Microalgae cultivation in view of resource and energy recovery

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Doctoral thesis

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CERTIFICAMOS:

Que el ingeniero químico SERGI CARBONELL CHACÓN ha realizado bajo nuestra dirección, el Trabajo que con título **"Microalgae cultivation in view of resource and energy recovery"**, se presenta en esta memoria, y que constituye su Tesis para optar al Grado de Doctor por la Universitat Autònoma de Barcelona dentro del Programa de Doctorado en Ciencia y Tecnología Ambiental.

Y para que se tenga conocimiento y conste a los efectos oportunos, presentamos a la Escuela de Doctorado de la Universitat Autònoma de Barcelona la presente tesis, firmando el presente certificado en

Bellaterra, 6 de marzo de 2019

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Resum

La present tesi doctoral té com objectiu la millora del cultiu de microalgues amb la finalitat de millorar l'obtenció de productes orgànics d'alt valor afegit. L'elaboració de la mateixa està englobada dins de dos projectes europeus Mar3 i Oli-PHA que tenien per objectiu la bioremediació de les marees negres mitjançant l'ús d'agents gelificants i l'obtenció de PHA destinat a l'ús en el packaging a l'industria alimentaria, respectivament.

El capítol 4 de la tesi doctoral es focalitza en els paràmetres que afecten al disseny d'un fotobioreactor (FBR). La intensitat de llum, la seva qualitat, la profunditat del reactor o els cicles de llum/obscuritat aplicats durant el cultiu de les microalgues afecten significativament al metabolisme de les mateixes. L'estratègia de cultiu, ja sigui fotoautotròfica, heterotròfica o mixotròfica també juga un paper important en disseny d'un FBR. La hidrodinàmica del reactor, l'òptima forma d'homogeneïtzar el líquid durant el cultiu i a transferència de massa i energia són també investigades en aquest capítol de la memòria. Una extensa recerca bibliogràfica permet assolir un criteri de disseny adequat per al disseny d'un FBR eficient destinat al cultiu de *Chlorella vulgaris*.

El capítol 5 de la memòria desenvolupa un model cinètic amb l'objectiu de descriure el comportament de la cianobacteria *Synechocystis sp.* PCC 6803 durant el seu cultiu destinat a la producció de PHA. El model cinètic proposat en base a l'especiació dels nutrients i el pH descriu correctament els resultats experimentals obtinguts durant la fase experimental de la investigació i es determinen les diferents constants de semi-saturació per als diferents nutrients emprats en el creixement del microorganisme. Es confirma tant experimental com teòricament què el creixement de la cianobacteria és significativament influenciat per la concentració de fòsfor inicial al medi. Finalment, s'explota el model proposat amb simulacions canviant el cicle llum/obscuritat amb la finalitat de determinar el comportament del sistema en funció d'aquestes condicions.

El capítol 6 es focalitza en l'obtenció d'energia a partir de l'excedent de biomassa de la cianobacteria *Synechocystis* sp. PCC 6803 usada per a la producció de PHA. Per primer cop es reporta la producció de biogàs a partir d'aquesta cianobacteria i els resultats obtinguts són similars als trobats a la bibliografia en estudis previs usant biomassa microalgal. S'empren pretractament tèrmics i la co-digestió amb un altre substrat,

aigües residuals del procés d'obtenció de l'oli d'oliva en aquests cas, amb l'objectiu de determinar l'augment de la producció de biogàs gràcies a l'aplicació d'aquests tractaments. Finalment, es determinen les constant cinètiques del procés mitjançant l'ús de l'equació de Gompertz.

Resumen

La presente tesis doctoral tiene como objetivo la mejora en el cultivo de microalgas con la finalidad de obtener productos orgánicos de alto valor añadido y energía. La elaboración de la misma está englobada dentro de dos proyectos europeos Mar3 y Oli-PHA que tenían por objetivo la bio-remediación de mareas negras mediante el uso de agentes gelificantes y la obtención de PHA destinado al uso en el packaging en la industria alimentaria, respectivamente.

El capítulo 4 de la tesis doctoral se focaliza en los parámetros que afectan al diseño de un fotobiorreactor (FBR). La intensidad de luz, su calidad, la profundidad del reactor o los ciclos de luz/oscuridad aplicados durante el cultivo de las microalgas afectan significativamente al metabolismo de las mismas. La estrategia de cultivo, ya sea fotoautotrófica, heterotrófica o mixotrófica también juega un papel importante en diseño de un FBR. La hidrodinámica del reactor, la manera óptima de homogeneizar el líquido durante el cultivo y la transferencia de masa y energía son también investigadas en este capítulo de la memoria. Una extensa investigación bibliográfica permite alcanzar un criterio de diseño adecuado para el diseño de un FBR eficiente destinado al cultivo de *Chlorella vulgaris*.

El capítulo 5 de la memoria desarrolla un modelo cinético con el objetivo de describir el comportamiento de la cianobacteria *Synechocystis* sp. PCC 6803 durante su cultivo destinado a la producción de PHA. El modelo cinético propuesto en base a la especiación de los nutrientes y el pH describe correctamente los resultados experimentales obtenidos durante la fase experimental de la investigación y se determinan las diferentes constantes de semi-saturación para los diferentes nutrientes empleados en el crecimiento del microorganismo. Se confirma tanto experimental como teóricamente que el crecimiento de la cianobacteria es significativamente influenciado por la concentración de fósforo inicial al medio. Finalmente, se explota el modelo propuesto con simulaciones cambiando el ciclo luz/oscuridad con el fin de determinar el comportamiento del sistema en función de estas condiciones.

El capítulo 6 se focaliza en la obtención de energía a partir del excedente de biomasa de la cianobacteria *Synechocystis* sp. PCC 6803 usada para la producción de PHA. Por primera vez se reporta la producción de biogás a partir de esta cianobacteria y los resultados obtenidos son similares a los encontrados en la bibliografía en estudios

previos usando biomasa microalgal. Se emplean pretratamientos térmicos y la codigestión con otro sustrato, aguas residuales del proceso de obtención del aceite de oliva en este caso, con el objetivo de determinar el aumento de la producción de biogás gracias a la aplicación de estos tratamientos. Finalmente, se determinan las constantes cinéticas del proceso mediante el uso de la ecuación de Gompertz.

Abstract

The objective of this doctoral thesis is to enhance the production of microalgae with the aim to obtain organic products of high added value and energy. The elaboration of this thesis is part of two European projects, Mar3 and Oli-PHA, which had as their objective the bio-remediation of oil spills through the use of gelling agents and production of PHA for use in packaging in the food industry, respectively.

Chapter 4 of the doctoral thesis focuses on the parameters affecting the design of a photobioreactor (FBR). The intensity of light, its quality, the depth of the reactor or the light/dark cycles applied during the culture of the microalgae significantly affect their metabolism. The culture strategy, whether photoautotrophic, heterotrophic or mixotrophic also plays an important role in the design of a FBR. The hydrodynamics of the reactor, the optimal way to homogenize the liquid during culture and the transfer of mass and energy are also investigated in this memory chapter. An extensive bibliographic research allows reaching a suitable design criterion for the design of an efficient FBR for the *Chlorella vulgaris* culture.

Chapter 5 of the memory develops a kinetic model with the aim of describing the behaviour of cyanobacteria *Synechocystis* sp. PCC 6803 during its cultivation for the production of PHA. The proposed kinetic model based on nutrient speciation and pH correctly describes the experimental results obtained during the experimental phase of the research and determines the different semi-saturation constants for the different nutrients used in the growth of the microorganism. It is confirmed both experimentally and theoretically that the growth of cyanobacteria is significantly influenced by the concentration of initial phosphorus in the medium. Finally, the proposed model is exploited with simulations changing the light/dark cycle in order to determine the behaviour of the system as a function of these conditions.

Chapter 6 focuses on obtaining energy from the surplus biomass of the cyanobacteria *Synechocystis* sp. PCC 6803 used for the production of PHA. For the first time the production of biogas from this cyanobacterium is reported and the results obtained are similar to those found in the bibliography in previous studies using microalgal biomass. Thermal pre-treatments and co-digestion with another substrate, wastewater from the olive oil production process in this case, are used in order to determine the increase in

biogas production thanks to the application of these treatments. Finally, the kinetic constants of the process are determined using the Gompertz equation.

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1 Introduction

1.1 Background

The rapid increasing of human population and their technological advancements have led to rising energy demands, which is projected to increase by 50% or more by the year 2030 (Maness et al., 2009). The natural petroleum resources cannot compensate the current consumption rate which is already reported to be hundred times faster than nature can restore (Rittmann, 2008). Rittmann (2008) described the danger of depending on fossils fuels from three points of view: i) depleting of fossil-fuel reserves dwindling resources, ii) leading to geopolitical conflicts between those lands rich in petroleum resources such as Norway, Saudi Arabia or Russia and economic superpowers such as United States of America and China; and iii) climate change resulting from the increasing atmospheric CO_2 concentration.

Moreover, the use of fossil fuels is hard-harming to the environment through greenhouse gas (GHGs) emissions produced during the combustion of the fossils fuels and the consequent global warming found during the last decades after the petroleum products demand increasing (Shuba and Kifle, 2018). Global warming has become an issue of current concern in our society. Scientists are looking for solutions through the mitigation of GHGs emission, where CO_2 plays the most important role as the main non-renewable waste produced at the petroleum refining and treatment plants (Sierra et al., 2008; Wu et al., 2018). In particular, the combustion of fossil fuels as an energy source in power plants is an important source of the CO_2 released into the atmosphere (Knoop et al., 2010) which needs to be mitigated in order to reduce the environmental harmful created by society. The reduction of non-renewable energy resources on Earth has encouraged researchers to explore and develop novel alternative energy sources. Therefore, due to the concern of energy crisis and the negative environmental effects of burning fossil fuels, renewable energy with low CO_2 emissions has been widely explored in the last years.

Sustainable and renewable energy sources such as hydroelectricity, solar and wind energy, wave power or geothermal energy are able to produce clean electricity, improve conventional energy efficiency and power up small cities (He et al., 2012). Among of these potential sources of energy, biomass and biofuels are seen as real means of achieving the goal of replacing fossil fuels in short term (Chisti, 2008).

1.2 CO_2 mitigation

The mitigation of the CO_2 generated by fossil fuel combustion in power plants can be achieved through three different ways: i) some process improvements, ii) the use of alternative renewable fuels and iii) the CO_2 capture and storage technologies (Markou and Nerantzis, 2013).

1.2.1 Alternative renewable fuels

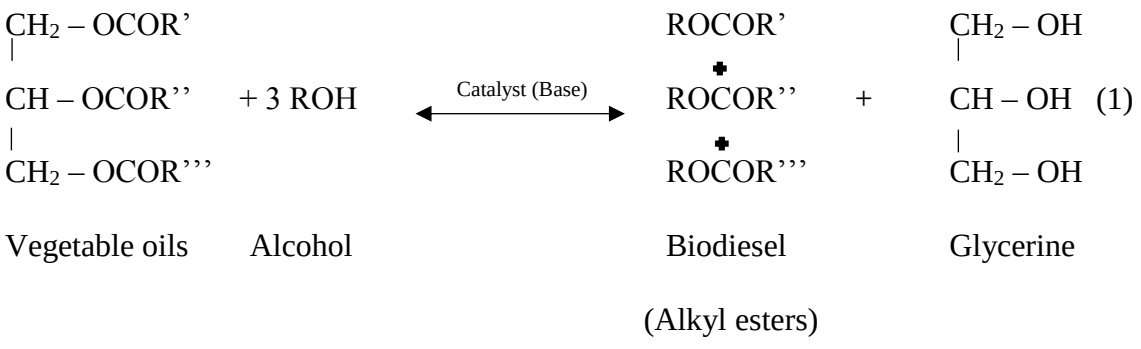
The use of alternative and clean energy sources could reduce the demand of conventional fossil fuels according to various investigations reported in the literature. Moreover, seeking alternative fuels is also necessary considering the current decline in world oil reserves (Jankowska et al., 2017; Lee, 2012). Several clean fuels are proposed for this purpose and, among those, biodiesel is considered one of the most promising alternatives. Biodiesel is defined as mono-alkyl esters with a long chain of fatty acids derived from starch, animal fats or used cooking oils, algal biomasses or vegetable oils. Biodiesel is readily available, renewable, flammable, non-toxic and environmentally friendly (Chisti, 2008; Lee, 2012). Some advantages of biodiesel have been highlighted in comparison to conventional petroleum-based products such as increased flash point, biodegradability, improved cetane number and reduced exhaust emissions (Colling Klein et al., 2018). Depending on the current feedstock types used and their current/future availability, biodiesel is categorized into 1^{st}, 2^{nd}, 3^{rd} and 4^{th} generation biofuels (Chisti, 2008).

Unfortunately, the present biofuels projections are actually based on feed-stocks that are food commodities and resources suitable for conventional agriculture. Prajapati et al. (2014) have pointed out that substitution of diesel by biodiesel involves the use of lands used to produce food and the fiscal incentives by governments are decreasing the land available for food production. Although no agreement has yet been reached on the equitable distribution of land for food production and renewable energy crops, both

governments and environmental associations are putting a great deal of effort in it (Prajapati et al., 2014).

There exist three main basic routes to biodiesel production from oils and fats which are: (i) a base catalysed transesterification of the oil, (ii) the direct acid catalysed transesterification and (iii) first conversion of the oil into its fatty acid and then, fatty acid transformation into biodiesel. Most biodiesel is produced using the first route as it is the most economical process conducted at low temperatures and pressures with yields around 98% (Chen et al., 2011).

A triglyceride is composed by a glycerine molecule and three long chain fatty acids attached. The characteristics of these triglycerides are determined by the nature of the fatty acids bonded to the glycerine molecule. During the biodiesel production process, the triglyceride reacts with alcohol, usually methanol or ethanol, in the presence of a catalyst. The reaction catalyst is usually strong alkaline like sodium or potassium hydroxide which are the cheapest bulk alkaline products in market (Tan et al., 2018). Eq. (1) represents the transesterification reaction involved in biodiesel production (Sun et al., 2018). The reaction between the triglyceride and the alcohol is strongly reversible and for this reason the alcohol must be added in excess to drive the reaction towards the biodiesel production ensuring the complete conversion of triglyceride into ester.



Biodiesel can be synthetized from different oils, but not all of them can be scaled-up to industrial processes. Only those ones with high oil production yields and non-toxic environmental effects are selected to produce this alternative renewable fuel. Some of them are represented in the Table 1.1.

Table 1.1. Comparison of different candidates for biodiesel production due to their high triglycerides production (Chisti, 2008).

Natural oil Producer	Annual Oil Production (L/Ha)
Corn	172
Soybean	446
Canola	1190
Jatropa	1892
Coconut	2689
Palm Oil	5950
Microalgae[1]	136.900
Microalgae[2]	58.700

1 70% Oil w/w in biomass

2 30% Oil w/w in biomass

Among all the presented alternatives, microalgae have a clear advantage over the other oil sources: algae are the only one natural source allowed to be grown virtually anywhere with enough available light (Hu et al., 2013) as they do not compete with food crops for arable land and water and their productivities as can be observed in Table 1.1 are several times higher than terrestrial crops (Canter et al., 2015).

1.2.2 CO_2 bio-capture to GHG's mitigation

Common CO_2 atmospheric management methods include carbon capture and storage through geo-sequestration and ocean-sequestration, enhanced oil and gas recovery, enhanced coal bed methane recovery, chemical methods, physical methods (i.e. cryogenic distillation or membrane filtration) and biological mitigation methods through terrestrial plants, macro and microalgae, microbes and biochar (Seyed Hosseini et al., 2018). Generally, higher plants can assimilate atmospheric CO_2 and produce organic matter through photosynthesis. Some microorganisms are allowed to conduct the same process: microalgae and/or cyanobacteria can convert CO_2 into high-valuable organic compounds for society but more efficiently than the terrestrial plants.

In microalgae, the CO_2 capturing mechanism is a biological adaptation that assists photosynthetic productivity in microalgae cells. Carbonic anhydrase enzyme has been reported as the dominant role in this CO_2 sequestration (Alaji et al., 2017).

Carbonic anhydrase is a zinc-containing enzyme that facilitates the fixation of carbon dioxide by a nucleophilic attack by a hydroxide ion bound to a zinc atom. This reaction is followed by the ionization of a water molecule bound to zinc, regenerating the active site and the production of a proton from the active site as represented in Eq. (2).

$$CO_2 + H_2O \overset{\text{Enzyme}}{\Longleftrightarrow} HCO_3^- + H^+ \overset{\text{Enzyme}}{\Longleftrightarrow} CO_3^{2-} + 2H^+ \quad (2)$$

Cyanobacteria and microalgae have developed their own exclusive photosynthetic carbon concentrating mechanism to aid ribulose-1,5-bisphophate carboxylase/oxygenase (RuBisCO) in efficient CO_2 capture. Hence, the role of carbonic anhydrase in carbon fixation is to convert CO_2 into bicarbonate (HCO_3^-), which is the substrate required from RuBisCO, the primary carbon fixation enzyme in cyanobacteria and microalgae.

Among all the species able to sequester CO_2, microalgae and cyanobacteria are preferable to macroalgae because they can be grown in an easier way using conventional mass culture processes (He et al., 2012). The biological fixation of CO_2 by microalgae can be accompanied with other processes such as wastewater treatment or production of high-valuable products, which increased the attention of several researchers in their use for CO_2 sequestration. This fact would be advantageous for providing more economic viability and environmental sustainability for the society.

Therefore, the combination of CO_2 capture, wastewater treatment and biofuel production provides a promising route to close the carbon loop using microalgae as the main responsible to make it possible (Wang et al., 2012). Figure 1.1 represents a diagram of the expected sustainable cycle of microalgae cultivation. According to Figure 1.1, expected products for the sustainable loop of microalgae are renewable energy production, protein and high-valuable organic compounds that should increase its utilization in all the industries interested in replacing the current fossil fuels.

