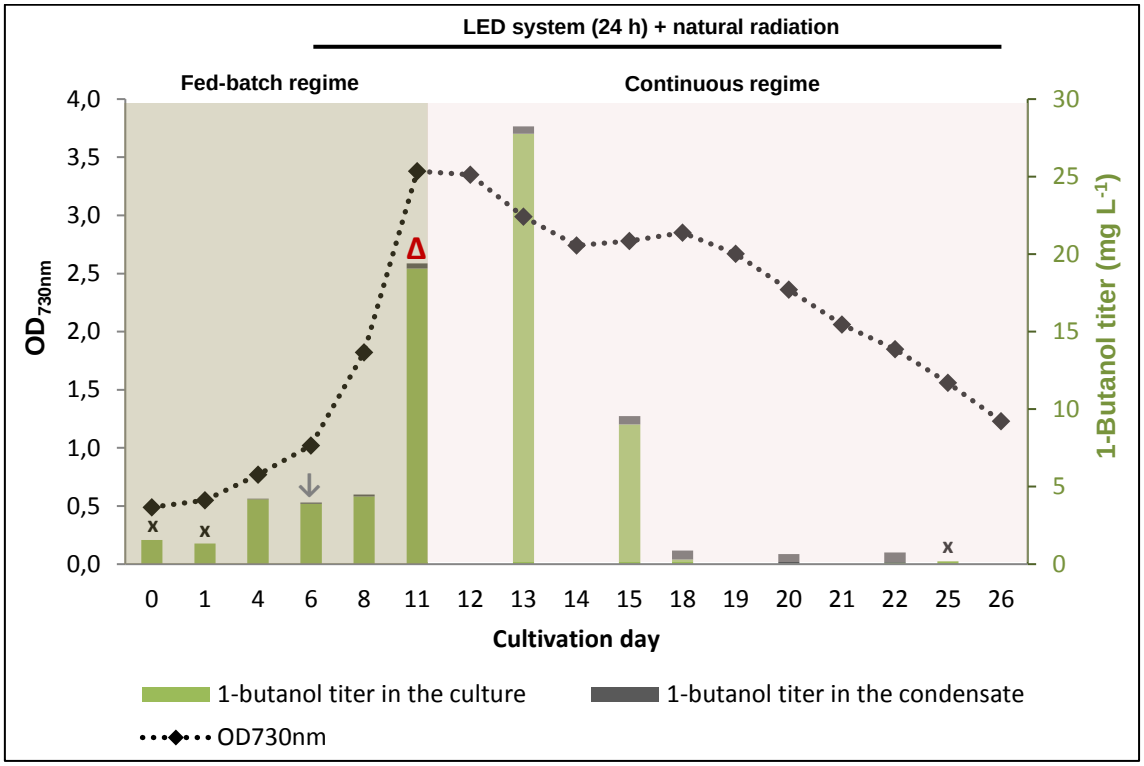

Figure 24 | Growth of SynBuOH-44 indicated by OD_{730nm} and 1-butanol titer (mg L^{-1}) presented in the culture and in the condensate accumulated into a collection bottle during 26 days of cultivation in a flat-panel photobioreactor at pilot-scale. The cumulative values of 1-butanol titer in the condensate are calculated in relation to the volume of culture in the photobioreactor at the same time. From the beginning of the assay until the 11th day of cultivation, SynBuOH-44 was grown under the fed-batch regime. On the 11th day of cultivation, the photobioreactor started to be operated under continuous cultivation regime and remained until the end of the test. At the 11th day of cultivation, also fungi were observed in the culture (**Δ**). On the 6th day of cultivation, beyond natural radiation a LED system was also added to the culture (↓) working 24 h per day. **X** indicates that no condensate sample was collected and analyzed.