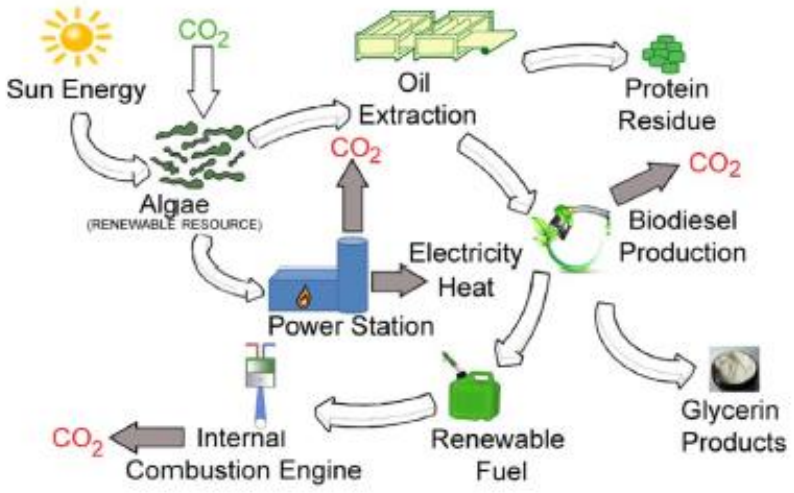


Figure 1.1. Schematic diagram of the sustainable cycle of the expected microalgae cultivation in near future (Yeo et al. 2018)

The use of microalgae to treat wastewater (especially for inorganic nitrogen and phosphorus removal) is a promising application that could substitute the conventional wastewater treatment processes (WWTPs) used only if more exhaustive investigations confirm the microalgae potential (Sforza et al., 2014). Microalgae can assimilate the nitrogen and phosphorus dissolved in wastewater reducing their concentration to reach the desired concentration of both pollutants at the end of the process. In opposite, economic aspects play an important role and more experimental researches are needed before replacing conventional WWTPs (Wang et al., 2012). Some investigations (Table 1.2) reports the benefits of the microalgae application in wastewater treatment and management to reduce the inorganic pollutants from wastewater.

According to the experimental assays represented in Table 1.2, microalgal strains such as *Chlorella sp.* show a high potential of inorganic pollutants removal when it is used in wastewater treatment. Furthermore, these microorganisms use the inorganic matter to grow achieving promising biomass productivities as reported by Min et al. (2011), where *Chlorella sp.* was used to treat swine manure wastewater. Therefore, some investigations consider microalgae to be a green technological means of eliminating wastewater pollution although it is not yet applicable in WWTPs.

Table 1.2. Microalgae applications in wastewater treatment

Wastewater type	Microalgae strain	Pollutants removal	BP ($g/m^2/d$)	Reference
Swine manure wastewater	*Chlorella sp.*	COD: 7.21$g/m^2/d$ TP: 0.067$g/m^2/d$ TN: 3.19$g/m^2/d$	14.59	(Min et al., 2011)
Municipal wastewater	*H. rubescens*	TP: 1.53mg/L/d TN:5.52mg/L/d	6.3	(Shi et al., 2014)
Municipal wastewater	*Chlorella sp., Cryptomonas sp., Scenedesmus sp.*	COD: 84% TP:95% TN:93%	3.5 - 22.7	(Novoveská et al., 2016)
Municipal wastewater	*Desmodesmus sp.*	COD: >50% TN: >90%	14.1 - 20	(Carney et al., 2014)

There are several reasons for the culture of these microorganisms to treat wastewater as pointed out by Santos-Ballardo et al. (2016):

1. Cost-effective treatment
2. Low power consumption
3. Reduction of sludge formation
4. Production of algal biomass rich in valuable organic compounds.

These advantages to conventional WWTPs make the microalgae cultivation one of the best routes to reduce the current problem of society related to climate change and decreased availability of non-renewable fuels resources and wastewater treatment (Jacquel et al., 2008; Wang et al., 2012).

1.3 Organic products from microalgae

Microalgae (unicellular, filamentous or colonial) are simple microorganisms in structure and the energy received as light intensity (LI) is directly converted into organic matter without establishing or maintaining complex tissues and organs in their cells structures (Borowitzka, 2013; Takaichi, 2013). In general, microalgae offer the prospect of high biomass productivities (BPs) without requiring any arable land and have the potential of being cultivated in different environmental conditions. Moreover, some microalgal strains can be grown with high BPs under saline and wastewater environments, making them a most promising feedstock than terrestrial crops (He et al., 2012; Santos-Ballardo et al., 2016; Srinuanpan et al., 2018). There are several organic compounds produced during the microalgae cultivation which have an interest for different industries such as pharmaceutics, nutritional or medical.

1.3.1 Pigments

Pigments are colourful chemical compounds that absorb and reflect certain wavelengths of visible light. Pigments participate in the photosynthesis of microalga acting as light energy absorber. The main pigments are grouped in chlorophylls, carotenoids and phycobilins. Pigments are high-valuable organic compounds that can be used as additives and health-promoting supplements in human nutrition. Their concentration in microalga biomass depends on cultivation conditions. Especial secondary pigments are accumulated in higher amounts under stress conditions during the microalgae cultivation, while chlorophylls in general are reduced under stress and therefore their content in biomass decreases drastically (Del Campo et al., 2007; Takaichi, 2013; Ting et al., 2017).

Pigments pharmacological potential includes their activity as antioxidants, anti-inflammatory, neuro-protective and heap-protective agents (Eriksen, 2008). Some microalgal strains with high pigments productivities reported in the literature are represented in Table 1.3.

Table 1.3. Pigments content in some microalgal strains cultivated under stress conditions

Microalgae species	Astaxantin (%)	β-carotene (%)	Lutein (%)	Phycobilins (%)	Reference
C.zofingiensis	1.5				(Del Campo et al., 2004)
Chlorococcum sp.	0.71				(Ma and Chen, 2001)
H.pluvialis	4				(Boussiba et al., 1999)
Muriellopsis			4.3		(Del Campo et al., 2007)
S. almeriensis			4.5		(Del Campo et al., 2007)
C. ptotothecoides			4.6		(Shi et al., 2006)
D. salina		12			(Del Campo et al., 2007)
E. polyphem		5			(Li et al., 2012a)
V. stellata		5.9			(Li et al., 2012b)
Nostoc sp.				20	(Sekar and Chandramohan, 2008)
S.platensis				9.6	(Sekar and Chandramohan, 2008)
Spirulina sp.				17.5	(Sharma, 2014)

The investigations reported in Table 1.3 were conducted under stress conditions, i.e. high LI with low light/dark (L/D) periods, high concentrations of CO_2 or nutrients starvation with the aim to enhance the BPs and the organic target product productivity.

With the exception of some microalgal compounds that are already produced at commercial level, the rest of the microalgae high-value compounds are either not established in the market or are still not commercialized. However, it seems that clear market opportunities for new high-value products exist as is represented in Table 1.4.

Table 1.4. Global market for selected high-value compounds produced by microalgae (Borowitzka, 2013)

High-value compound	**Global market (M$/year)**	**Production (kTn/year)**	**Price ($/kg)**
Carotenoids	1200		
β-carotene	261		300-700
Lutein	233		
Astaxantin	240		2000-7000

Beside the constrain of the consumption of nutrients, water and land occupation, one major constrain of the concept of cultivating biomass for high-value products and biofuel production, is that the potential market of these products might be saturated quickly, decreasing the economic viability of the biorefinery concept (Chisti, 2008).

1.3.2 Fatty Acids (FA)

Lipids are one of the main organic compounds produced by microalgae with particular interest for biofuel production companies as it has been mentioned previously in section 1.2.1. However, not all FA are suitable to produce biodiesel. Only the neutral FA are currently used to produce biodiesel (Kim et al., 2013). Several microalgal strains accumulate FA as energy storage compounds and their accumulation is usually enhanced under stress conditions such as nutrient deficiency in Kwon et al. (2012) or salinity stress in Takagi et al. (2006). The strategy used to cultivate the microalgae i.e. photoautotrophic, heterotrophic or mixotrophic cultivation influences positively the BPs and lipid productivity (LP) reached in the bioreactors. Some of the microalgal strains used to produce FA reported in the literature are represented in Table 1.5.

Table 1.5. BP and LP for some microalgal strains depending on cultivation strategy

Microalgae	Cultivation strategy	BP (g/L/d)	LP (mg/L/d)	Reference
Chlorella sp.	Phototrophic	0.1	6.9	(Dar et al., 2009)
C.vulgaris	Heterotrophic	0.15	27-35	(Liang et al., 2009)
C. vulgaris	Phototrophic	0.18	7.4	(Gouveia and Oliveira, 2009)
D. tertiolecta	Phototrophic	0.1	60.6 - 69.6	(Takagi et al., 2006)
Nannochloropsis sp.	Phototrophic	0.17	60.9	(Rodolfi et al., 2009)
Scenedesmus sp.	Phototrophic	0.22	20.7	(Dar et al., 2009)
Spirulina maxima	Phototrophic	0.21	8.6	(Gouveia and Oliveira, 2009)
T. suecica	Phototrophic	0.28	36.4	(Rodolfi et al., 2009)

The major part of the investigations reported previously were conducted under phototrophic cultivation strategy achieving a maximum BP of 0.28g/L/d in Rodolfi et al. (2009). The highest LP was achieved by Takagi et al. (2006) 69.6mg/L/d. This value corresponded to 0.6g/g which is 1.8 times higher than the reported by Dar et al. (2009). It means that *D. tertiolecta* seems a promising microalga to produce lipids due to it is able to accumulate more than the 60% of FA.

1.3.3 Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHA) are organic compounds accumulated by some microorganisms in response to stressing growth conditions. The most simple and recognized compound of PHA is polyhydroxybutyrate (PHB).

PHB is a thermoplastic which has similar properties than petroleum-based plastics (Panda et al., 2006; Shrivastav et al., 2010). Some researchers have focused their investigations in the bio-production of PHB as an alternative to these petroleum-based plastics, mainly used in active packaging of food. The production of PHB has been studied for several cyanobacterial strains as is represented in Table 1.6.

According to the PHB content reported in Table 1.6, the higher values were achieved with the cyanobacteria *N. muscorum* (0.35g/g) as is reported in Sharma and Mallick, (2005b) and *Synechocystis sp.* PCC 6803 (0.38g/g) as is reported in Panda and Mallick, (2007) when both cyanobacteria strains are cultivated in mixotrophic conditions.

Table 1.6. PHB content in some cyanobacteria strains investigated to produce bio-plastics

Cyanobacteria	Cultivation strategy	PHB (g/g)	Stress type	Reference
S. subsala	Phototrophic	0.147	Salinity	(Shrivastav et al., 2010)
N. muscorum	Phototrophic	0.08		(Sharma and Mallick, 2005b)
N. muscorum	Mixotrophic	0.35		(Sharma and Mallick, 2005a)
Synecocystis sp. PCC 6803	Mixotrophic	0.38	P starvation	(Panda and Mallick, 2007)
Synecocystis sp. PCC 6803	Phototrophic	0.11	N, P starvation	(Panda et al., 2006)
Synecocystis sp. PCC 6803	Mixotrophic	0.29	P starvation	(Panda et al., 2006)

1.4 Photobioreactors (PBRs) for microalgae cultivation

A photobioreactor (PBR) is a culturing recipient which incorporates some type of light source. Virtually any translucent container could be called a PBR, however the term is most commonly used to define a closed system, as opposed to an open tank or pond. A PBR can be operated in batch mode but it is also possible to introduce a continuous stream of sterilized water containing nutrients, air and carbon dioxide making the operation semi-continuous. A typical PBR used to culture microalgae is represented in Figure 1.2.

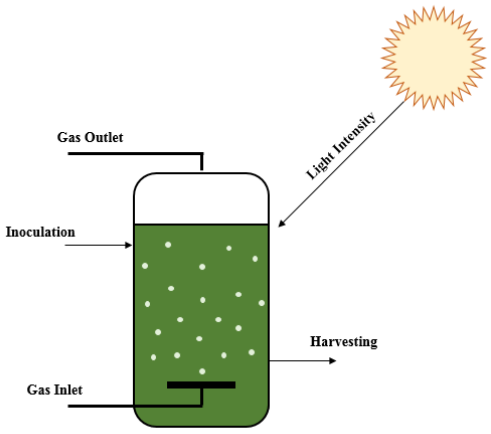


Figure 1.2. Diagram of a conventional PBR used in microalgae cultivation

There are several parameters affecting the microalgae cultivation in the PBRs such as pH, LI, heat and mass transfer and mixing. These parameters should be considered during the PBR design to prevent limitation of microalgae growth at the cultivation stage (Borowitzka, 2013; Huang et al., 2017). PBRs can be mainly divided into open and closed systems. In addition, each one of them can be subdivided into particular PBR depending on the bioreactor geometry.

1.4.1 Open PBRs

The cultivation of microalgae in open ponds has been widely studied in last decades. Open cultivation systems include natural, circular and raceway ponds. The benefits of these systems with respect to the closed PBRs are their simple design, low construction cost and high production capacity (Santos-Ballardo et al., 2015).

Inorganic pollutants, mainly nitrogen and phosphorus, in the effluents of WWTPs could be used as nutrients for microalgae cultivation in PBRs. However, open PBRs are strongly sensitive to environmental conditions and monitoring of growth parameters is not an easy task when these configurations are applied. Open PBRs cultivation often shows culture contamination because it directly exchanges gases with the atmosphere and does not have any barrier preventing the contact of the culture and the environmental contaminants. Although open ponds do not require high maintenance costs, their utilization is restricted for commercial production of microalgae due to their susceptibility to contaminants.

Other drawbacks influencing the utilization of open PBRs at full scale are: i) inefficiency of the mixing which causes low mass and heat transfer during the microalgae cultivation, ii) uncontrollable LI applied to the PBRs affecting the microalgae metabolism, iii) temperature gradient along the PBR which modifies the microalgae kinetic reactions and iv) requirement of a large amount of LI to irradiate all the cultivation surface.

1.4.1.1 Circular Pond

A typical circular pond is presented in Figure 1.2. This configuration is mainly used in China for *Chlorella sp.* cultivation in biodiesel production processes. The idea of using a rounded pond with a long rotating arm was inspired in the circular reactor used in WWTP.

The idea of using a rounded pond with a long rotating arm was inspired in the circular reactor used in WWTPs. This type of configuration is always 20-30cm in depth and 40-50m in diameter obtaining high volumes of PBRs. The long rotating arm is set in the centre of the pond and it is used to increase the turbulence of the system favouring the heat and mass transfer inside the PBR.

Figure 1.2. Circular open pond used in biodiesel production processes (Kiran et al., 2014)

As can be observed in Figure 1.3. the culture is exposed directly to the environment and, thus, contamination is unavoidable in this PBR configuration. For this reason, this configuration is being disused. According to the research literature consulted, the BPs achieved in circular ponds are in the range 8.5 - 21g/m^2/d (Colling Klein et al., 2018).

1.4.1.2 Raceway pond

Open raceway ponds are usually made of a closed-loop recirculation channel with 0.3 m of depth which is similar than the used in circular ponds. The construction of these bioreactors is made of concrete or compacted earth, sometimes lined with white plastic and equipped with paddlewheel trying to enhance the mixing and circulation of the microalgae cultivated in these PBRs. This PBR configuration achieves higher BPs than the circular ponds mentioned in section 1.4.1.1. due to the closed-loop, which increases the mixing of the culture contained in the PBR, enhances the heat and mass transfer capability of the system. A simple representation of a raceway pond is displayed in Figure 1.3.

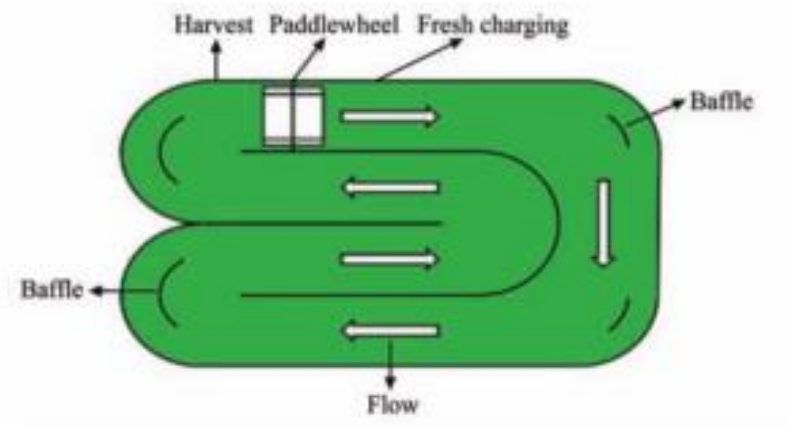


Figure 1.3. Open raceway pond scheme (Jorquera et al., 2010)

Fresh nutrients and high-density microalgae inoculum are introduced to the PBR just in front of the paddlewheel according to Figure 1.3. The paddlewheel is always in movement to prevent culture sedimentation. The largest biomass production plant based on raceway ponds is located in Calipatria, CA (USA) where *Spirulina platensis* is cultivated for its uses as nutritional supplementation (Oswald and Goleuke, 1967). This PBR occupies an area of $4.4 \cdot 10^5 m^2$ and it is considered one of the higher cultivation systems still in use around the world. Some investigations (Table 1.7) reported the efficiency of these PBRs configurations applied in WWTPs.

The efficiencies of the open PBRs are well demonstrated according to Table 1.7. Chemical oxygen demand (COD) is reduced in the range 56-88% while N and P are removed over 95% for all the investigations represented in Table 1.7. Although the efficiency is high, their use is limited because open systems are significantly affected by environmental conditions and contamination.

Table 1.7. Efficiencies achieved using open PBRs to treat wastewater from different sources

PBR configuration	Wastewater Type	Microalgae strain	Operation strategy	Efficiency (%)	Reference
Circular pond	Municipal wastewater	*C. pyrenoidosa*	Batch	COD: 87.93 N: 98.17 P: 96.87	(Sfez et al., 2015)
Circular pond	Piggery wastewater	*Chlorella sp* *Scenedesmus sp.*.	Semi-continuous	N: 95.76	(Nwoba et al., 2016)
Raceway pond	Municipal wastewater	*Scenedesmus sp.*	Semi-continuous	COD: 84 N: 79 P:57	(Posadas et al., 2015)
Raceway pond	Piggery wastewater	*Scenedesmus sp.*	Semi-continuous	COD: 56 N: 98	(de Godos et al., 2010)

1.4.2 Closed PBRs

Closed bioreactors are capable of cultivating several strains of microalgae with longer retention times and avoiding contamination because they are closed systems. Furthermore, they offer a number of advantages against previously described in section 1.4.1. open systems, including (i) the exhaustive control of operational parameters, and (ii) the absence of carbon dioxide losses during the microalgae cultivation. Different designs types of closed PBRs have been studied for microalgae cultivation, but two main designs dominate the market: tubular and flat-plate PBR.

The conventional materials used in the construction of these PBRs are glass or plastic-based materials. The most common plastic materials are polymethylmetacrylate (PMMA), polycarbonate and reinforced fibber glass polyethylene. The use of transparent plastic or glass facilitates the penetration of light required for photosynthesis when this configuration is used to cultivate the microalgae.

Based on the liquid flow circulation inside the bioreactor, vertical tubular PBRs can be divided into bubble column and airlift reactors. In addition, other classification can be applied as a function of their orientation to LI. Closed PBRs can be vertical, horizontal or near-horizontal. Aeration/mixing are usually performed by means of an air pump an it is introduced to the PBR using a sparger placed at the bottom part of the reactor allowing the vertical circulation of the liquid.

This arrangement also improves mass transfer and ensures the overall removal of oxygen produced at the dark respiration period. The O_2 produced could induce the limitation of the microalgae growth by affecting their metabolic pathways.

1.4.2.1 Tubular PBRs

Tubular PBR is one of the most popular configurations of PBRs used at full scale. Depending on their orientation to LI, tubular PBRs can be divided into horizontal, inclined or vertical arrangement according to previous description in section 1.4.2. Horizontal PBRs are capable to achieve a greater surface area to volume ratio than their vertical orientation due to the ability to decrease the diameter of tubes without affecting the overall structural integrity. Horizontal PBRs also have better angle for incident light in comparison to vertical PBRs, allowing for more efficient light harvesting. However, this configuration also causes overproduction of heat, which often requires an elevated investment in temperature control systems (Qiang et al., 1998).

Hence, horizontal PBRs are especially difficult to of scale-up, where large areas occupied require a sophisticated temperature control to maintain the microalgae cultivation in adequate temperature conditions. Often, a heat-exchanger is incorporated into the design to maintain an optimal growth temperature (Watanabe and Hall, 1995). Some of the tubular configurations reported in the literature are represented in Figure 1.5.

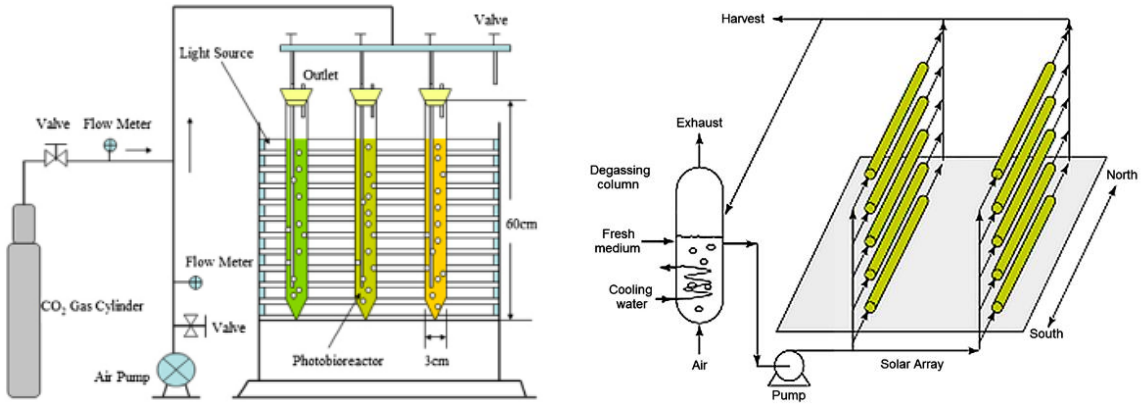


Figure 1.4. Tubular PBRs configuration depending on their orientation to LI. (a) Vertical tubular PBR (Li et al., 2012b) and (b) horizontal tubular PBR (Wang et al., 2012)

Several tubular PBRs have been used during the last decades WWTPs and production of high-valuable organic compounds, since the open ponds have been in disuse. Some investigations (Table 1.8.) reported the efficiency of tubular PBRs applied in WWTPs. The efficiency of tubular PBRs reducing contamination from wastewater is clearly demonstrated according to the values reported in Table 1.8. For all investigations, the N and P reduction achieved was higher than 80%.

Table 1.8. Efficiency of contamination reduction and BPs for some tubular PBRs used in WWTPs

PBR	**Two Plexiglas tubular PBR**	**Transparent PMMA tubular airlift PBR**	**Tubular bubble column PBR**	**Tubular PBR**
Wastewater type	Tertiary treatment	Urban wastewater	Piggery wastewater	Fish farm wastewater
Algae species	*Scenedescmus sp.*	*Scenedesmus obliquus*	*Chlorella zofingiensis*	*Tetraselmis suecica*
Operation mode and scale	Batch Pilot scale	Batch Pilot scale	Batch Lab scale	Continuous Pilot scale
Influent (mg/L)	N: 7.43 P: 16.23	N: 24.92 P: 26.16	N: 148 P: 156	N: 40.7 P:4.96
Removal (%)	N: 90 P: 80	N: 94.9 P: 94	N: 82.7 P: 98.17	N: 95.7 P: 99.7
BP (g/L/d)	0.03-0.3	0.082	0.1-0-29	0.32-0.54
Reference	(Di Termini et al., 2011)	(Garrido-Pérez et al., 2013)	(Hiltunen et al., 2013)	(Michels et al., 2014)

1.4.2.2 Flat-plate PBRs

Flat panel PBRs are closed photobioreactors with a narrow light path and are characterized by large illuminated surface to volume ratio (S/V) in comparison to tubular PBRs. These PBRs can be oriented into the direct path of light to obtain maximum exposure to solar energy.

They are divided into two main categories according to the means of mixing: pump-driven flat-plate or airlift. Pump-driven configuration depends on the flow of liquid created by pumping to generate the required turbulence for mixing, while airlift flat-plate depends on the supply of compressed air or air enriched with pure CO_2 to ensure the completely mixed conditions inside the PBR.

Flat-plate PBRs often suffers bio-fouling problems, cells adhesion into wall surface of the PBR, when the mixing of the reactor is not enough. Moreover, if the PBR is directly exposed to sunlight, sometimes photoinhibition phenomenon is found during the microalgae cultivation. Hence, it was pointed out that mixing control and light

inhibition are two major challenges in designing flat-plate PBR. The two possible configurations of a flat-plate PBRs are represented in

Figure 1.5.

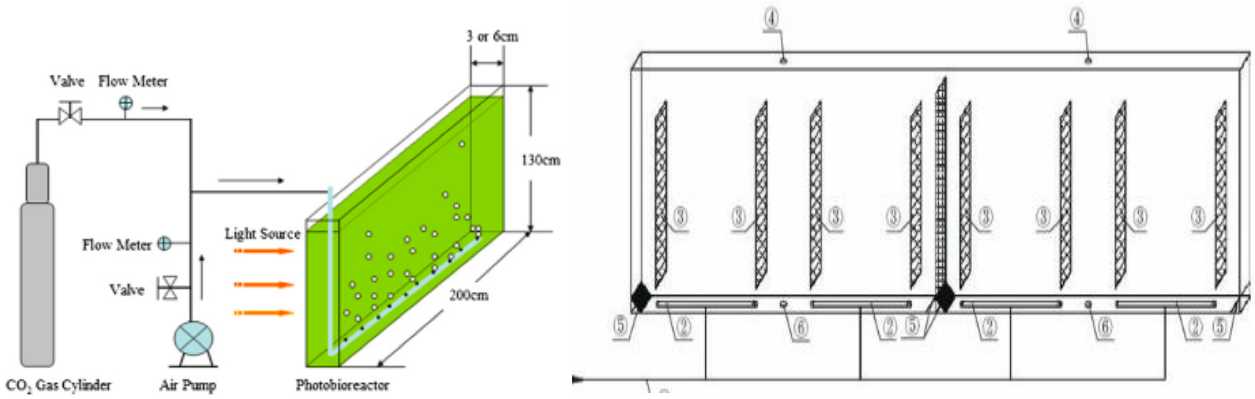


Figure 1.5. Flat-Plate configurations depending on the mixing technique applied. (a) Pump-driven flat-plate (Li et al., 2012b) and (b) Airlift flat-plate (Huang et al., 2016)

Some investigations (Table 1.9.) reported the efficiency of flat-plate PBRs in the pollutants removal of wastewater. The efficiency of flat-plate PBRs to reduce wastewater contamination is demonstrated in these works: N removal is higher than 90% while inorganic phosphorus reduction is in the range 71-97%.

Table 1.9. Contamination removal efficiency for some flat-plate PBRs reported in the literature

PBR	Wastewater type	Algae species	Operation mode and scale	Removal (%)	Reference
Transparent PMMA flat panel	Urban wastewater	*S. obliquus*	Batch Lab scale	N:93.4	(Ruiz et al., 2013)
Vertical flat-plate PBR	Digested starch wastewater	*C. pyrenoidosa*	Batch Lab scale	N: 97 P: 71	(Tan et al., 2014)
Airlift flat-panel PBR	Urban wastewater	*Chlorella protothecoides*	Batch Lab scale	N: 96 P: 97	(Olkiewicz et al., 2015)

1.4.3 Closed PBRs comparison

The benefits and drawbacks of each closed PBR configuration are discussed in this section. Table 1.10. presents the advantages and disadvantages of each configuration. According to the information reported, flat-plate PBRs are the best selection to cultivate microalgae because their only drawbacks are the scale-up difficulty and the bio-fouling formation due to cells adhesion to surface wall when the mixing inside the PBR is insufficient.

Table 1.10. Benefits and drawbacks for closed PBRs depending on their configuration

Closed system	Advantages	Disadvantages
Tubular PBR	Large illumination area	Requires large land space
	Suitable for outdoor cultures	Photoinhibition is common
	Good BP	Poor mass transfer
Column PBR	High mass transfer, photosynthetic efficiency, potential for salability	Small illumination area
	Reduced photoinhibition and photo-oxidation	Low surface to volume ratio
	Low cost, compact, easy to operate	Expensive compared to open pond
	Greater gas hold-up	
	Best exposure to light/dark cycles	
	Reduced land use	
	Promising for large scale cultivation of algae	
Flat plate PBR	Large illumination surface area	Difficult to scale-up
	High area to volume ratio	Algae adheres to walls
	Suitable for outdoor cultures	
	High biomass productivities	
	Uniform distribution of light	
	Inexpensive	
	Easy to build, maintain and clean	
	High photosynthetic efficiency	
	Massive production of microalgae	

1.5 PBR design criteria

The efficiency of PBRs is determined by the integration of different factors affecting the microalgae cultivation. An efficient PBR design should accomplish the following criteria: (i) harvest as much light as possible, which means an enhancement of the photosynthetic efficiency of the system, (ii) maintain a convenient and precise control of all the physical and chemical parameters affecting the microalgae cultivation, (iii) minimize as much as possible the investment and operational costs of the proposed PBR and (iv) reduce the energy consumption and the environmental impact of the designed bioreactor.

1.5.1 Light

Microalgae that perform oxygenic photosynthesis can only reach a theoretical maximum conversion efficiency of 8 to 10% solar-to-biomass energy (Posten, 2009). Maximize conversion efficiency is a challenge during the PBR design phase. There are some factors involved on light affecting the microalgae cultivation i.e. LI, light quality and PBR depth (light path). LI is the most affecting parameter related to this issue. An insufficient light supply produce photo-limitation growth of the microalgae, while and excessive LI produce photoinhibition, which reduces the BP obtained at the end of the microalgae cultivation.

1.5.2 Mixing

Mixing is also an important issue to consider during the design phase of a PBR. It can not only reduce nutrients, pH, and temperature gradient in the culture broth, but an efficient mixing also prevents cell sedimentation, the emergence of dead zones, cell clumping and cell attachment to the walls in reaction containers (Carvalho and Meireles, 2006). In addition, mixing guarantees equal exposure to LI for all cells and enhances mass transfer between the different phases. However, excessive mixing may produce cell damage, known as shear-stress, resulting in the culture collapse and a reduction of BPs obtained during the microalgae cultivation (Wang et al., 2012).

1.5.3 Temperature

The system temperature could influence severely on the microalgae growth. Depending on the PBR location, a considerable variation in temperature will be experienced during the cultivation of microalgae due to diurnal and seasonal behaviours.

Therefore, an exhaustive temperature control system is required in order to keep the culture temperature within a favourable range permitting to obtain an appropriate BPs during the microalgae cultivation.

1.5.4 pH

Most of the microalgae strains reported in the literature have an optimal pH range of 8.2 to 8.7 but some of them can also be cultivated in more basic or acid mediums in the range 5 to 9. In any case, it is recommended to keep the pH within the microalgal strain optimal value to prevent the limitation of the culture growth due to the metabolic alteration and possible cell disruption when the microalgae are cultivated far away from their optimal pH.

1.5.5 Nutrients

Some microalgae such as *Chlorella vulgaris* or *Synechocystis sp.* PCC 6803 can enhance their organic target products accumulation when both are cultivated under stress conditions (nutrients depletion). *Chlorella vulgaris* has shown in several investigations an increasing content of lipids under mixotrophic cultivation, while *Synechocystis sp* enhanced its PHA production when the cyanobacterium is cultivated under N and P starvation. Hence, finding a compromise between nutrients stress conditions and organic product of interest production is a challenge during the PBR design phase.

1.6 Kinetic models for microalgae growth

Photoautotrophic microalgae cultivation has been extensively investigated with diverse purposes as LP or pigments synthesis for food supplementary agents during the last century. As the potential mass culture increases, kinetic modelling of microalgae growth has become of significant importance because an accurate model is a prerequisite for designing an efficient PBR, predicting the process performance and optimizing operating conditions (Yun and Park, 2003).

Microalgal BP is the net result of photosynthesis, photorespiration and dark respiration. Describing the rate of these mechanisms during the indoor or outdoor cultivation is challenging due to microalga activity is affected by several factors such as LI, temperature, pH, dissolved oxygen (DO) and CO_2 and nutrients concentration (Mata et al., 2010). All of these factors should be exhaustive evaluated to develop a dynamic model predicting properly the system evolution during the microalgae cultivation.

Several models have been reported in the literature evaluating the influence of each one of the parameters affecting the microalgae growth during the cultivation phase. Béchet et al. (2013) proposed a state of the art about the influence of light and temperature on the microalgae growth. The investigation also gives some advices about the different equations used to describe the microalgae behaviour under light stress strategy. Heo et al. (2018) also proposed a dynamic kinetic model to simulate the microalgae growth under nutrient full and deplete conditions. The kinetic model proposed shows a strongly effect of cell death on LP, reducing it drastically. García-Camacho et al. (2012) proposed a model of microalgae growth considering the photo-acclimation when the microalga is inoculated into the PBR.

1.7 Anaerobic digestion (AD) of microalga biomass

Microalgae contain a range of organic macromolecules i.e. lipids, proteins or carbohydrates that can produce energy by generating biogas via anaerobic digestion (AD) processes (Zhang et al., 2019). Scaling-up microalgae production to the industrial level presents challenges including the disposal or reuse of excess biomass once the organic target product is extracted (Hii et al., 2014). One of the methods proposed to recover energy and reuse this residue is the AD process. However, further investigations are required to fully develop this re-use way.

Waste biomass coming from biodiesel manufacturing processes present potential problems for the AD process. *Chlorella sp.*, which is the usual microalgae used to produce biodiesel, is highly resistant to AD. The major inconvenient presented by this strain is its cell wall robustness preventing the complete degradation of the cells during the anaerobic process (Mussgnug et al., 2010). Therefore, disruption of the *Chlorella sp.* cell wall has been proposed as an important step for efficient AD of the biomass coming from biodiesel production processes (Rodriguez et al., 2015). Numerous studies have focused on methods to enhance anaerobic biodegradability of microalgae, by means of physical, chemical and biological pre-treatments (Passos et al., 2014, 2013; Rodriguez et al., 2015).

Microalgae with high carbohydrates and proteins content, usually residual biomass wastes in biodiesel production processes, are theoretically poorer substrates for methane production than other wastes lipid-rich biomass (Mendez et al., 2015).

The optimal C/N ratio (w/w) for AD process is 25-30, but this ratio is only 6.7 in *Chlorella sp.* (Hiltunen et al., 2013). Due to the low C/N ratio in microalgae biomass, the addition of a carbon-rich co-substrate could enhance the methane yield achieved during the AD process (Meneses-Reyes et al., 2018).

1.8 Thesis motivation

This thesis was conducted in parallel with two European Projects called MAR3 (grant agreement No. RDNET-11-2-0003) ("*Valorisation of hydrocarbon sludges for the production of additives for marine remediation and biomass for energetic purposed"*) and Oli-PHA (grant agreement No. 280604) ("*A novel and efficient method for the production of polyhydroxyalkanoate polymer-based packaging from olive oil wastewater"*) under the EU 7th framework programme. The company involved in the projects, *Técnicas para la fijación del carbono S.L. (FCTecnics)* was granted also with a TEM grant (grant agreement No. 2010 TEM 65) allowing the possibility for an investigator to conduct an industrial PhD programme.

The aim of MAR3 project was to develop a gelling agent capable to flocculate and coagulate the hydrocarbons present in the oil spills during the refining of oil production processes at the sea-platforms. The contribution of the author of the present thesis was to design, construct and operate an optimized PBR allowed to cultivate the microalgae *Chlorella vulgaris*. This microalgae strain was selected due to its high BP potential and its high lipid content (g/g). Once extracted the fatty acids (FAs) from the cells, they were completely transformed into esters (gelling agent) to recover the coagulated hydrocarbons from the oil spills.

The aim of the Oli-PHA project was to produce an innovative bioplastic for a suitable packaging of food. Among the candidates, PHA represented the best alternative to achieve this goal. The contribution of the author in this project was to design, construct and operate a suitable PBR allowed to cultivate the cyanobacteria *Synechocystis sp.* PCC 6803. This microorganism has been extensively investigated because it is considered the most promising producer of PHB. A kinetic model predicting the cyanobacterium behaviour during the cultivation phase was also

developed in this investigation. Moreover, the exceeding biomass (60-70%) remaining once extracted the organic target product (PHA) was reused to produce energy in form of biogas by utilizing AD processes.

Thermal treatments and co-digestion techniques were conducted during the AD processes with the aim to enhance the biogas production potential (BPP) of the *Synechocystis sp.* PCC 6803.

The author of the present thesis was granted with a TEM grant (grant agreement No. 2010 TEM 65) that permitted the thesis development and the work for a private company at the same time. Hence, the present investigation is not only focused on investigation, but there are other affecting issues such as commercial market for the developed products and operating conditions established to achieve the optimal BPs of the systems developed during the European projects.

1.9 Thesis overview

This thesis is divided into seven chapters. The first chapter is a general introduction about the current status of fossil fuels and the intention of the worldwide population to replace them by renewable bio-resources reducing GHGs emissions and as a consequence the global warming by using microalgae. It also includes a brief resume about the PBR types and the BPs achieved in previous investigations using microalgae in WWTPs. The second chapter describes the initial objectives for the present thesis. The third chapter is focused on the description of the material and methods used at the experimental phase. The fourth chapter is focused on the design of a tailor-made PBR with the aim to produce great BPs and LPs using the microalgae *chlorella vulgaris.* It also permits to produce at the end of the process an emulsifier for the bioremediation of oil spills.

The fifth chapter aims to the development of a kinetic model for describing the growth of the cyanobacterium *Synechocystis sp.* PCC 6803 considering all the parameters involved in this cyanobacterium cultivation. The sixth chapter is focused on the energy recovery by AD of the remaining cyanobacterium biomass once the high-valuable product PHA, destined to be used as active packaging production in food industry, was extracted from *Synechocystis sp.* PCC 6803. The seventh and last chapter

of the present thesis gives an overview of the main achievements and points out the topic for future investigations derived from this thesis.