At the beginning of the assay, the culture had an OD_{730nm} of 0.5 (0.15 g L^{-1} DW) (**Figure 24**), which was considered a low cell concentration to start the continuous cultivation regime since this regime would cause a dilution in the culture and maybe lead to its photoinhibition. The photobioreactor was then operated in a fed-batch cultivation regime until the 11th day of cultivation. At this time, the cell concentration of the culture was considered to be suitable to start the continuous cultivation regime. In order to accelerate the growth of the culture to allow the start of the chemostat regime as early as possible, on the 6th day of cultivation, a LED lighting system was added to the photobioreactor for 24 h in addition to the naturally occurring radiation. **Figure 24** evidences a marked increase in OD_{730nm} from the 6th day of cultivation to the 11th corresponding to the day in which the continuous culture regime was initiated influencing the cell concentration in the culture. It was also on the 11th day of cultivation that fungi were observed under the microscope (**Figure 25**), precisely on the day in which the chemostat regime was initiated. After detecting fungi in the culture it was observed a slight increase in the 1-butanol titer followed by an expected abrupt fall in 1-butanol concentration (**Figure 24**). The 1-butanol production by the culture between the 11th and 13th days was higher than its consumption by the small fungal population at this time what led to the slight increase in 1-butanol after the appearance of fungi. The possible 1-butanol consumption by contaminating microorganisms was discussed in **3.2.1.**. The maximum 1-butanol titer obtained during this assay was 27.76 mg L^{-1}, on 13th day of cultivation (**Figure 24**). However, due to the presence of contaminants in the culture throughout the chemostat culture regime the 1-butanol titers obtained during this period were considered to be biased.

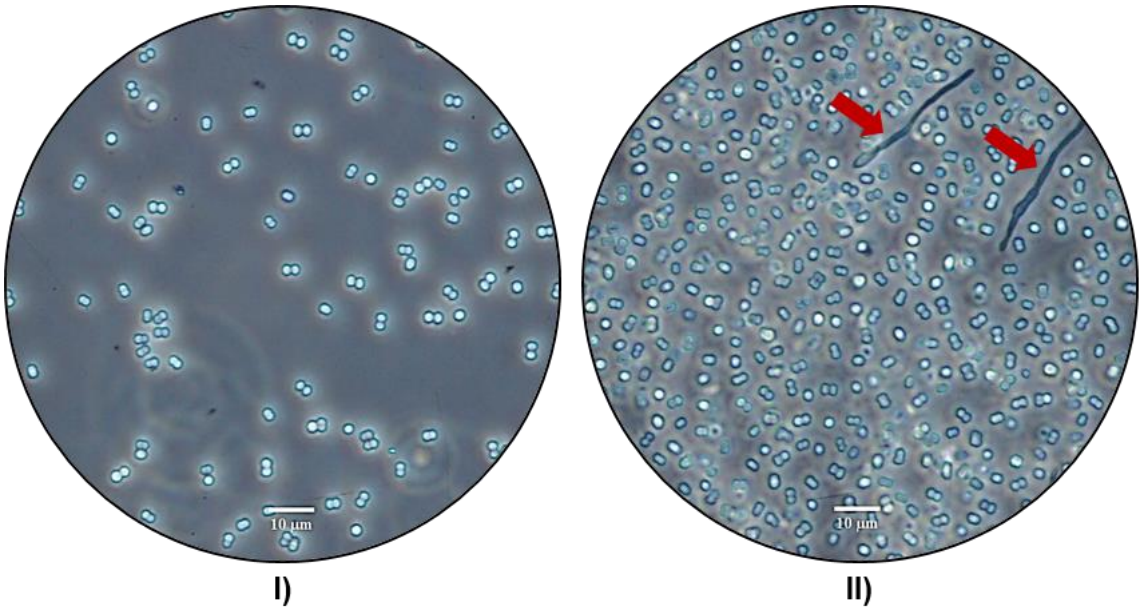

Figure 25 | Microscopic observation of SynBuOH-44 culture grown at pilot-scale (phase contrast). I) Non-contaminated culture. II) Culture contaminated with fungi. Red arrows indicate fungi.