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2 Objectives

The main objective of the present thesis is the enhancement of the microalgae cultivation with the aim to produce high-value organic compounds and energy at the end of the cultivation.

Furthermore, this objective is divided into the five specific objectives:

1. To conduct an exhaustive literature research about the parameters involved in the microalgae cultivation in view of designing an efficient PBR. The design criteria comprises the influence of light, carbon source, nutrients, cultivation strategies, mixing and reactor hydrodynamics over the microalgae growth. The literature revision will provide guidelines for the design of an efficient low-cost PBR for cultivating the microalga *Chlorella vulgaris* and for enhancing its BP and LP. The same guidelines will be also used to design and construct an efficient PBR for the cultivation of the cyanobacterium *Synechocystis sp.* PCC 6803 with the aim to achieve 2 kg of DW of biomass per week.
2. The development of a kinetic model to describe the cyanobacterium *Synechocystis sp.* PCC 6803 behaviour during its cultivation in the PBR. This model should account for the influence of nutrients and the ion speciation over the cyanobacterium growth. To gain insight on the process, the effect of two parameters will be experimentally investigated: pH and phosphorus concentration. Moreover, some simulations will be conducted to show the model response under different scenarios.
3. To conduct AD tests in view of promoting energy recovery for the waste biomass released after the PHA extraction from the cyanobacterium *Synechocystis sp.* PCC 6803. To determine, for a first time, the BPP of this cyanobacterium biomass.
4. To enhance the BPP of the *Synechocystis sp.* PCC 6803 with different thermal pretreatments such as ultrasonication or microwaving. This enhancement should be analysed through an energy balance to determine the feasibility of full-scale implementation of thermal treatments for biogas production enhancement.

5. To use a co-substrate (i.e. oil mill wastewater) with a higher C/N ratio than that of the cyanobacteria with the aim of enhancing the biogas production in the AD of *Synechocystis sp.* PCC 6803.

6. To describe the BPP when both treatments, co-digestion and microwaving, are conducted at the same time and determine the enhancement of BPP obtained.

7. To describe the kinetic behaviour during the AD process of *Synechocystis sp.* PCC 6803

3 Materials and Methods

3.1 Materials

3.1.1 *Chlorella vulgaris* culture medium

The microalgal strain *Chlorella vulgaris* used in the experimental set-up of the investigation conducted (See chapter 4, *Guidelines for a tailor-made photobioreactor design: The case of Chlorella vulgaris*) was cultivated in a 150mL Erlenmeyer flasks prior its inoculation in the PBRs using Bold Basal medium (Ho et al., 2016). The Bold Basal medium consisted of 0.25g/L $NaNO_3$, 0.025g/L $CaCl_2 \cdot 2H_2O$, 0.075g/L $MgSO_4 \cdot 7H_2O$, 0.075g/L K_2HPO_4, 0.175g/L KH_2PO_4, 0.025g/L NaCl, 0.05g/L Na_2EDTA, 0.031g/L KOH, 4.98mg/L $FeSO_4 \cdot 7H_2O$, 11.42mg/L H_3BO_3, 8.82mg/L $ZnSO_4 \cdot 7H_2O$, 1.44mg/L $MnCl_2 \cdot 4H_2O$, 0.71mg/L MoO_3, 1.57mg/L $CuSO_4 \cdot 5H_2O$ and 0.49mg/L $Co(NO_3)_2 \cdot 6H_2O$.

Micro and macronutrients were prepared separately in a 1L bottles previously autoclaved at 121ºC for 1h. Osmotic water was used to dissolve the micro and macronutrients during the solutions preparation. Nutrient solutions were kept tempered in a refrigerator (4ºC) and coated with aluminium paper preventing them from the direct light exposure prior to their use during the experimentation procedures.

3.1.2 *Synechocystis sp.* PCC 6803 culture medium

Axenic cultures of *Synechocystis sp*. PCC 6803 utilized in the experimental set-up of the experimentation conducted (see chapter 5, *Kinetic model development for Synechocystis sp. PCC 6803*) were grown in 250mL Erlenmeyer flasks prior to their inoculation in the PBRs using BG-11 medium (Kim et al., 2011). The BG-11 medium consisted of 1.5g/L $NaNO_3$, 0.02g/L Na_2CO_3, 0.075g/L $MgSO_4 \cdot 7H_2O$, 0.036g/L $CaCl_2 \cdot 2H_2O$, 0.04g/L KH_2PO_4, 0.06g/L citric acid, 0.06 g/L ferric ammonium citrate, 0.01g/L Na_2EDTA, 1.81mg/L $MnCl_2 \cdot 4H_2O$, 0.039mg/L Na_2MoO_4, 2.86mg/L H_3BO_3, 0.079mg/L $CuSO_4 \cdot 5H_2O$, 0.04mg/L $Co(NO_3)_2 \cdot 6H_2O$ and 0.22mg/L $ZnSO_4 \cdot 7H_2O$.

Micro and macronutrients were prepared simultaneously in a 1000mL bottles previously autoclaved at 121ºC for 1h. Osmotic water was used to prepare the micro and macronutrients solutions.

Nutrient solutions were kept tempered in a refrigerator (4ºC) and coated with aluminium paper preventing them from the direct light exposure prior to their use during the experimentation procedures.

3.1.3 PBR for *Chlorella vulgaris* cultivation

The design and the detailed description of the airlift PBR used in *Chlorella vulgaris* cultivation is explained in a specific section of the Chapter 4 (*Features of the designed PBR*).

LI was provided by a sunlight simulating device (Gavita, LEP300) providing 150μmol/m^2/s of photosynthetic active radiation (Bernardi et al., 2014; Pei et al., 2016). Light/dark (L/D) cycles were set at 20:4h according to the consulted literature at the designing phase of the PBR (Atta et al., 2013; Blanken et al., 2016; Ma et al., 2017). The microalga growth strategy selected was mixotrophic cultivation using glucose (10g/L) as organic carbon source and pure CO_2 (10%v/v) as inorganic carbon source according to the literature consulted at the designing phase (Gonçalves et al., 2016; Liang et al., 2009; Sakarika and Kornaros, 2017). Mixing was achieved by aeration and pure CO_2 injection (when it was necessary) at the bottom zone of the PBR due to a sparger located at the inner cylinder of the PBR in order to maintain the pH in the adequate range of 8-8.5. The aeration flow was set at 0.1v/v (15L/min) after the hydrodynamic investigation conducted during the system start-up. Temperature was controlled due to a transparent silicone-jacket covering the working volume of the PBR. Temperature set-up was fixed at 30ºC according to previous investigations reported in the literature (Cheirsilp et al., 2016; Liang et al., 2009). pH, Temperature, dissolved oxygen (DO) and light intensity was continuously monitored during the experimentation procedures.

3.1.4 PBR for *Synechocystis sp.* PCC 6803 cultivation

The PBR designed for the purpose of cultivating *Synechocystis sp.* PCC 6803 aimed to improve the light distribution within the PBR, then, a new illumination concept based on light emitting diodes (LEDs) was used for this bioreactor.

The PBR consisted of a vertical flat-plate made of polymethyl methacrylate PMMA body (IRIS S.L., Spain) with a total capacity of 4L (lab-scale PBR). Four equal PBRs were used in the different assays conducted.

The PBR was designed according to the design criteria obtained in Chapter 4, hence, the airlift configuration was proposed to ensure that PBR was under completely mixed conditions.

At the bottom part of the PBR, an air diffusor was used to aerate the bioreactor. The air flow was continuous while depending on the measured pH (8.5 – 9.5) pure CO_2 was added as handle variable in an on/off pH control loop. Linear-fluorescent lamps (TL-D36W, Phillips) were placed on both sides of the PBRs to provide sufficient light quality to the cyanobacteria at those wavelengths were LEDs were not emitting (400-550nm). The light intensity applied to the PBRs was fixed at 100μmol/m^2/s. During initial phases of cultivation, photo-acclimation of cyanobacterium, L/D cycles were set at 12:12h. A sampling port was located at the top of the PBR to extract samples for the determination of the different parameters involved in the *Synechocystis sp.* PCC 6803 growth.

3.1.5 PBR sanitisation, inoculation and cyanobacterium acclimation

The PBR was sanitized by washing it once with deionized water containing 0.04%v/v of NaOCl and 0.2%w/v of NaOH. After several rinses with sterile deionized water, the PBR was loaded with 1L of concentrated inoculum and 3L of BG-11 culture media. After the PBR inoculation, the air supply was turned on and the CO_2 dosage was regulated following the previous detailed procedure in section 3.1.4.

3.1.6 Thermal treatments in AD tests

Microwaving consisted of heating the cyanobacterium powder once extracted the organic compound for 40s at 900W inside a laboratory microwaving system (same that commercially available one) while ultrasonication process consisted of placing the biomass powder inside of an ultrasound bath for 10min at 2000W. Both treatments conducted were according to previous investigations on the enhancement of BPP by disrupting the cells with thermal treatments (Calicioglu and Demirer, 2016; Passos et al., 2013).

Both resulting solutions after thermal treatment were kept for an hour at room temperature to cool them down. After that, both solutions were lyophilized to reduce their humidity and were kept in a freezer at -82ºC prior to its use in AD assays.

3.2 Methods

3.2.1 Total solids (TS) and volatile solids (VS) determination

Total solids (TS) represented the total amount of organic and inorganic matter in the mixed liquor sample. Volatile solids (VS) corresponded to the volatile organic matter which is close to the amount of biomass. Both were analysed according to Standard Methods (APHA,1995). 100mL of well-mixed sample was add into a dish previously dried up and weighed (w_0). Then, the dish was introduced into the oven at 100ºC overnight and put in the desiccator for 2h before weighing it again (w_1). After that, the dish was introduced into the furnace using ceramic bowl for 30 minutes at 550ºC and in the desiccator for 2 hours previously weighing it (w_3).

The difference between w_0 and w_1 indicated the TS. Conversely, VS was the result of the difference between w_1 and w_3. Both were expressed as mass per volume of filtered sample (i.e. g/L). All samples were conducted per triplicate to normalise the results obtained. Regular sampling of the bulk liquid contained in the PBR was used to determine through off-line analysis the optical density, the μ and the BP for each one of the PBRs assays conducted during the experimentation procedures.

3.2.2 Optical density measurement and specific growth rate (μ) determination

Specific growth rate (μ) was measured thorough an optical measurement based in the optical density achieved by the culture. Samples were introduced into glass optical cuvette and the optical density determination was conducted in a spectrophotometer Perkin-Elmer lambda 1050 UV/Vis. Optical density was determined at the wavelengths 600nm for *Chlorella vulgaris* and 750nm for *Synechocystis sp.* PCC 6803 according to previous investigations consulted (Kamravamanesh et al., 2017; Rashid et al., 2015).The μ for each microalgal strain can be determined using the corresponding Eq. (1).

$$\mu = \frac{\mathrm{Ln}(\mathrm{OD}_2) - \mathrm{Ln}(\mathrm{OD}_1)}{t_2 - t_1} \quad (1)$$

Where OD_2 corresponds to the optical density measured at time 2 while OD_1 is the optical density measured at time 1. t_2 is the time when sample 2 was took from the PBR while t_1 is the time when the previous sample was extracted from the PBR. μ is

represented in 1/d. All measurements were conducted per triplicate for all optical density measurements.

3.2.3 Dry weight (DW) and Biomass productivity (BP) determination

Dry weight (DW) was determined in accordance to TS and VS determinations previously described in section 3.2.1. A dish with a filter paper was weighted (w_0). Then, 10mL of sample containing biomass (previously homogenised by mixing it vigorously) was deposited over the dish and it was introduced in a furnace for 1 hour at 150ºC. After that, the dish was introduced for 2 hours in the desiccator before weighing it again (w_1).

The difference between w_1 and w_0 indicated the DW of biomass. DW is expressed as mass per volume (i.e. g/L) as concentration. All measurements were conducted per triplicate for all DW determinations.

Biomass productivity (BP) can be determined using the DW obtained in each measurement conducted and the sample time using the corresponding Eq. (2).

$$BP = \frac{DW_2 - DW_1}{t_2 - t_1} \quad (2)$$

Where DW_2 corresponded to the biomass concentration inside the PBR at time 2 while DW_1 was the biomass concentration measured at time 1. BP was expressed as concentration per time (i.e. g/L/d). Measurements were conducted per triplicate and error bars were represented in their corresponding figures.

3.2.4 Biogas production potential (BPP) determination

A portable pressure indicator was used to determine the overpressure produced daily on the digesters during the AD assays with the cyanobacterium *Synechocystis sp.* PCC 6803. This overpressure was directly converted into biogas production potential (BPP) (mL/gVS) at the standard temperature using the corresponding Eq. (3).

$$BPP = \frac{P_m \cdot V_g \cdot T^0}{P_a \cdot T_w} \quad (3)$$

where P_m was the pressure measured by the sensor (bar), V_g was the volume occupied by the gas in the digester bottles (mL), T^0 was the standard temperature (0ºC, 273K), P_a was the atmospheric pressure (1bar) and T_w was the working temperature (37ºC, 410K)

BPP rate was commonly referred to the initial volatile solid (VS) concentration; hence it was expressed in mL/gVS. Thus, the resulting value determined in Eq. (3) was then divided by VS content in biomass. These calculations were conducted according to the literature consulted (Mussgnug et al., 2010). All biogas determinations were conducted per triplicate and error bars were represented in their corresponding figures.

3.2.5 Total inorganic nitrogen (TIN) and total inorganic phosphorous (TIP) determination

Samples of 10mL extracted from the PBRs were introduced in a specific inorganic nitrogen analyser, AMTAX, which measured the nitrogen content in the sample. The values were expressed in mg-N/L. Then, the value observed was corrected to mg-NO_3^-/L by using the molecular weight NO_3^-.

The procedure conducted to determine TIP was similar to TIN determination. Samples of 10mL extracted from the PBRs were introduced in a specific inorganic phosphorus analyser, PHOSPAX, which measured the phosphorous content in the sample. The values were expressed in mg-P/L. Then, the value observed was converted into mg-HPO_4^{2-}/L by using the molecular weight of HPO_4^{2-}.

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4 Guidelines for a tailor-made photobioreactor design: The case of *Chlorella vulgaris*

Abstract

The research conducted in this chapter was framed in the European project (Manunet - MAR3). MAR3 was a large project dealing with the bioremediation of oil spills consisting of several stages. In particular, the main objective in this chapter was the design, construction and operation of an optimised PBR capable of increasing the production of biomass, particularly FA productivity, by cultivating the microalga *Chlorella vulgaris*. To this end, an exhaustive bibliographic review was carried out on the main parameters affecting the design of the PBR. Guidelines for appropriate reactor design were established and, once all these performance variables had been defined, a new reactor concept was developed and tested cultivating the proposed microalga.

4.1 Introduction

4.1.1 MAR3 project overview

Manunet – MAR3 was a large European project that aimed at the bioremediation of oil spills produced during the oil refining process at the sea-platforms. Oil spills are considered a hard pollutant and its impact over the sea ecosystem is very severe (Davies et al., 2004).

An innovative process (consisting in the bio-synthesis of a flocculent or coagulant agent) trying to overcome this problem was developed. The different stages of the MAR3 project are shown in Figure 4.1.

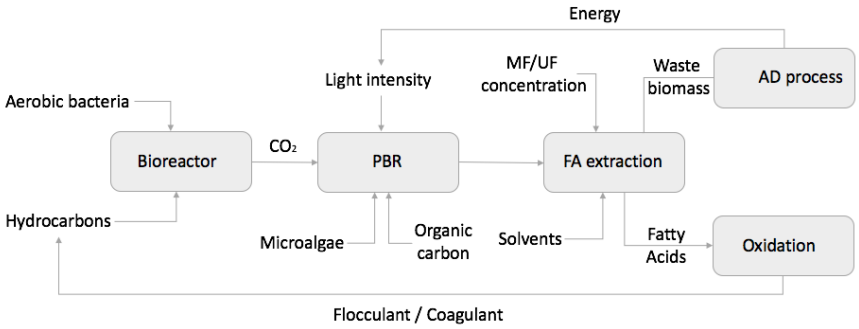

Figure 4.1. Processes involved in the bioremediation of oil spills developed during the MAR3 project

4.1.2 Biodegradation stage

The biodegradation stage consisted of the partial degradation of hydrocarbons from the wastewater produced during the oil refining process. This oxidation was conducted by an aerobic bacteria consortium in a semi-industrial scale bioreactor. The bioreactor had a wide range of design possibilities regarding materials, shape and operational conditions. The need of reaching a commercial product at the final stage of the project constrained the design of this prototype according to certain parameters such as simplicity, replicability and low-cost manufacturing. The prototype used had a capacity of 100L with a hydrocarbon alimentation rate of 10L/h. Therefore, the hydraulic residence time (HRT) used during the partial oxidation was set at 10h.

4.1.3 FA production stage

The FA production stage consisted of the cultivation of an optimal microalgae consortium with high BP and LP. In our case, we selected the microalga *Chlorella vulgaris* since it has been largely reported as a feedstock for the production of biofuels due to its potential to accumulate high lipid content (g_L/g_{DW}) under certain operational conditions (Chen et al., 2011; Markou et al., 2013; Shuba and Kifle, 2018). The PBR developed presented some improvements in comparison to the commercially available devices aiming at increasing the BP and LP of the microalga suggested. Partial of the CO_2 produced at the biodegradation stage could be used together with an organic carbon source to enhance the cultivation yield.

4.1.4 FA extraction stage

The extraction of FA from the microalgae consisted of a multiple stage process with several consecutive operations aiming at obtaining high purity on the products extracted. The first step consisted of a microfiltration followed by an ultrafiltration to concentrate the biomass produced in the PBR. Then, ultrasonication was applied with the aim to disrupt the cells and release the FA. Last stage based on a liquid-liquid extraction process with an appropriate organic solvent (i.e. n-hexane or n-toluene) was used to recover the FA released. The remaining biomass after was digested anaerobically to produce energy in view of reducing the energy consumption in the whole MAR3 project.

4.1.5 Emulsifier production stage

The emulsifier production stage consisted of the completely transformation of the FA extracted into an emulsifier gel. This product synthetized should be used to flocculate and coagulate the hydrocarbons released during the oil refining process. More details about the processes conducted (i.e. kinetic reactions suggested and emulsifier formulation) cannot be shared at this stage since this information is protected by a non-disclosure agreement signed by the author of the present thesis.

The main contribution of the author in MAR3 was the design of an optimized PBR in the second stage of the project with the aim to achieve a BP and LP using the microalga *Chlorella vulgaris*.