During the operation of the photobioreactor in a chemostat regime, the flow rate used during the week was 9 L day^{-1} chosen based on an assay carried out by the project partners with the same cyanobacterial strain (data not published). However, at the weekend, the flow rate was 5 L day^{-1} due to the limitations of the capacity of the available jerricans (20 L) to be used in this assay as accumulators of fresh culture medium or culture removed from the photobioreactor, which are unable to sustain the operation of the chemostat at a flow rate of 9 L day^{-1} during the whole weekend time.

The cell concentration of the culture was decreasing during the chemostat cultivation regime (**Figure 24**) and the hypothesis that the flow rates used would not be the most indicated arose. The culture growth rate (**Equation 1**) between the 6^{th} and 11^{th} day of cultivation (before starting the continuous regime) was 0.14 g L^{-1} day^{-1} and the biomass dilution rates (**Equation 2**) on the first day of the chemostat regime were 0.18 g L^{-1} day^{-1} (week days) and 0.10 g L^{-1} day^{-1} (weekend days). During the week days, as the biomass dilution rate was higher than the culture growth rate, more culture was being lost than was being produced, what would lead to culture washout (Doran, 2013; Ziv *et al*., 2013). The continuous cultivation using a chemostat assumes that the biomass dilution rate has to be lower than the maximum specific growth rate (Novick and Szilard, 1950; Ziv *et al*., 2013). Therefore, considering the growth rate at which the culture was growing, the use of a flow rate of 5 L day^{-1} seems to be more indicated than 9 L day^{-1}. Furthermore, the regular change in flow rate (from 5 to 9 L day^{-1} and *vice versa*) may make it impossible for the culture to reach the steady-state, as it is expectable in a chemostat regime, for lack of time. The steady-state, stage in which the culture is forced to divide to keep up

with the dilution (Dunham, 2010), implies that the specific growth rate of the cells is equal to the rate at which the culture is diluted (Ziv *et al*., 2013). Additionally, the peristaltic pumps available in the pilot unit do not allow an easy and accurate flow rate adjustment when required and did not appear to work exactly at the flow rate for which they were set leading to a constant change of the culture volume. A chemostat assumes a constant volume in the reactor by the maintenance of continuous and identical in- and outflow rates through the use of appropriate technical devices (Doran, 2013; Harmand *et al*., 2017).

Regarding to 1-butanol evaporated from the culture and condensed by condensing system coupled to the photobioreactor, until the day when contaminants were detected in the culture, on average, only 0.41 % of the 1-butanol present in the culture was daily lost and condensed (**Equation 4**). This value is slightly lower than those obtained in the other assays performed using the condensing system already presented in this internship report, however, all of them are below 1 %.

The high risk of contamination in a continuous regime due to a large number of pumping and fitting processes is a challenge to face in a chemostat cultivation (Blunt *et al*., 2018). The appearance of contaminants in the culture during the continuous cultivation regime made it impossible to achieve the objective of this assay. Therefore, this test should be repeated to get conclusions about what is the cultivation regime which leads to the enhancement of 1-butanol production and accumulation in the culture. However, according to the literature, continuous cultivation can achieve higher productivity in comparison to discontinuous processes (Braunegg *et al*., 1995; Koller and Braunegg, 2015; Meyer *et al*., 2017) since the culture is maintained at a moderate but constant growth rate for extended time periods avoiding the non-productive phase required for harvest, cleaning, sterilization, cooling, and culture inoculation in a discontinuous cultivation regime (Blunt *et al*., 2018).

3.2.3. Continuous cultivation regime at alkaline pH

The appearance of contaminants in all the SynBuOH-44 cultures cultivated at pilot-scale, even in previous studied by the A4F – Algafuel, S.A. team (results not published), led to the need to find solutions to control it since it seems to influence the 1-butanol production and accumulation in the culture. Considering the positive effects that alkaline pH seems to have on the control of contaminants (Touloupakis *et al*., 2016) and the fact that high pH cultivation does not appears to impair 1-butanol productivity, according to the laboratory results presented above, the culture in this assay was grown at high pH (around 10.5) as a preventive measure for the emergence of contaminants. Thus, as

reliable data was not obtained from the performed chemostat (Monod, 1950; Novick and Szilard, 1950) assay due to culture contamination, a second assay in continuous regime was carried out at high pH. For this purpose, two jerricans and peristaltic pumps attached to the photobioreactor, as illustrated in **Figure 10**, were used as well to operate the photobioreactor according to a continuous culture regime (chemostat). According to the results obtained in the pilot-scale assay also performed under the chemostat regime, the 5 L day^{-1} flow rate was chosen to operate the chemostat in this assay. Due to lack of an available automatic probe for pH control, the pH was manually controlled using CO_2 injections and $NaHCO_3$ additions at pH 10.5 in order to decrease and increase the pH of the culture, respectively. A 24h-LED lighting system was also attached to the photobioreactor in order to facilitate the pH control since under these conditions the CO_2 uptake for photosynthesis is higher than the CO_2 evolved by cellular respiration, which leads to an increase in pH (Hopkins, 2006). The culture conditions are presented in **Table 11**.

The photobioreactor and all its constituents coupled used to operate the culture system according to a continuous culture regime are presented in **Figures 17** and **23**, respectively.

Table 11 | Experimental conditions of the assay aimed to determine whether the continuous cultivation regime (chemostat) enhances 1-butanol production at alkaline pH.

System	Culture regime	Flow rate	Culture medium	Initial volume	pH set-point	Light	Temperature set-point	Carbon source
Flat-panel PBR	Chemostat	5 L day^{-1}	A4F-IM	57 L	10.5	Natural and natural + LED system	30 ºC	CO_2 + $NaHCO_3$

The growth curve and the 1-butanol titer obtained during the 29 days of cultivation are presented in **Figure 26**.