4.2 Objectives

The main objective of this chapter was to give a comprehensive overview on all the parameters involved in the design of a PBR used in microalgae cultivation. An exhaustive literature research was conducted aiming at providing some guidelines for the design of tailor-made PBR. This knowledge obtained was used to develop the PBR required in the second stage of the MAR3 project targeting high biomass and FA production using *Chlorella vulgaris.*

4.3 Materials and Methods

We conducted a preliminary study to understand the volumetric mass transfer coefficient, K_La, variations inside the PBR under different conditions. Some hydrodynamic parameters such as superficial gas velocity (U_G), bubble diameter (ϕ_B) and gas hold-up (ε_G) influence the mass transfer inside the PBRs.

A hydrodynamic study was conducted at different aeration flows (8, 10, 12 and 15L/min corresponding to 0.05, 0.07 0.8 and 0.1v/v) to describe its influence over the K_La. The gas hold-up (ε_G) corresponding to the volume occupied by the gas in a biphasic column can be determined as Eq. (1).

$$\varepsilon_G = \frac{H_G - H_L}{H_G} \quad (1)$$

where H_G is the height of the liquid + gas column and H_L corresponds to the height of the liquid column.

Airlift bioreactors operation consist of agitating the liquid contained in the reactor pneumatically using gas. There are two different channels within an airlift reactor, one channel for gas liquid up-flow (riser) and other channel for gas/liquid down-flow (downcomer). In our case, the PBR consisted of two concentrically disposed cylinders where inner cylinder actuated as riser whereas the external one was the downcomer channel. The liquid flew from the riser to the downcomer through small windows located at the top part of the inner cylinder.

We estimated the hold-up by observing the liquid height reached at the window for a period of constant airflow that lasted at least two minutes. This experiment was conducted by triplicate under different aeration flows. The experimental results of the hold-up reached as different aeration flows are represented in Figure 4.2. A linear relationship between both parameters ($\varepsilon_G = 4.85 \cdot U_G + 5.6 \cdot 10^{-3}$) is also shown in Figure 4.2.

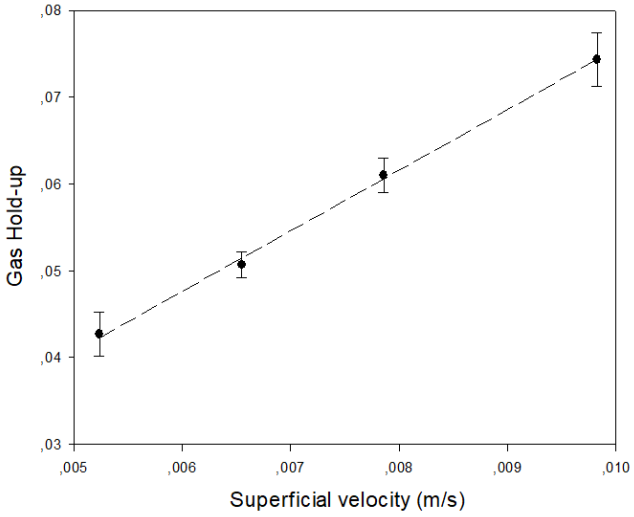

Figure 4.2. Gas hold-up (ε_G) as function of aeration flows selected in the experiment

The tests served to confirm (i) an increasing of the aeration flow over the bioreactor caused an increasing gas volume on the liquid column according to the literature consulted (Babcock et al., 2016; Huang et al., 2016; Razzak et al., 2016) and (ii) the maximum aeration flow permitted for the designed PBR was 25L/min due to higher flows caused a collapse at the inner tube (windows blocking) reducing the mixing efficiency of the system.

We also wanted to estimate K_La_{CO2} to understand and try to enhance the CO_2 transfer from the gas to the liquid phase. The K_La_{CO2} can be determined as a function of the K_La_{O2} according to Eq. 2.

$$k_L a_L(CO_2) = \sqrt{\frac{D_{O_2}}{D_{CO_2}}} K_L a_L(O_2) \quad (2)$$

where D_{O2} is the O_2 diffusivity, $2 \cdot 10^{-9} m^2/s$ and D_{CO2} is the CO_2 diffusivity at 25ºC $2.41 \cdot 10^{-9} m^2/s$. The K_La_{O2} was calculated in our case by using a perturbation experiment. Aeration was suddenly changed and the DO profile was monitored. A mass balance of the DO leads to Eq. 3.

$$ln\left(\frac{C^* - C_0}{C^* - C}\right) = k_L a(O_2) \cdot (t - t_0) \quad (3)$$

K_La values obtained at different aeration flows are represented in Figure 4.3. Flow changed from laminar to turbulent regime (Figure 4.3) at aeration flow of 0.008m/s.

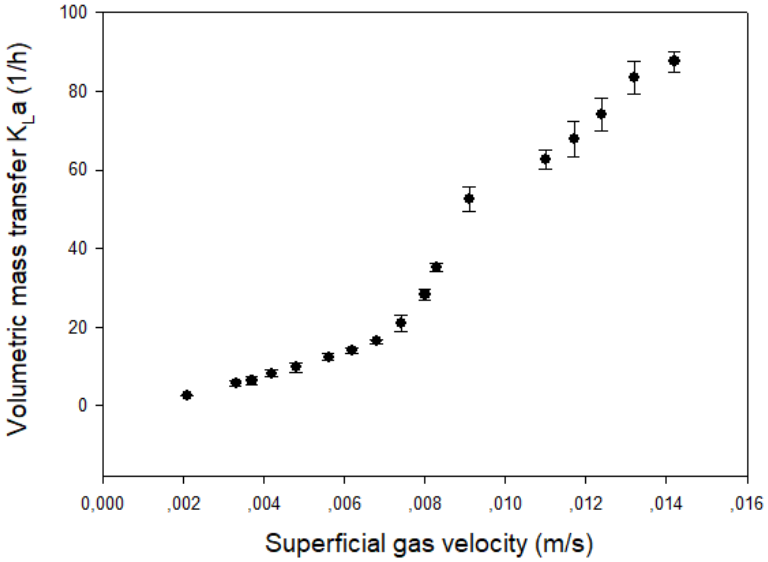

Figure 4.3. Experimental K_La determination at different aeration flows

The K_La experimental determination served to determine the maximum aeration flow permitted for the proposed PBR. According to literature consulted, high turbulence could reduce the biomass yield because of shear stress induced over the cells and, thus, it is suggested to operate the PBRs at laminar flow regime (Babcock et al., 2016; Razzak et al., 2016). Therefore, with the aim to reduce the operational costs and the shear stress on the microalga, we suggested to operate the PBR at aeration flow of 15L/min (U_G=0.067m/s) corresponding to a K_La of 16.65(1/h) according to the experimental values represented in Figure 4.3.

4.4 Results and discussion

An industrial scale PBR is considered efficient when it accomplishes the following requirements:

i. Microalga needs light intensity to conduct the photosynthesis; therefore, the PBR must be able to provide enough light to the cells.
ii. Microalga also requires macro and micronutrients to synthetize new cells, then, an efficient PBR should facilitate the necessary nutrients to be assimilated.
iii. Microalga operates in a narrow range of physico-chemical conditions and the PBR must control all the parameters affecting the microalgae metabolism i.e. pH, CO_2 and O_2 exchange between liquid-gas phase and mixing.

An efficient PBR should also requires a low investment cost due to the optimal relation between materials-durability-cost. In addition, operational costs (i.e. microalgae harvesting and CO_2 nutrients and heat costs) must be minimized as much as possible to facilitate the full-scale PBR implementation.

4.4.1 Light

Light is the main the resource required by the microalgae to conduct the photosynthesis. There are several parameters affecting the light availability during the microalgae cultivation such as: light intensity (LI), light path, light/dark (L/D) cycles or light quality. Estimating the optimal value of these parameters is the most difficult engineering challenge at the PBR designing phase.

4.4.1.1 Light intensity (LI)

The growth of microalgae and the biochemical composition of the cells is greatly dependent on the LI.

LI is normally quantified as the photosynthetic active radiation (PAR) in $\mu mol/m^2/s$. Each microalga has an optimal LI in which the biomass yield is maximized. LIs far away from the optimal could significantly affect to the microalgae metabolism. When cells are exposed for a sufficient time to high levels of light, photo-oxidation of cells is detected (Ma et al., 2017). Photo-oxidation is a metabolic mechanism involving the O_2 excitation due to the excess of energy. Then, this excited O_2 reacts with the fatty acids of the cells to form lipid peroxides which are detrimental to the cell membrane, and can even lead to the cell decay (Carvalho et al., 2011).

In contrast, when cells are exposed to low levels of LI, the biomass productivity (BP) is highly reduced due to the lack of energy to conduct the photosynthesis. This phenomenon is reported in the literature as photo-limitation (Carvalho et al., 2011; Posten, 2009). Several studies (Table 4.1.) investigated the BP of different microalga strains as function of the LI applied to the PBR.

Table 4.1. BP achieved depending on LI applied at the PBR during the cultivation of *N. salina*, *S. Obliquus* and *C. vulgaris*

Microalga strain	Parameters investigated	Experimental results					Reference
N. salina	LI (μmol/m^2/s)	50	120	150	550	1100	(Bernardi et al., 2014)
	BP (g/L/d)	0.08	0.31	0.33	0.23	0.1	
S. obliquus	LI (μmol/m^2/s)	150	650				(Barbera et al., 2015)
	BP (g/L/d)	0.37	0.81				
Chlorella vulgaris	LI (μmol/m^2/s)	30	90	150	200	300	(Pei et al., 2016)
	BP (g/L/d)	0.088	0.126	0.208	0.121	0.150	

The high biomass yield achieved in Bernardi et al. (2014) (0.33g/L/d) and Pei et al. (2016) (0.208g/L/d) was obtained at LI of 150μmol/m^2/s. Low levels of light (i.e. 30 – 50μmol/m^2/s) investigated reduced the BP in both reports showing a clear photo-limitation. Moreover, low biomass yields are observed at high levels of LI (i.e. 300 – 600μmol/m^2/s) corresponding to the photo-oxidation of microalgae cells.

4.4.1.2 Light path

Light path or light depth is referred to the nominal distance from the light source to the PBR superficial wall. It is usually measure in cm due to the distance between both elements is limited. The depth of the PBR influences the growth metabolism because of a higher light depth results in less LI available for microalgae becoming in a photo-limited culture (Reyna-Velarde et al., 2010).

Barbera et al. (2015) reported the light path influence over the BP of *Scenedesmus obliquus*. The distance between PBR and light was varied from 1 to 10cm and the high biomass yield (1.85g/L/d) was reached at the minimum light path. In contrast, Gao et al. (2017) suggested a light path of 20cm for the *Chlorella vulgaris* cultivation according to the experimental results achieved (0.26g/L/d). In general, the light path recommended varies from 1 to 20 cm for all the citations consulted. Considering this range of distances and the fact that in our case we were trying to design a semi industrial scale PBR, we decided to set a reactor depth of 12.5cm.

4.4.1.3 Light/Dark (L/D) cycle ratio

L/D cycle ratio corresponds to the ratio of the length of the period when microalga is exposed to continuous illumination (light period) versus the period under completely darkness (dark period). There are three competing metabolic processes during the photoautotrophic growth of microalgae:

i) Photosynthesis: microalgae use light to fix CO_2 and to produce O_2 as a by-product.

ii) Photorespiration in the light period: microalgae use some O_2 produced during the photosynthesis to conduct the Calvin cycle.

iii) Photorespiration in the dark period: microalgae consume O_2 and free-organic compounds to produce energy and to release CO_2

Hence, it is required to find an optimal L/D that enables the adequate performance of the three metabolic processes. Some investigations (Table 4.2.) reported the influence of L/D ratio over the biomass concentration (DW) and lipid productivity (LP) for different microalgae strains.

Table 4.2. L/D cycle ratio influence over DW and LP for the microalga *Scenedesmus sp.* and *Chlorella vulgaris*

Microalgae	**Parameter investigated**	**Experimental results**					**Reference**
Scenedesmus sp	L/D ratio (h)	0:24	8:16	12:12	16:8	24:0	(Ma et al., 2017)
	DW (g/L)	0.48	0.82	1.23	1.48	1.72	
	LP (mg/L/d)	18.42	34.65	56.82	42.13	36.26	
Chlorella vulgaris	L/D ratio (h)	12:12	16:08	24:00			(Atta et al., 2013)
	μ (1/d)	1.15	1.20	1.18			
	Lipid content (g/g)	0.18	0.21	0.19			

The final DW and LP is enhanced when the L/D ratio is increased up to 12:12h according to the results shown in Table 4.2. It has been reported that the BP and LP are decreased if the dark periods are longer than light periods (Atta et al., 2013; Blanken et al., 2016; Ma et al., 2017). Ma et al. (2017) reported the high DW at L/D of 24:0h, indicating that the cells were continuously illuminated.

In contrast, Atta et al. (2013) reported the maximum lipid content (0.21g/g) at L/D of 16:8h. Considering that the cells need a certain period of complete darkness to conduct dark respiration and that continuous illumination increases the operating costs of the system, the L/D cycle suggested for the operation of our PBR was set at 20:4h.

4.4.1.4 Light quality

Light quality is defined as the adequate energy and wavelength in which the microalgae are capable to synthetize the target organic product. Sunlight is the preferred option as the energy source for microalgae cultivation (Chisti, 2008) because:

- sunlight is free whereas artificial light sources are expensive (Chen et al., 2011).
- solar energy contains the full spectrum of light needed for microalgae cultivation, and through a specific UV filter, it can provide a suitable absorption wavelength for both microalgae cells and organic target product (Chen et al., 2011).

Although sunlight is preferred to cultivate microalgae in front of artificial light sources, there are practical cases when artificial light is required. In our case, we could not leave the reactor outdoors to use sunlight since both rainy periods and large temperature differences between day and night were detrimental for the PBR performance. Large temperature gradients between day-night resulted in the need of temperature control equipment and, thus, an exhaustive energy consumption and operational cost. Several PBRs have been investigated to improve light quality and biomass production performance using artificial light. Blažej et al. (2004) proposed a PBR that was designed by combining a light receiving equipment and a reflection sheet to transfer light in an efficient way. Some researchers also developed a PBR comprising three concentric glass cylinders with incandescent lamps placed directly into the PBR (Hu and Sato, 2017). Some other investigations using artificial light for *Chlorella vulgaris* cultivation are represented in Table 4.3. Mujtaba et al. (2012) reported the highest BP (0.145g/L/d) and LP (77mg/L/d). The BP is in the same range the reported in Pei et al. (2016) (Table 4.1.) although the LI applied in their investigation was lower (150μmol/m^2/s) than the reported in Mujtaba et al. (2012).

Table 4.3. BP and LP reported cultivating *Chlorella vulgaris* using artificial light sources

Light Supply	LI (μmol/m²/s)	BP (g/L/d)	LP (mg/L/d)	Reference
Artificial	80	0.114	20.81	(Wong, 2017)
Artificial	270	0.144	30.2	(Pruvost et al., 2011)
Artificial	---	---	27.38	(Heredia-Arroyo et al., 2011)
Artificial	200	0.145	77	(Mujtaba et al., 2012)
Artificial	150	0.104	6.91	(Khoo et al., 2016)

Some improvements were proposed in our design in terms of light quality. A focus simulating the sunlight spectrum was proposed in order to provide the optimum light spectrum to the microalgae trying to obtain better results than those reported in the table 4.3. In addition, some glass reflectors were added to the prototype system at the opposite face of the PBR to ensure the correct light distribution in the cultivation bioreactor.

4.4.2 Carbon source

4.4.2.1 Photoautotrophic cultivation

Nowadays, the most common procedure for cultivating microalgae or cyanobacteria (which is a variety of microalgae) is at photoautotrophic conditions. Photoautotrophic cultivation requires light, CO_2, nutrients (nitrogen, phosphorus and minerals) to convert CO_2 into biomass due to photosynthetic mechanisms. If the CO_2 assimilated comes from the atmosphere of from flue gas emissions, this autotrophic growth results in a reduction of the concentration of this greenhouse gas in the atmosphere (Gonçalves et al., 2016).

Gonçalves et al. (2016) reported the BPs of different microalgae strains cultivated at various levels of CO_2 concentrations. According to the values reported in Table 4.4., the optimal CO_2 concentration to grow *Chlorella vulgaris*, *M. Aeruginosa* and *Synechocystis salina* was at 7%v/v meanwhile for *P. subcapitata* the optimal CO_2 concentration was at 9%v/v. Comparing BP achieved for *Chlorella vulgaris*, Gonçalves et al. (2016) (0.164g/L/d) productivity is in the same range than in Pei et al. (2016)

(0.208g/L/d) and Mujtaba et al. (2012) (0.145g/L/d), both investigations conducted in photoautotrophic conditions.

Table 4.4. BP achieved at different CO_2 concentrations for the microalgae strains *C. vulgaris*, *P. subcapitata*, *S. salina* and *M. Aeruginosa*

Microalgae	**Parameter investigated**	**Experimental results**					**Reference**
Chlorella vulgaris	CO_2 (%v/v)	3	5	7	9	10	(Gonçalves et al., 2016)
	BP (g/L/d)	0.131	0.122	0.164	0.150	0.148	
P. subcapitata	CO_2 (%v/v)	3	5	7	9	10	(Gonçalves et al., 2016)
	BP (g/L/d)	0.118	0.098	0.100	0.129	0.0783	
S. salina	CO_2 (%v/v)	3	5	7	9	10	(Gonçalves et al., 2016)
	BP (g/L/d)	0.149	0.139	0.173	0.136	0.108	
M. Aeruginosa	CO_2 (%v/v)	3	5	7	9	10	(Gonçalves et al., 2016)
	BP (g/L/d)	0.127	0.136	0.154	0.118	0.119	
Chlorella vulgaris	CO_2 (%v/v)	2	3	6	8	10	(Hossain et al., 2018)
	BP (g/L/d)	0.087	0.081	0.078	0.053	0.049	

4.4.2.2 Heterotrophic cultivation

An alternative to photoautotrophic cultivation is heterotrophic cultivation. The main difference between both cultivation strategies is the carbon source. Photoautotrophic cultivation uses inorganic CO_2 as carbon source meanwhile in heterotrophic cultivation is used organic carbon. The most common organic carbon sources used in heterotrophic cultivations are sugars, acetate and glycerol (Liang et al., 2009; Pei et al., 2016; Shen et al., 2015).

The main advantages of photoautotrophic cultivation in front of heterotrophic cultivation are (i) the operational costs are lower since the organic carbon source is more expensive than CO_2 and (ii) the CO_2 capture reduces the global emissions of this greenhouse gas. In contrast, the BPs and LPs achieved under photoautotrophic conditions are lower than those obtained using the heterotrophic cultivation. Some

investigations (Table 4.5.) reported the BP for different microalgae strains and organic carbon sources cultivated under heterotrophic conditions.

Table 4.5. BPs for *S. quadricauda* and *C. vulgaris* cultivated under heterotrophic conditions with different organic carbon sources

Microalgae	Parameter investigated	Experimental values	Reference
Scenedemus quadricauda	Xylose (g/L)	4	(Song and Pei, 2018)
	BP (g/L/d)	0.110	
Chlorella vulgaris	Glucose (g/L)	0.935	(Babaei et al., 2018)
	BP (g/L/d)	0.05	
Chlorella vulgaris	Glycerol (g/L)	2	(Ge et al., 2018)
	BP (g/L/d)	0.08	
Chlorella vulgaris	Glucose (g/L)	10	(Sakarika and Kornaros, 2017)
	BP (g/L/d)	0.502	
Chlorella vulgaris	Acetate (g/L)	4.7	(Shen et al., 2015)
	BP (g/L/d)	0.137	

High BP was achieved in Sakarika and Kornaros, (2017); 0.502g/L/d. Among the different organic carbon sources tested to cultivate *Chlorella vulgaris* under heterotrophic conditions, glucose led to the maximum BP 0.502g/L/d.

Sakarika and Kornaros, (2017) reported more efficient BP than other organic carbon sources used such as glycerol Ge et al. (2018) and acetate Shen et al. (2015).The BP achieved in Sakarika and Kornaros, (2017) is 2.5 times higher than the maximum BP achieved in photoautotrophic cultivation 0.208g/L/d in Pei et al. (2016). Although the BP is increased under heterotrophic conditions, it should be noted than the cost of nutrients for this cultivation strategy are higher than in photoautotrophic cultivation due to the cost of glucose.

4.4.2.3 Mixotrophic cultivation

Mixotrophic cultivation is an intermediate solution between photoautotrophic and heterotrophic cultivation. Mixotrophic cultivation utilizes both carbon sources, CO_2 such as in photoautotrophic cultivation and supplementary organic carbon. This cultivation strategy contributes in reducing the CO_2 emissions and reduces the nutrients

operational costs derived from heterotrophic cultivation. Moreover, mixotrophic cultivation reduces the organic carbon source consumption. Some investigations (Table 4.6) reported the BP of *Chlorella vulgaris* cultivated under mixotrophic conditions.

Table 4.6. BP of *Chlorella* vulgaris cultivated under mixotrophic conditions

Microalgae	**Culture conditions**	**BP (g/L/d)**	**Reference**
C. vulgaris	Mixotrophic	0.027	(Lin and Wu, 2015)
C. vulgaris	Mixotrophic	0.254	(Liang et al., 2009)
C. vulgaris	Mixotrophic	0.2	(Correia et al., 2018)

Liang et al. (2009) achieved the highest BP, 0.25g/L/d, cultivating *Chlorella vulgaris* under mixotrophic conditions according to the represented values in Table 4.6. This yield was obtained using 1%w/v of glucose as organic source. This BP (0.254g/L/d) is 1.22 times higher than the reported in Pei et al. (2016), 0.208g/L/d.

Otherwise, Liang et al. (2009) BP is 1.97 times lower than that reported in Sakarika and Kornaros, (2017) 0.502g/L/d under heterotrophic conditions using 10g/L of glucose concentration as organic carbon source. Although heterotrophic cultivation seems the optimal strategy to obtain great BPs when cultivating *Chlorella* vulgaris, we selected to cultivate the microalgae under mixotrophic conditions. CO_2 is produced during the hydrocarbons oxidation in previous processes involved in the Mar3 project as is mentioned previously (See section 4.1, *bioreactor for partially degradation of hydrocarbons*) and we could use it for microalgae growth in view of a circular economy scenario.

4.4.3 Nutrients

Nutrients have an important role during the microalgae cultivation since microalgae assimilate macronutrients (mainly C, N, P, O and S) to produce new biomass. One of the main characteristics of microalga is that it can adapt its metabolism to synthetize certain compound of interest (i.e. pigments, lipids or polyhydroxyalkanoates, PHAs) depending on the nutrient availability. Takagi et al. (2006) reported the enhancement of 1.3 times the lipid content (g/g) in the microalga *Dunaliella Salina* cultivated under N-limited conditions. Panda et al. (2006) also

demonstrated the enhancement (from 0.2 to 0.38g/g) of the PHA content in the cyanobacterium *Synechocystis sp.* PCC 6803 cultivated under N and P-limitation.

Enhancing LP has been one of the main researches focuses when aiming at biodiesel production from microalgae. N-starvation in *Chlorella vulgaris* cultivation stimulates the carbon flow from protein to lipid synthesis increasing, thus, the lipid content in the cells (Paranjape et al., 2016; Scarsella et al., 2013; Takagi et al., 2006). Some investigations (Table 4.7) reporting the BP and LP enhancement in *Chlorella vulgaris* cultivated under N-limitation are represented in Table 4.7. Sakarika and Kornaros, (2017) achieved the highest BP and LP, 0.502g/L/d and 98mg/L/d, according to the results achieved in Table 4.7.

Table 4.7. BP and LP for *Chlorella vulgaris* cultured under N-limited conditions

Cultivation strategy	**Stress condition**	**BP (g/L/d)**	**LP (mg/L/d)**	**Reference**
Mixotrophic	N-limitation	0.475	75	(Scarsella et al., 2013)
Heterotrophic	N-limitation	0.225	62	(Scarsella et al., 2013)
Mixotrophic	N-limitation	0.1	37	(Paranjape et al., 2016)
Heterotrophic	N-limitation	0.137	66	(Shen et al., 2015)
Heterotrophic	N-limitation	0.502	98.93	(Sakarika and Kornaros, 2017)

The lipid content (g/g) in the *Chlorella vulgaris* cells in Sakarika and Kornaros, (2017) was 0.197g/g, which corresponded to a 20% of lipid accumulation inside the cells. In contrast, Paranjape et al. (2016) reported low BP and LP, 0.1g/L/d and 37mg/L/d, as can be noted in Table 4.7. The lipid content obtained in Paranjape et al. (2016) was 0.37g/g, which is 1.86 times that achieved in Sakarika and Kornaros, (2017), corresponding to 37% of lipid accumulation inside the cells. An enhancement of the lipid content in *Chlorella vulgaris* represents a decreasing of BP of the microalgae. Finding an optimal nutrient concentration that enhances both the biomass and lipid content is not a simple decision during the PBR operation. It is impossible to maximise both BP and LP only choosing the nutrient content of a certain feed since both use the

inlet carbon. The objective is to find certain experimental conditions where both processes are balanced with relatively high BPs and LPs.

4.4.4 Mixing

Mixing is an important feature to consider during the PBR design. An efficient mixing is necessary to accomplish the following microalga requirements:

i. Improve the CO_2 and O_2 exchange between the gas and liquid phase. It has been previously mentioned (see section 4.4.2., *Carbon source*) that the microalga requires CO_2 to conduct the photosynthesis. If the CO_2 is not efficiently transferred to the liquid phase, the metabolism of the cells is affected negatively and therefore, the BP is drastically reduced. Also, if the O_2 in not well exchanged, an O_2 combination to high levels of LI could results into photo-inhibition of the cells.

ii. Ensure that the cells have a uniform exposure to light and nutrients. When the mixing is not homogeneous, there exist a light gradient along the PBR. If cells are exposed excessively to high levels of LI they are subjected to photo-inhibition meanwhile photo-limitation occurs when the cells are exposed to low levels of LI (see section 4.4.1., *Light*). Both phenomena influence negatively to the microalga metabolism resulting in a reduction of the BP.

iii. Prevent sedimentation microalgae at the bottom zone of the PBR. Sedimentation of microalgae at the bottom zone of the PBR occurs when the mixing is insufficient. Under this situation, the cells cannot assimilate light (cells are not exposed equally to LI) and CO_2 (only superficial cells are allowed to capture CO_2) affecting negatively their BP.

iv. Facilitate heat transfer by reducing the thermal gradient along the PBR. Kinetic reactions involved in microalgae metabolism are strongly-dependent to temperature. Thermal gradients during the microalgae cultivation could affect negatively the cells metabolism reducing the BP.

Depending on the cultivation system and scale, mixing could be addressed by aeration, pumping, mechanical agitation or combining these techniques. It should be noted that not all algal strains can tolerate vigorous mixing.

Usually, mechanical agitation is discarded since it produces a hydrodynamic stress to the microalgae. This stress results in an inhibitory effect on microalgae growth and on metabolic (Sharma and Mallick, 2005; Yun and Park, 2003). In many occasions, finding a balance between the microalgae requirements and the economic aspects of the project is a major engineering challenge.

Furthermore, considering that the cells do not tolerate large turbulences and, based on the mass transfer tests performed, we suggest operating the PBR in laminar regimes.

For this purpose, we decided to set the aeration flow at 15L/min corresponding to a K_La of 16.65(1/h) which was in the same range than other volumetric mass transfer coefficients reported in the literature for tubular PBRs (Babcock et al., 2016; Razzak et al., 2016; Reyna-Velarde et al., 2010).

4.4.5 Features of the designed PBR

The guidelines for the design of a tailor-made PBR have been obtained from an exhaustive literature research. These guidelines were used for the design of PBR aiming at producing 4Kg of FA per week with *Chlorella vulgaris* under the project Manunet – MAR3.

The PBR consisted into two concentrically disposed cylinders where the inner one actuated as a riser (liquid/gas up-flow) and the outlet one was the downcomer (liquid/gas down-flow) as an airlift system. The PBR design considered the maximum distance between the light source and the PBR according to the literature consulted. The inner diameter of the PBR was fixed at 0.125m avoiding the photo-limitation occurred when the light path was up-to 0.15m. The inner cylinder had at top zone some holes (rectangular windows of 0.1m of height) allowing the liquid flow from the riser area to the downcomer zone. Figure 4.4 represents a render of the prototype designed to cultivate *Chlorella vulgaris*.

The LI and quality required for *Chlorella vulgaris* was considered also during the design phase. A sunlight simulator focus was selected as light source due to its high-quality spectrum of light.

Moreover, some glass reflectors were installed in the opposite face of the PBR with the aim to enhance the light transmission into the PBR. Figure 4.5 represents the distribution of the PBR with the glass reflectors used to increase the light transmission.

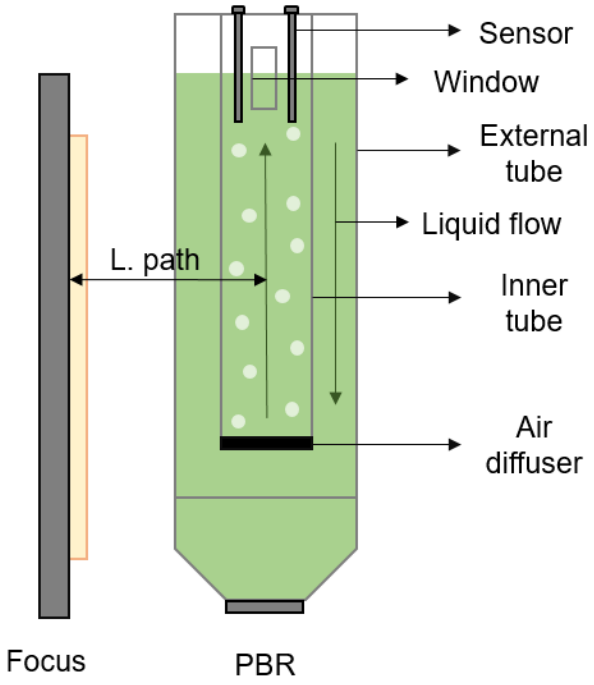

Figure 4.4. PBR features including airlift configuration (liquid/gas circulation and windows), light path and sunlight simulating focus

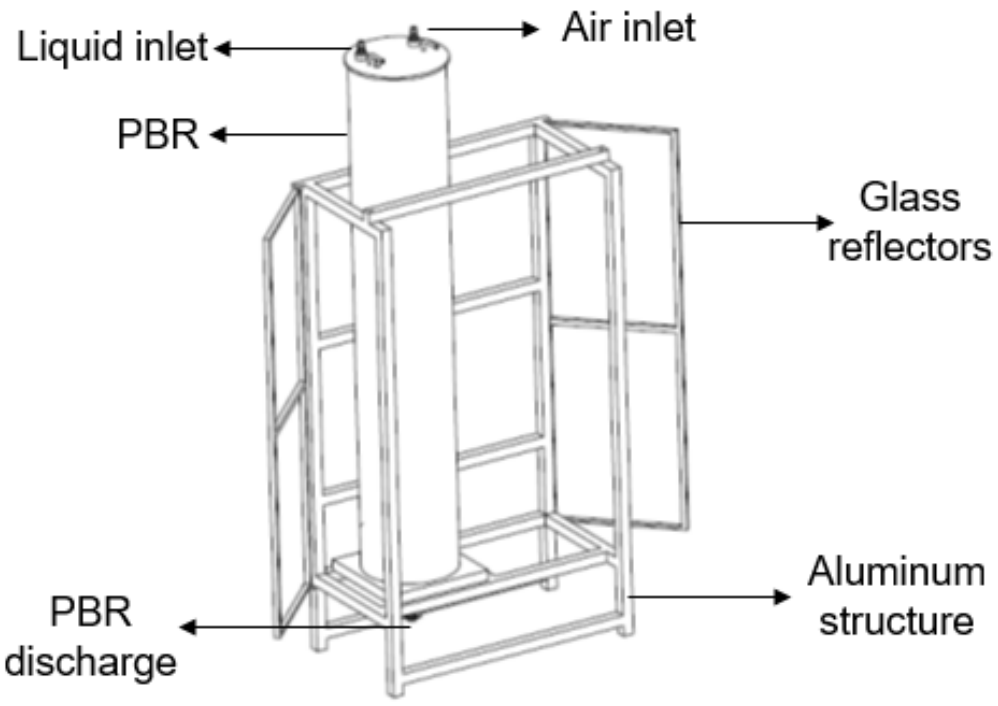

Figure 4.5. PBR features including glass reflectors, aluminium structure and PBR inlet and outlet connections

In addition, we suggested to operate the PBR prototype setting the LI at 150μmol/m^2/s with a L/D cycle of 20:4h trying to enhance the BP and LP of the microalgae in comparison to previous investigation related to the *Chlorella vulgaris* cultivation. We also recommended to cultivate the microalga under mixotrophic conditions using the CO_2 produced in the biodegradation stage of oil spills bioremediation and glucose as supplementary organic carbon according to the enhancement reported in previous investigations reported in the literature. pH, DO and light parameters (L/D cycles, LI) were recommended as the main parameters to monitor during the microalga cultivation since they have a significant influence over the microalgae *Chlorella vulgaris* BP and LP.

4.5 Conclusions

The exhaustive research conducted during this investigation permitted to obtain some knowledge about the design criteria of an efficient PBR for the microalga *Chlorella vulgaris* cultivation. There exist several parameters influencing BP and LP of the microalgae.

Light and CO_2 are the main parameters involved on the photosynthetic activity of the microalgae. An adequate design considering LI, light quality, L/D cycles and light path is required to enhance the BP production of *Chlorella vulgaris*. In accordance, cultivation strategies could enhance the BP of the microalga since it has been demonstrated during the review elaborated. Mixotrophic cultivation has reported the highest BPs and LPs but it is required to find an equilibrium between the biomass enhancement and cost associated to the cultivation.

Mixing and nutrients plays also an important role during the microalgae cultivation. An inefficient mixing combined to high levels of LI could results in a photo-inhibition of the culture. In opposite, a non-sufficient mixing in combination to low levels of LI could results in a photo-limitation of the cells. Therefore, it is required to find in each case the optimal mixing conditions permitting the microalgae cultivation avoiding possible problems related to mixing.

Nutrients deficiency can enhance the target organic product productivity as it has been described but it is impossible to enhance the biomass and organic product at the same time. Therefore, it is required to find a certain experimental condition where both processes are balanced with relatively high BPs and LPs.

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5 Kinetic model development for the *Synechocystis sp.* PCC 6803

Abstract

A multicomponent kinetic model was developed with the aim to better understand the cyanobacterium *Synechocystis sp.* PCC 6803 behaviour during its cultivation in an efficient PBR. Model described accurately the different assays conducted at the experimentation phase adjusting the main parameters involved in the cyanobacterium metabolism. The μ adjusted by the kinetic model was 0.962(1/d) and the half-saturation constants for nutrients were K_{HCO3}=1.47mg/L, K_{NO3}=31.76mg/L and K_{HPO4}=7.72mg/L. Assays by varying the initial TIP concentration were conducted and it was confirmed the reduction of cyanobacteria growth at low levels of P. Simulations changing the light/dark (L/D) cycles in the PBR were conducted with the aim to describe the kinetic model response on these scenarios. At L/D cycle of 20:4h the system achieved the pseudo-steady state at 150h while in L/D cycle of 8:16h the pseudo-steady state was never reached during the simulation.

5.1 Introduction

Unicellular algae (commonly referred as microalgae) and cyanobacteria cultivation has been extensively investigated for producing a variety of high-value products (i.e. fuel, fish and animal feed, nutraceuticals, pharmaceutical…) due to its high yield potential and carbon-neutral production (Markou and Nerantzis, 2013; Martínez et al., 2011). These oxygenic photosynthetic prokaryotes are able to assimilate inorganic carbon (CO_2) to synthetize some valuable products, which represent a tremendous impact on the global market (See chapter 1, *Organic products from microalgae*) (Hu et al., 2008; Martínez et al., 2011).

However, photobiological processes are difficult to optimize because of the complex interactions between fluid dynamics and biochemical reactions during the microalgae cultivation (See chapter 4, *Guidelines for a tailor-made PBR design: The case of Chlorella vulgaris*). Moreover, environmental factors i.e. temperature and light quality can induce a diverse range of biological responses and require complex strategies to control them at full scale cultivation (Panda et al., 2006; Prajapati et al., 2013). In addition to light and carbon, cyanobacteria need other macro and micro nutrients to conduct the cellular metabolism such as nitrogen, phosphorus, potassium and sodium. However, some researchers demonstrated the acclimation of some microalgae to environments with low availability of these nutrients (Rodionova et al., 2017; Shuba and Kifle, 2018).

For example, *Synechocystis sp.* PCC 6803 increases the production of PHB under nitrogen or phosphorus deficiency. This acclimation to adverse cultivation conditions is extensively reported in the literature (Panda et al., 2006; Panda and Mallick, 2007). The cyanobacterium *Synechocystis sp.* PCC 6803 is a promising candidate for large-scale biomass production due to its robustness to a wide range of environmental conditions such as salt concentration, pH, temperature, UV light and CO_2 concentration, and also when nutrients are depleted (Chen et al., 2011). One of the main characteristics of this cyanobacterium is its high yield of lipid production when it is grown under specific nutritional conditions in comparison to conventional routes of oil production. Hence, it could be a potentially promising way to replace other conventional forms of biofuel production (Hu et al., 2008; Jacquel et al., 2008).