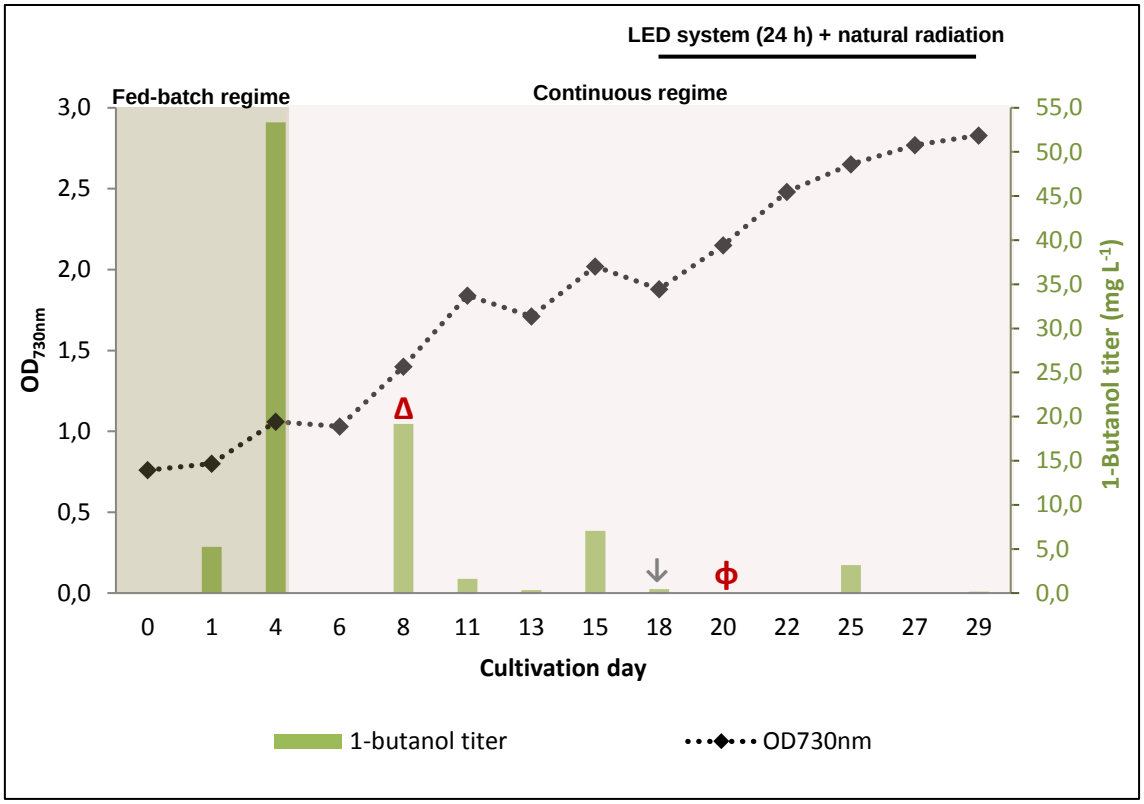

Figure 26 | Growth of SynBuOH-44 indicated by OD_{730nm} and 1-butanol titer (mg L^{-1}) accumulated in the culture during 29 days of cultivation in a flat-panel photobioreactor at pilot-scale. From the beginning of the assay until the 4th day of cultivation SynBuOH-44 was grown according to the fed-batch regime. On the 4th day of cultivation, the photobioreactor started to be operated under continuous cultivation regime and remained until the end of the test. At the 8th day of cultivation, fungi were observed in the culture (Δ) and then on the 20th day of culture bacteria were also observed in the culture (ϕ). On the 18th day of cultivation, beyond natural radiation a LED lighting system was added to the culture (↓) working 24 h per day.

This assay started with the culture being operated according to a fed-batch cultivation regime. On the 4th day of cultivation, the culture regime was changed to a chemostat regime having remained until the end of the assay. The culture evidenced growth throughout the assay and did not reach the steady-state (**Figure 26**), therefore, the flow rate of 5 L day^{-1} seems not to have been the most suitable for this test. The maximum 1-butanol titer obtained in the analyzed samples for the 1-butanol content in this assay, 53.36 mg L^{-1}, was achieved on the same day in which the continuous cultivation regime was initiated (**Figure 26**). Then, on 8th day of cultivation, fungi were observed in the culture and 12 days later bacteria were also observed through microscopic observation (**Figure 27**). The presence of contaminants in the culture seems to cause the 1-butanol titer to drop to near zero (**Figure 26**). Bacteria and fungi have been found to coexist, however, the emergence of bacteria led to a gradual decrease in the fungal population as well as an increase in bacteria over time (data not shown). The emergence of the contaminants did not lead to immediate end of the assay so that it was possible to

optimize the technical part of the operation of the photobioreactor according to a continuous culture regime.

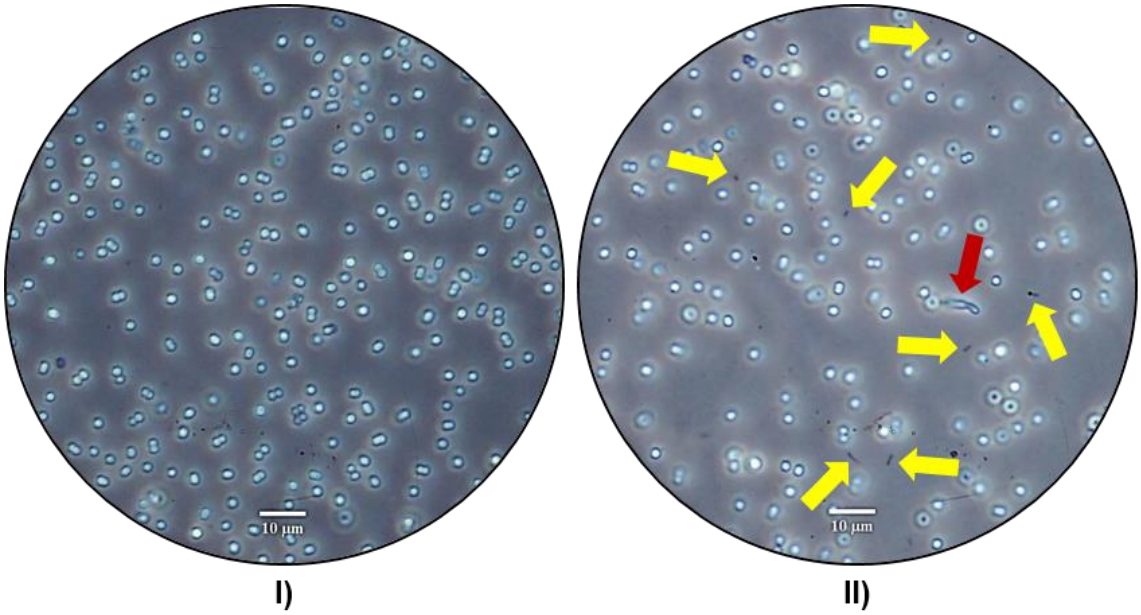

Figure 27 | Microscopic observation of SynBuOH-44 culture grown according to a chemostat culture regime at high pH at pilot-scale (phase contrast). I) Non-contaminated culture. II) Culture contaminated with fungi and filamentous bacteria. Red arrows indicate fungi. Yellow arrows indicate bacteria.

The pH control at alkaline values was quite complicated without an automatic pH control probe. The constant oscillation of the daily average pH values between values near to 8 and 11 throughout the assay is evidenced in **Figure 28.**