The specific growth rate (μ) of this photoautotrophic organism depends mainly on light intensity (LI), temperature, pH and concentration of total inorganic carbon (TIC), nitrogen and total inorganic phosphorus (TIP) , which are defined as physical PBR parameters (Kamravamanesh et al., 2017; Reddy et al., 2003). Nutrient concentrations are controllable factors at lab scale and their supply can be well adapted to the rate of biomass synthesis. However, some improvements should be made to achieve the reproducibility of lab scale results in full scale implementation (Wang et al., 2013). The maximum μ of *Synechocystis sp.* PCC 6803 reported in the literature is in the range of 2.0-2.5(1/d), while when the cyanobacterium was grown under stress conditions, the μ is drastically reduced to 1(1/d)(Kim et al., 2011; Mendez et al., 2015).

5.1.1 Existing kinetic models describing microalgae growth

Several kinetic models have been proposed to describe the culture of *Synechocystis sp.* PCC 6803 and other cyanobacteria and microalgae. Some of the existing kinetics models proposed to describe the parameters affecting the microalgae cultivation are represented in the Table 5.1.

Table 5.1. Existing kinetic models describing microalgae and cyanobacteria growth reported in the literature (Goli et al., 2016)

Parameters	Model	Reference
TIC concentration	$\mu = \mu_{max} \frac{S_C}{K_C + S_C}$	Hsueh et al., (2007); He et al., (2012)
Nitrogen concentration	$\mu = \mu_{max} \frac{S_N}{K_N + S_N}$	Aslan and Kapdan, (2006)
TIP concentration	$\mu = \mu_{max} \frac{S_P}{K_P + S_P}$	Aslan and Kapdan, (2006)
LI	$\mu = \mu_{max} \frac{S_I}{K_I + S_I}$	Chae et al., (2006); Chojnacka and Zielińska, (2012); Sasi et al., (2011)
LI considering multiple factors	$\mu = \mu_{max} \frac{S_I}{K_I + S_I}\left(\frac{S_C}{K_C + S_C}\right) f_t$ $f_t = \frac{I_{av}}{I_s} exp\left(1 - \frac{I_{av}}{K_{SI}}\right)$	Yang, (2011)
pH	$\mu = \frac{A' e^{\left(\frac{-E}{RT}\right)}[H^+]}{[H^+] + K_{OH} + \left(\frac{[H^+]^2}{K_H}\right)}$	Mayo, (2006)
Temperature	$\mu = T\mu_T \frac{S}{(K_S + S)}$	Sterner and Grover, (1998)

Pruvost et al. (2011) proposed the kinetics of nutrient uptake (P and CO_2) and cell growth, considering a nonlinear model to assess the optimal concentration of TIC, TIP and LI for CO_2 uptake and microalgae growth. Hsueh et al., (2007); He et al., (2012) described the influence of TIC over μ where K_C (mgC/L) corresponded to the half-saturation constant for carbon and S_C (mgC/L) was the carbon available.

Aslan and Kapdan, (2006) described the influence of phosphorus and nitrogen over μ. K_P and K_N (mgN or P/L) were the TIP and ammonium semi-saturation constants while S_N and S_P (mgN or P/L) corresponded to the available TIP and ammonium.

Chae et al., (2006); Chojnacka and Zielińska, (2012); Sasi et al., (2011) described the influence of LI over μ according to a Monod-based equation where K_I (μmol/m^2/s) corresponded to the half-saturation constant for LI and S_I (μmol/m^2/s) was the LI available for the microalgae culture. Yang, (2011) described the influence of TIC and LI over μ. Saturation LI I_S (μmol/m^2/s) was the LI at which the microorganism suffers photo-oxidation. It also considered the available light for the microorganism as I_{AV} (μmol/m^2/s). Mayo, (2006) described the influence of pH over μ according to water ionization, K_H and K_{OH} (mmol/L) reaction represented in Table 5.1 Finally, Sterner and Grover, (1998) described the influence of temperature over μ where K_s (K) corresponded to the half-saturation constant for temperature and S (K) was the environmental temperature in which the microalgae was cultivated.

There are more specific kinetic models aiming to describe the cyanobacterium *Synechocystis sp.* PCC behaviour during its cultivation. Among them, Kim et al., (2015) proposed a kinetic model for *Synechocystis sp.* PCC 6803 to describe kinetic parameters without considering TIC speciation and LI influence over the cyanobacterium growth. More recently, Carpine et al. (2018) proposed a model to predict the cyanobacterial growth and PHB production for *Synechocystis sp.* PCC 6803 without determining the effect of pH on TIC and TIP speciation, which it has been demonstrated that influence over the cyanobacterium metabolism.

This chapter proposed a new dynamic model to describe this cyanobacterium cultivation under different light/dark (L/D) cycles and considering nutrients speciation, concretely TIC and TIP as a function of pH trying to improve previous cyanobacterium behaviour reported in the literature. The model developed was also used to describe the experimental results obtained during the assays conducted under the different operating conditions proposed in our investigation. The tests were conducted using PBRs subjected to different (L/D) cycles and using an specific culture medium similar to the BG-11 medium defined by Stanier et al., (1971) but with different initial concentrations of TIP.

5.2 Materials and Methods

5.2.1 Experimental set-up for *Synechocystis sp.* PCC 6803 cultivation

The experimental devices (4 lab-scale PBRs) used to cultivate the cyanobacterium *Synechocystis sp*. PCC 6803 during the experimental stage of this investigation are fully described in chapter 3 (*PBR for Synechocystis sp. PCC 6803 cultivation*).

5.2.2 PBR sanitisation, inoculation and cyanobacterium acclimation

The PBR sanitisation, culture medium preparation, cyanobacterium inoculation and acclimation were conducted according to the procedures described in chapter 3 (*PBR sanitisation, inoculation and cyanobacterium acclimation*).

5.2.3 Correlation of dry weight (DW) and turbidity

Turbidity and dry weight (DW) were determined in several samples to obtain a direct correlation between them allowing the DW determination based on turbidity measurements. Turbidity was measured by UV – Vis spectrophotometer (PerkinElmer, UV-Vis Lambda 1050). DW was measured according the procedure detailed in chapter 3 (*Total solids and volatile solids determination*).

A sample containing only BG-11 medium was used as a blank. The absorbance measured in the blank test was subtracted from each one of the turbidity measurements. All DW determinations were conducted by triplicate. The mean values obtained in these calculations are represented in Table 5.2. The experimental values and linear correlation are shown in Figure 5.1.

Table 5.2. Experimental turbidity and DW measurements obtained for their relationship determination

Sample	NTU	DW (g/L)
1	221.0±7.3	0.564
2	83.0±5.7	0.252
3	26.5±5.2	0.109
4	14.6±3.1	0.044
5	2.6±1.2	0.004

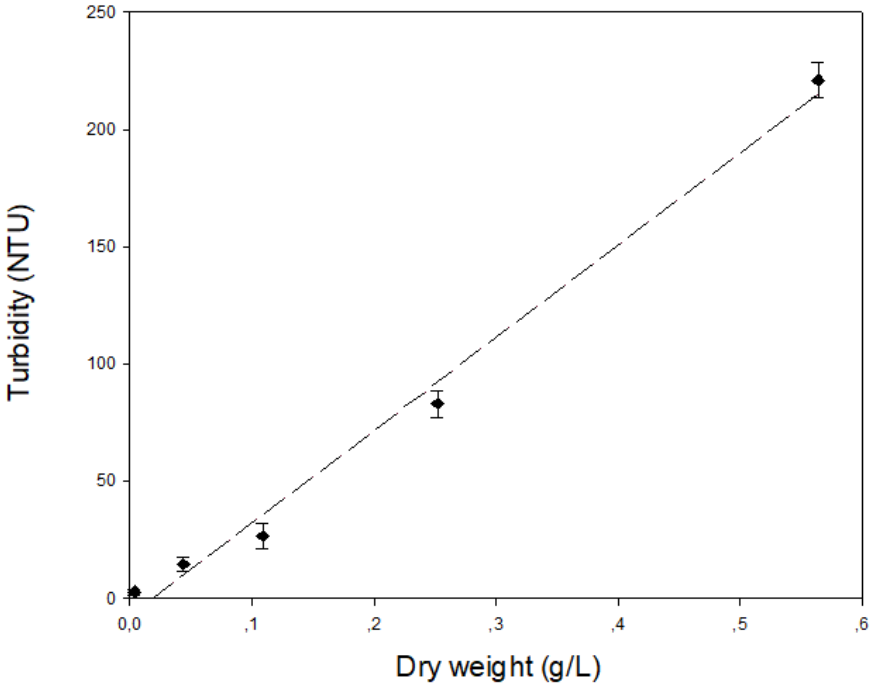

Figure 5.1. Linear correlation between turbidity and DW (T=395.23·DW – 7.38, R^2 = 0.991)

The proposed method to determine DW as a function of turbidity was validated with additional samples extracted from the bulk liquid during cultivation tests. The results obtained are represented in Table 5.3. The method was considered acceptable as the largest deviation between experimental and determined DW observed during method validation was 2.31%, which was lower than the maximum acceptable deviation of 5%.

Table 5.3. Validation of the method for DW determination based on turbidity measurements. DW_{CAL} was the calculated DW and DW_{EXP} the experimental DW

	NTU	**DW_{CAL} (g/L)**	**DW_{EXP} (g/L)**	**Error (%)**
PBR_1	42.4 ± 8.7	0.110	0.112	1.78
PBR_2	35.6 ± 9.2	0.093	0.094	1.06
PBR_3	74.7 ± 8.6	0.194	0.198	2.02
PBR_4	81.0 ± 7.4	0.211	0.216	2.31

5.2.4 Volumetric mass transfer (K_La) determination

A preliminary hydrodynamic study was made in view of determining the mass transfer efficiency of the PBR according to the procedures described in Chapter 4 (*Volumetric mass transfer determination*). The relation between the superficial gas (U_G) velocity and the volumetric mass transfer (K_La) is represented in Figure 5.2.

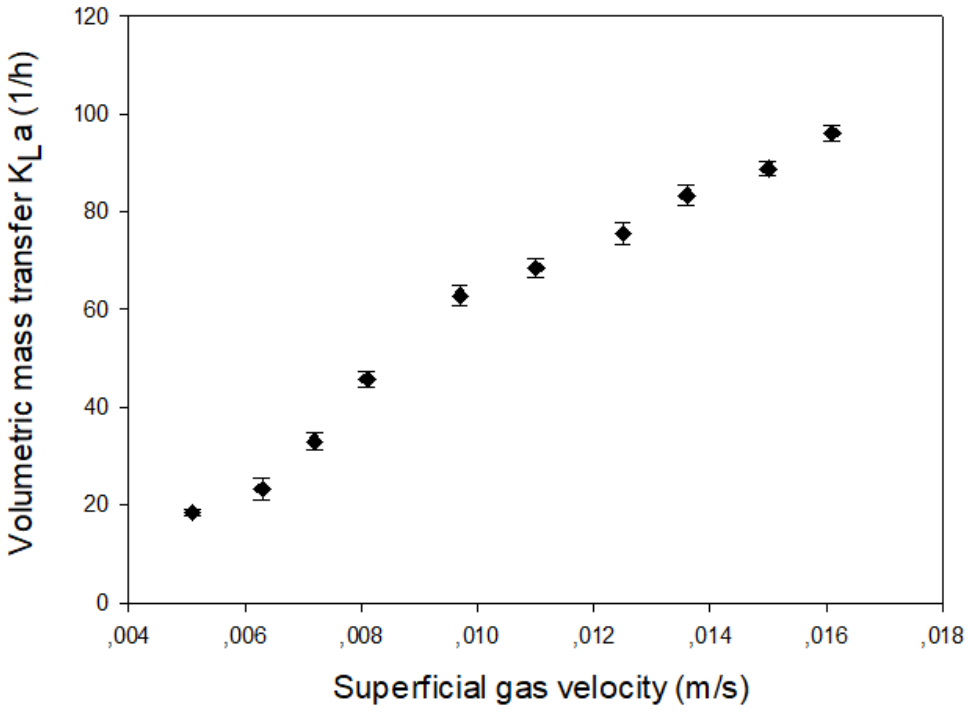

Figure 5.2. Correlation between U_G and K_La. Error bars indicates standard deviation of experimental values

A turbulent regime applied to the culture can results in a reduction of the biomass productivity (BP) of the system due to the share-stress induced over the microorganism. It was decided to operate the PBR under laminar regime in order to reduce the energy consumption of the system and, thus, the operational cost of *Synechocystis sp*. PCC 6803 cultivation. According to that, the aeration flow was set at 40L/h, corresponding to a U_G of 0.0075m/s for all the assays conducted at the experimental phase. The resulting volumetric mass transfer determined from experimental assays was K_La = 26.65(1/h) according to the Figure 5.2.

5.2.5 Analytical determinations

The μ and biomass productivity (BP) of the *Synechocystis sp.* PCC 6803 were determined according to the procedures described in Chapter 3 (*Optical density measurement and specific growth rate (μ) determination and Dry weight (DW) and Biomass productivity (BP) determination*). pH, TIN and TIP were also determined according to the procedures described in Chapter 3 (*Total inorganic nitrogen (TIN) and total inorganic phosphorus (TIP) determinations*).

5.2.6 Experimental assays description

5.2.6.1 First experimental assays

Initial batch tests were conducted with the aim to determine the efficiency of the PBR designed for the purpose of cultivating *Synechocystis sp.* PCC 6803. The LI was set at 50μmol/m^2/s with an L/D cycle ratio at 12:12h. Continuous aeration flow was set at 40L/h corresponding to 10L/h for each PBR (4 lab-scale PBRs were run at the same time). pH was continuously monitored and controlled by pure CO_2 supplementation according to the fixed pH set-points (7.5-9.5). The PBRs were submerged in a water bath to maintain their temperature controlled throughout the experiment. The temperature was measured twice a day and part of the liquid was daily refreshed to maintain the proper temperature at 28ºC, which is the recommended temperature in the *Synechocystis sp.* PCC 6803 cultivation (Kim et al., 2015; Panda et al., 2006). Samples were daily extracted and turbidity was measured and DW was estimated following the procedure described in section 5.2.3. for each sample aiming to monitor μ and DW.

5.2.6.2 Second experimental assays

The second set of batch experiments was conducted with the aim to calibrate the parameters of the proposed kinetic model. Also, after the evaluation of the results obtained in first experimental assays, we tried to increase μ and BP by changing LI applied to the PBR and the L/D cycles. The LI was set at 100μmol/m^2/s by adding linear fluorescent illuminating the PBRs with a different light spectrum than LEDs and L/D cycle was set at 24:0h reducing the dark period tested in the first tests aiming to the reduction of biomass loses due to dark respiration. pH was continuously monitored and controlled according to the same procedure conducted in previous assays. Temperature control and samples extraction were conducted according to the same procedure described in section 5.2.6.1.

5.2.6.3 Synechocystis sp. PCC cultivation under P-deficiency

Additional batch trials were conducted by changing the TIP concentration at the beginning of the tests to investigate its influence on the μ and DW. The initial TIP concentrations were set at 4, 8 and 15mg/L (15mg/L is the reported concentration of TIP in BG-11 medium). LI, L/D cycles applied to the PBR and temperature conditions were maintained as detailed in section 5.2.6.1. pH was not controlled with the aim to describe the pH influence over the μ and BP during the assays conducted.

5.2.6.4 Parameters estimation

In this work, a kinetic model was developed to describe the growth of *Synechocystis sp.* PCC 6803 in order to build a reliable tool that can be used to optimise the operation/design of PBRs. The kinetic expressions describing the behaviour of *Synechocystis sp.* PCC 6803 were implemented in MATLAB (R2018b) and solved using the *ode45* function, which uses a 4-5 order Runge-Kutta method with adjustable integration step time. Model parameters were calibrated using the *fminsearch* which minimizes an objective function. In our case, this objective function which was minimized was the sum of squared errors between predicted and experimental data.

5.3 Results and discussion

5.3.1 Kinetic model development

The kinetic model aimed to describe the influence of several physical and physicochemical factors on the μ and BP of *Synechocystis sp.* PCC 6803. The kinetic model for cyanobacteria growth was developed using Monod-type terms. The nutrients speciation was considered according to pH in order to obtain more reliable results. The main species considered were: nitrogen as nitrate, TIP, composed by PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$ and H_3PO_4, where the main species consumed was HPO_4^{2-}), TIC, composed by CO_2, HCO_3^- and CO_3^{2-}, where the main specie consumed was HCO_3^-).

This parameters selection was based on previous investigations on cyanobacteria growth (Heo et al., 2018; Kim et al., 2011; Panda et al., 2006). The expressions used to determine the nutrients speciation are represented in Table 5.4. The pKa_1 and pKa_2 values for inorganic carbon ions were 6.35, 10.35, while pKa_0, pKa_1 and pKa_2 for inorganic phosphorus ions were 2.3, 7.3 and 12.3 respectively. The molar

fractions (f_i) of each inorganic ion were estimated following the equations reported by Marcelino et al. (2009).

Acid phosphoric (H_3PO_4) was not considered in our case because the pH in the PBR never reached values below 2.3, in which H_3PO_4 is significant according to the acid-base equilibria.