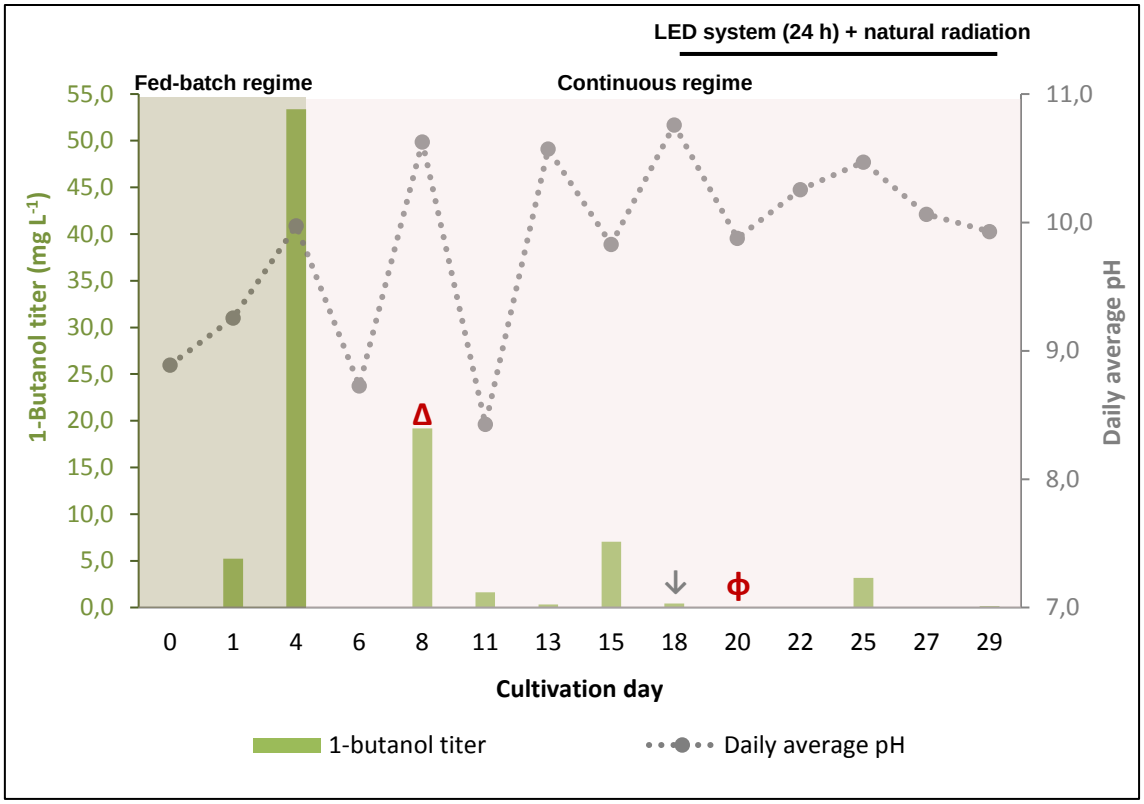

Figure 28 | Daily average pH variations and 1-butanol titer (mg L^{-1}) accumulated in the culture during 29 days of cultivation in a flat-panel photobioreactor at pilot-scale. **Δ** e **ϕ** indicate that fungi and bacteria were observed in the culture, respectively. ↓ indicate that a LED lighting system was added to the culture.

According to the literature, high pH seems to be incompatible with the growth and survival of some contaminants (Touloupakis *et al*., 2016). However, in this assay it was not possible to control the appearance of contaminants perhaps due to the fluctuation of pH values. It would be necessary to have an automatic pH control probe for better pH control to the desired values in order to clarify the effect that a constant alkaline pH can have on contaminants control, which was not achieved with this assay.

The inexistence of an alternative to the peristaltic pumps used in the previous assay, also performed according to a continuous culture regime, which were considered inefficient to that aim, led to the use of the same devices in this assay. Once again, the culture volume was not maintained constant as well as the dilution rate that depends on the culture volume, and therefore, it was not achieved a steady-state as is aimed in a chemostat regime (Doran, 2013). The cultivation of the culture according to the chemostat regime should be optimized to achieve all the assumptions involved in this type of culture regime in order to reach our proposed objectives. Although during this project it was not possible to determine which culture regime leads to a higher 1-butanol productivity, according to the literature, usually the continuous culture leads to higher productivity (Braunegg *et al*., 1995; Koller and Braunegg, 2015; Meyer *et al*., 2017).

When cells are grown in continuous cultures they are maintaining in a producing state for prolonged time periods leading to high productivities and reduce costs (Peebo and Neubauer, 2018). Unpublished results from the Photofuel project partners indicate that the chemostat cultivation regime leads to higher 1-butanol production and accumulation in the culture compared to the fed-batch cultivation.

Chapter 4.

Conclusion

4. Conclusion

This master's project was performed in the scope of the Photofuel European project, which aims to develop a next-generation technology for the sustainable production of low impact liquid transportation fuels using photosynthetic microbial cells. The present works' main goal was to optimize the culture conditions at pilot-scale of a genetically modified *Synechocystis* sp. PCC 6803 strain for 1-butanol production (SynBuOH-44) in order to enhance alcohol productivity.

The industrial medium developed and optimized by A4F – Algafuel, S.A. (A4F-IM) used for cultivating SynBuOH-44 was tested under laboratory conditions in order to ascertain the impact it could have on 1-butanol production and accumulation in the culture. These results were compared to the ones obtained cultivating the same strain in a standard laboratory medium for cyanobacteria (BG-11), which has been used by the project partners throughout this work for the cultivation of SynBuOH-44. The results obtained seem to indicate that both culture media can be used to grow SynBuOH-44 without impairing 1-butanol productivity.

Also in a laboratory environment, it was studied the effect that the cultivation of the SynBuOH-44 at an alkaline pH (around 10) can have on cell growth and on the 1-butanol accumulated in the culture when compared to the cultivation at pH commonly used in *Synechocystis* sp. cultures (pH 7 to 8). In terms of 1-butanol content, the results obtained indicate that 1-butanol production and accumulation do not seem to be impaired with SynBuOH-44 cultivation at high pH. However, due to the pH playing a crucial role in carbon supply the cell growth was negatively impacted by cultivation at alkaline pH and premature bleaching and death of the culture was observed in these conditions as well.

Considering the low 1-butanol yields obtained in assays performed by A4F – Algafuel, S.A., it was hypothesized that there could be losses of 1-butanol by evaporation during SynBuOH-44 cultivation at pilot-scale. The results obtained in this work evidenced 1-butanol losses from the flat-panel photobioreactor due to the aeration system, which deflates 1-butanol concentrations measured in the cultures. A control test with an initial 1-butanol concentration of 433.83 mg L^{-1} was performed and it was obtained a daily average of 1-butanol losses by evaporation of about 4 %. Of this 4 %, not even 1 % was condensed and possibly recovered through the condensing system coupled to the air outlet of the reactor, which consists of a coil condenser, a refrigeration system, and a condensate collection container. Thus, on a daily average, more than 3 % of the 1-butanol present in the photobioreactor was lost to the environment.

At pilot-scale, the impact of the cultivation regime in 1-butanol production and accumulation in the culture was also studied. However, due to the appearance of

contaminating microorganisms in the culture grown according to a chemostat regime, which leads to a sharp decrease in 1-butanol titer in the culture to values close to zero, it was not possible to conclude on which cultivation regime (fed-batch or chemostat) leads to a superior 1-butanol accumulation in the culture. Nevertheless, according to the unpublished results from the Photofuel project partners, the chemostat cultivation regime leads to higher 1-butanol production and accumulation in the culture compared to the fed-batch cultivation.

The maximum 1-butanol content measured in the cultures of the assays performed at laboratory- and pilot-scale were 165.01 mg L^{-1} and 53.36 mg L^{-1}, respectively. These values are far below what is known to be the potential of this strain under laboratory conditions (2.13 g L^{-1}) (Miao, 2018). There is still a long work of research and optimization of the cultivation of this strain ahead to considerably increase 1-butanol yield to make this sustainable fuel production process economically feasible.

The culture system used for SynBuOH-44 cultivation, in this case a flat-panel photobioreactor, directly affects the productivity of the intended product due to its volatility. Thereby, it will be necessary to attach a condensing system to the air outlet of the photobioreactor to avoid alcohol losses from evaporation. However, the condensing system used in this work does not seem to have enough capacity to condense all the evaporated 1-butanol. It will be necessary to acquire a larger capacity condenser or even to use several smaller condensers in series. As an alternative to the outside collecting system, a hydrophobic layer of organic solvent such as dodecane can be added to the top of the culture to capture the volatile product excreted by the cells (Newman *et al*., 2006; Lauersen *et al*., 2016).

The availability of axenic cultures is an essential condition for effective 1-butanol accumulation in the culture. One of the major challenges faced at pilot-scale is the operation in aseptic conditions and the maintenance of axenic cultures. The development of new strategies to improve sterilization procedures at pilot-scale, the continued application of good handling practices by the company's employees, and the inclusion of quality control steps are necessary to avoid culture contaminations, which affect 1-butanol production and accumulation.

Due to the challenges faced during the chemostat assays, these should be repeated but this time using efficient peristaltic pumps that operate at a constant flow rate and allow an easy and accurate flow rate adjustment, which should be chosen according to the specific growth rate of the culture under the conditions in which the assay is performed. In addition to this equipment, it would also be important to acquire an automatic pH control probe for tighter control to the desired values.

Also the routine nutrient analysis would be a good approach in order to be able to act before the culture dies for the eventual lack of nutrients.

Future works and research related to the future large-scale production of solar fuels through photosynthetic microbial cell factories should aim to improve the biomass and valuable compounds productivity, to reduce water, energy, and land-use footprints of the integrated operation, to improve nutrient requirement, to integrate biorefineries to reduce the production costs and constraints, among others (Laurens *et al*., 2017). The need to obtain higher-value co-products along with the biofuel is crucial to make the process commercially viable (Dutta *et al*., 2016). The sustainability and market competitiveness of the microalgae and cyanobacteria industry depends on the achievement of these goals.

Chapter 5.

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5. References

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