Table 5.4. Determination of speciation, acid-base equilibrium and molar fraction for nutrients involved in *Synechocystis sp.* PCC 6803 growth

Parameter	Equation	
TIC speciation	$\mathrm{TIC} = [\mathrm{CO_2}] + [\mathrm{HCO_3^-}] + [\mathrm{CO_3^{2-}}]$	(1)
TIC acid-base equilibria	$\mathrm{CO_{2(l)}} \rightarrow \mathrm{H_2CO_3} \overset{\mathrm{pKa_1}}{\Longleftrightarrow} \mathrm{HCO_3^-} + \mathrm{H^+}$	(2)
	$\mathrm{HCO_3^-} \overset{\mathrm{pKa_2}}{\Longleftrightarrow} \mathrm{CO_3^{2-}} + \mathrm{H^+}$	(3)
TIC molar fraction (Marcelino et al., 2009)	$\mathrm{f_{CO2}} = \frac{10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})}}{1 + 10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})} + 10^{(\mathrm{pKa_2}-\mathrm{pH})}}$	(4)
	$\mathrm{f_{HCO_3^-}} = \frac{10^{(\mathrm{pKa_2}-\mathrm{pH})}}{1 + 10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})} + 10^{(\mathrm{pKa_2}-\mathrm{pH})}}$	(5)
	$\mathrm{f_{CO_3^{2-}}} = \frac{1}{1 + 10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})} + 10^{(\mathrm{pKa_2}-\mathrm{pH})}}$	(6)
TIP speciation	$\mathrm{TIP} = [\mathrm{H_2PO_4^-}] + [\mathrm{HPO_4^{2-}}] + [\mathrm{PO_4^{3-}}]$	(7)
TIP acid-base equilibria	$H_3PO_4 \overset{pKa_0}{\Longleftrightarrow} H_2PO_4^- + H^+$	(8)
	$H_2PO_4^- \overset{pKa_1}{\Longleftrightarrow} HPO_4^{2-} + H^+$	(9)
	$HPO_4^{2-} \overset{pKa_2}{\Longleftrightarrow} PO_4^{3-} + H^+$	(10)
TIP molar fraction (Marcelino et al., 2009)	$\mathrm{f_{H_2PO_4^-}} = \frac{10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})}}{1 + 10^{(\mathrm{pKa_0}+\mathrm{pKa_1}+\mathrm{pKa_2}-3\mathrm{pH})} + 10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})} + 10^{(\mathrm{pKa_2}-\mathrm{pH})}}$	(11)
	$\mathrm{f_{HPO_4^{2-}}} = \frac{10^{(\mathrm{pKa_2}-\mathrm{pH})}}{1 + 10^{(\mathrm{pKa_0}+\mathrm{pKa_1}+\mathrm{pKa_2}-3\mathrm{pH})} + 10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})} + 10^{(\mathrm{pKa_2}-\mathrm{pH})}}$	(12)
	$\mathrm{f_{PO_4^{3-}}} = \frac{1}{1 + 10^{(\mathrm{pKa_0}+\mathrm{pKa_1}+\mathrm{pKa_2}-3\mathrm{pH})} + 10^{(\mathrm{pKa_1}+\mathrm{pKa_2}-2\mathrm{pH})} + 10^{(\mathrm{pKa_2}-\mathrm{pH})}}$	(13)

The μ of *Synechocystis sp.* PCC 6803 considering the influence of nutrients speciation and LI is represented in Table 5.5. μ in Eq. (11) was estimated by incorporating Eq. (15-18) to Eq. (14).

Table 5.5. μ as a function of nutrients and LI influence to the cyanobacterium growth

Parameter	Representing equation	
μ	$\mu = \mu_{max} \cdot M_C\, M_N\, M_P\, M_I$	(14)
TIC influence	$M_C = \frac{HCO_3^-}{K_{HCO_3^-} + HCO_3^-}$	(15)
Nitrogen influence	$M_N = \frac{NO_3^-}{K_{NO_3^-} + NO_3^-}$	(16)
TIP influence	$M_P = \frac{HPO_4^{2-}}{K_{HPO_4^{2-}} + HPO_4^{2-}}$	(17)
LI influence	$M_I = \frac{I_{AV}}{K_I + I_{AV}}$	(18)

I_{AV} is the available LI assimilated by the microorganisms in μmol/m^2/s and K_I is the half saturation constant of light intensity. I_{AV} can be described by the Beer-Lambert law for light distribution Eq. (19).

$$I_{AV} = \frac{I_0}{A \cdot X} \cdot (1 - e^{-A \cdot X}) \quad (19)$$

where I_0 is the incident light in μmol/m^2/s and A is the multiplication of the extinction coefficient by the PBR depth. Factor A was considered 14.7m^3/kg as a typical value for vertical flat-plate PBR reported in the literature (He et al., 2012).

The overall rate expressions describing the *Synechocystis sp.* PCC 6803 metabolism are summarized in Table 5.6. The μ was corrected considering the lag-phase detected during the cyanobacteria cultivation, by using Eq. (20), where τ was one of the parameters of the kinetic model estimated with the experimental data. This equation describes the typical response of a first order system to a step change in the input, and was found useful to describe the observed lag-phase during the cyanobacterium cultivation in our PBRs

Table 5.6. Overall rate expressions for lag-phase, biomass, nutrients and pH used to describe the cyanobacteria growth. Eq. (20-30)

Parameter	**Kinetic rate expressions**	
Lag-phase	$\mu = \mu \cdot (1 - e^{\left(\frac{t}{\tau}\right)})$	(20)
Biomass	$\frac{dX}{dt} = (\mu - k_d) \cdot X$	(21)
CO_2	$\frac{dCO_2}{dt} = k_L a \cdot (CO_{2x} - CO_2) + \frac{Q \cdot (CO_{2E} - CO_2)}{V} - \frac{\mu \cdot X}{Y_{X/C}} - r_{C1}$	(22)
HCO_3^-	$\frac{dHCO_3^-}{dt} = r_{c1} - r_{c2}$	(23)
CO_3^{2-}	$\frac{dCO_3^{2-}}{dt} = r_{c2}$	(24)
O_2	$\frac{dO_2}{dt} = k_L a \cdot (O_{2x} - O_2) + \frac{Q \cdot (O_{2E} - O_2)}{V} - \frac{\mu \cdot X}{Y_{X/O}}$	(25)
$H_2PO_4^-$	$\frac{dH_2PO_4^-}{dt} = -r_{p1}$	(26)
HPO_4^{2-}	$\frac{dHPO_4^{2-}}{dt} = r_{p1} - r_{p2} - \frac{\mu \cdot X}{Y_{P/X}}$	(27)
PO_4^{3-}	$\frac{dPO_4^{3-}}{dt} = r_{p2}$	(28)
NO_3^-	$\frac{dNO_3^-}{dt} = -\frac{\mu \cdot X}{Y_{N/X}}$	(29)
pH	$\frac{dH^+}{dt} = r_{c1} + r_{c2} + r_{p1} + r_{p2} - \frac{\mu \cdot X}{Y_{H/X}}$	(30)

r_{C1} and r_{C2} are the dissociation rates of CO_2 into bicarbonate and bicarbonate into carbonate respectively. r_{P1} and r_{P2} are the dissociation rates of dihydrogenphosphate into hydrogenphosphate and hydrogenphosphate into phosphate respectively. These kinetic reactions are represented in the Table 5.7.

Table 5.7. Kinetics for TIC and TIP equilibria depending on the culture media

Parameter	Kinetics	
HCO_3^- production	$r_{C1} = K_{C1} \cdot (CO_2 - 10^{(pKa1-pH)} \cdot HCO_3^-)$	(31)
CO_3^{2-} production	$r_{C2} = K_{C2} \cdot (HCO_3^- - 10^{(pKa2-pH)} \cdot CO_3^{2-})$	(32)
HPO_4^{2-} production	$r_{P1} = K_{P1} \cdot (H_2PO_4^- - 10^{(pKa3-pH)} \cdot HPO_4^{2-})$	(33)
PO_4^{3-} production	$r_{P2} = K_{P2} \cdot (HPO_4^{2-} - 10^{(pKa4-pH)} \cdot PO_4^{3-})$	(34)

Where K_{C1}, K_{C2}, K_{P1} and K_{P2} were considered 10^7 (fast kinetics for acid-base equilibrium). This assumption was selected aiming to set the immediate ionization of nutrients dissolved in the liquid phase depending on the culture pH.

The stoichiometry of *Synechocystis sp.* PCC 6803 growth, Eq. (35), was considered according to the determination conducted in Kim et al., (2011).

$$0.1681CO_2 + 0.0362NO_3^- + 0.0019HPO_4^{2-} + 0.0401H_2 + 0.1152H_2O \rightarrow 0.1681CH_{1.62}O_{0.4}N_{0.22}P_{0.01} + 0.25O_2 \quad (35)$$

The values of all the physical and nutritional parameters involved in the kinetic model describing *Synechocystis sp.* PCC 6803 behaviour during the cultivation are shown in Table 5.8.

Table 5.8. Parameters used to describe the *Synechocystis sp.* PCC 6803 behaviour during its cultivation

Parameter	Value	Reference
V (L)	4	This work
Q (L/h)	24	This work
LI ($\mu mol/m^2/s$)	100	This work
K_La (1/h)	26.65	This work
A extinction coefficient (m^3/kg)	14.7	(He et al., 2012)
Carbon		
pKa_1	6.35	
pKa_2	10.35	
Kc_1	$1 \cdot 10^7$	This work
Kc_2	$1 \cdot 10^7$	This work
CO_{2x} (mg/L)	13.5	(Eze et al., 2018)
CO_{2E} (mg/L)	15	This work
$Y_{X/C}$ (g/g)	1.94	(Kim et al., 2011)
f_{CO2}	Depending on pH (Eq. 4)	This work
f_{HCO3-}	Depending on pH	This work
f_{CO32-}	Depending on pH	This work
K_c (g/g)	1.476	This work
Phosphorus		
pKa_3	7.3	
pKa_4	12.3	
Kp_1	$1 \cdot 10^7$	This work
Kp_2	$1 \cdot 10^7$	This work
$Y_{X/P}$ (g/g)	66.67	(Kim et al., 2011)
$_{fH2PO4-}$	Depending on pH	This work
f_{HPO42-}	Depending on pH	This work
f_{PO43-}	Depending on pH	This work
K_p (mg/L)	7.72	This work
Nitrate		
K_N (mg/L)	31.7	This work
$Y_{N/X}$ (g/g)	7.75	(Kim et al., 2011)
Biomass		
μ (1/d)	0.962	This work

5.3.2 First experimental assays

The results obtained at the first experimental assays of *Synechocystis sp.* cultivation are represented in Figure 5.3. These results are the mean of the four experimental PBRs operated in parallel.

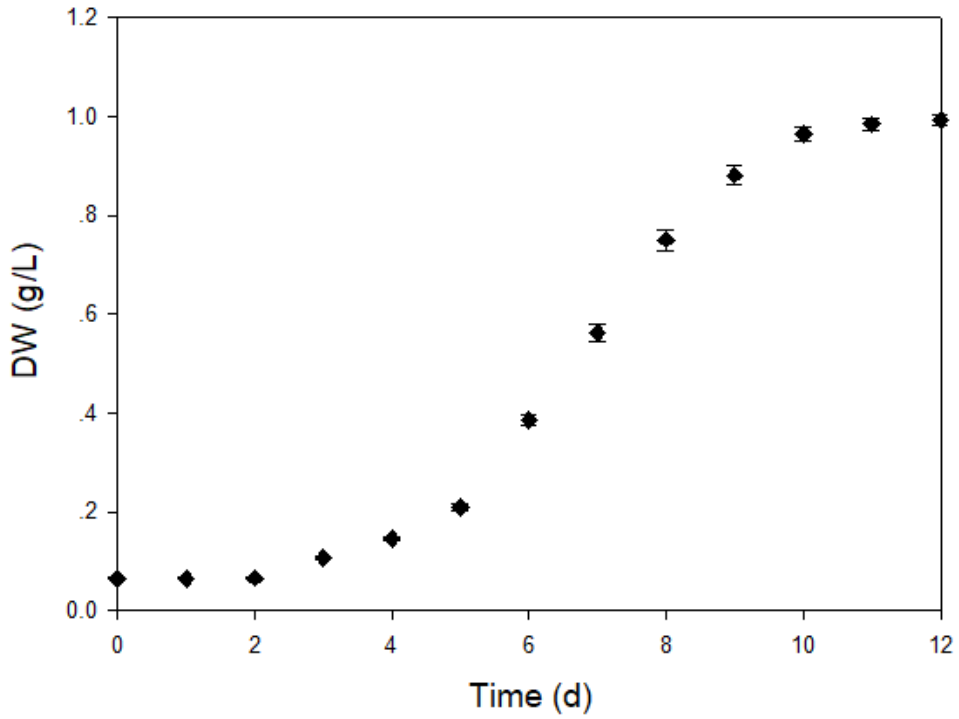

Figure 5.3. DW profile at the first experimental assay

The μ and BP for these experiments were 0.227d^{-1} and 0.077g/L/d, respectively. A BP of 0.077g/L/d is comparable to other microalgae strains reported in the literature by Carvalho and Meireles, (2006)(Table 5.9), although it was in the low range of these values.

Our BP was similar to that obtained with the microalga *Haematococcus pluvialis*, which was obtained 0.05g/L/d. The maximum BP reported for *Synechocystis sp.*PCC 6803 is 0.11g/L/d (Kim et al., 2011), which was 1.42 times higher than the obtained in our investigation. LI and L/D cycles applied in the PBR were increased, from 50 to 100μmol/m^2/s and from 12:12 to 24:0h respectively, in the second experimental assays aiming to increase the BP of the cyanobacterium *Synechocystis sp.* PCC 6803 and obtain a similar BP than the reported in Kim et al. (2011).

Table 5.9.Microalgal BP in different systems (Carvalho and Meireles, 2006)

Production system	**Microalgal strain**	**BP (g/L/d)**
Tubular bioreactor	*S.platensis*	3-17.8
Tubular bioreactor	*P. cmentum*	1.5
Tubular bioreactor	*P. tricornotum*	1.2
Bubble column PBR	*H. pluvialis*	0.05
Tubular PBR	*H. pluvialis*	0.06
Tubular PBR	*S. platensis*	0.41
Tubular PBR	*Arthrospira sp.*	1.15
Flat plate PBR	*Nannochloropsis sp.*	0.27
Flat plate PBR	*Chlorella sp.*	3.8
Flat plate PBR	*Chlorella sp.*	3.2
Column bioreactor	*Tetraselmis sp.*	0.42
Dome PBR	*Chlorococcum sp.*	0.1
Open pond reactors	*S. platensis*	0.18-0.32

5.3.3 Second set of experimental assays

A second set of tests changing the LI applied to the PBR (LI was set at 100µmol/m^2/s in comparison to 50 µmol/m^2/s used in the initial set of experimental assays) and L/D cycle (a reduction of dark period by modifying the L/D cycle from 12:12 to 24:0h) was proposed in view of increasing the µ and BP obtained during the first set of experimental assays. It was expected to obtain a BP according to previous investigations conducted with the same cyanobacterium reported in the literature. The DW profile obtained for these tests is represented in the Figure 5.4.

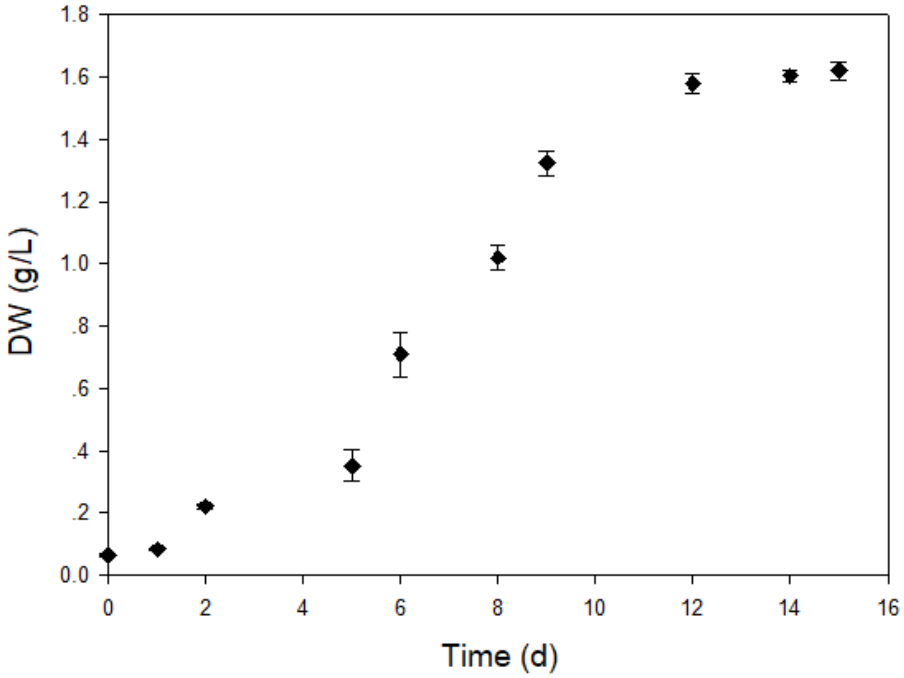

Figure 5.4. DW profile at the second set of experimental assays

The cyanobacteria growth is usually divided into four phases. These phases are: 1) lag-phase: cyanobacterium is self-acclimated to the culture media and PBR, 2) exponential phase: cyanobacterium has a quick-response to nutrients achieving the highest μ, 3) linear phase: the nutrients start to be limited and the growth of the cyanobacterium is proportional to the nutrients concentration and 4) deceleration phase: limitation of cyanobacterium growth due to the complete depletion of nutrients.

The μ and BP measured during each phase of the second set of assays are presented in Table 5.10. The mean μ achieved in these assays was 0.291(1/d). μ_{MAX} was obtained during the exponential growth of the *Synechocystis sp.* PCC 6803, 0.94(1/d). Kwon et al. (2012) reported a μ of 1.11(1/d) for *Synechocystis sp.* wild type using a flat plate PBR, although they operated the PBR 2 times more LI than the applied in our work. Kim et al. (2011) reported with the same cyanobacterium grown under non-limiting conditions a μ of 1.7(1/d) during the exponential growth phase using also a flat-plate PBR. According to these investigations, the μ obtained in our investigation was in the same range than the reported in previous investigations using the cyanobacterium *Synechocystis sp*. PCC 6803.

Table 5.10. μ and BP during the second set of experimental assays

Growth phase	Exp. Time (d)	μ (1/d)	μ_{MAX} (1/d)	BP (gDW/L/d)
Lag-phase	0 – 1	0.12		0.01
Exponential phase	1 – 6	0.59	0.94	0.18
Linear phase	6 – 12	0.16		0.13
Deceleration phase	12 - 15	0.02		0.07
Mean value		**0.291**		**0.131**

The mean BP obtained in our investigation was 0.131(g/L/d). Other works have reported the BP for other microalgae strains, obtaining values between 0.06 and 0.23 (g/L/d) as shown in Table 5.11.

Table 5.11. BP reported in the literature for different microalgae strains

Microalgae strain	BP (g/L/d)	Reference
Chlorella sp.	0.23	Rodolfi et al. (2009)
C. vulgaris	0.10	Liang et al. (2009)
D. tertiolecta	0.12	Gouveia and Oliveira, (2009)
Nannochloropsis sp.	0.17	Rodolfi et al. (2009)
S. obliquus	0.06	Maness et al. (2009)
Synechocystis sp. PCC 6803	0.131	This work

The BP of *Synechocystis sp.* PCC 6803 obtained in our work was in the same range than the reported in the literature for other microalgae strains according to the BPs represented in Table 5.11. Only the microalgae *Chlorella sp.* which it has been several times reported as the microalgae with the higher BP potential reported a BP 2 times higher than the obtained in our investigation.

5.3.4 Model fit

The second set of experimental assays were used to calibrate the parameters involved in the developed kinetic model. The values selected were μ_{MAX}, K_c, K_P and K_N, corresponding to the μ of *Synechocystis sp.* PCC 6803 and the half-saturation constants of nutrients.

The model prediction with the fitted parameters is presented in Figure 5.5 and compared to the experimental data. The model provided a good description of the experimental results during the experiment according to the DW profile displayed in Figure 5.5. The kinetic parameters fitted by the model, compared to the initial parameters used for the optimization, are presented in Table 5.12.

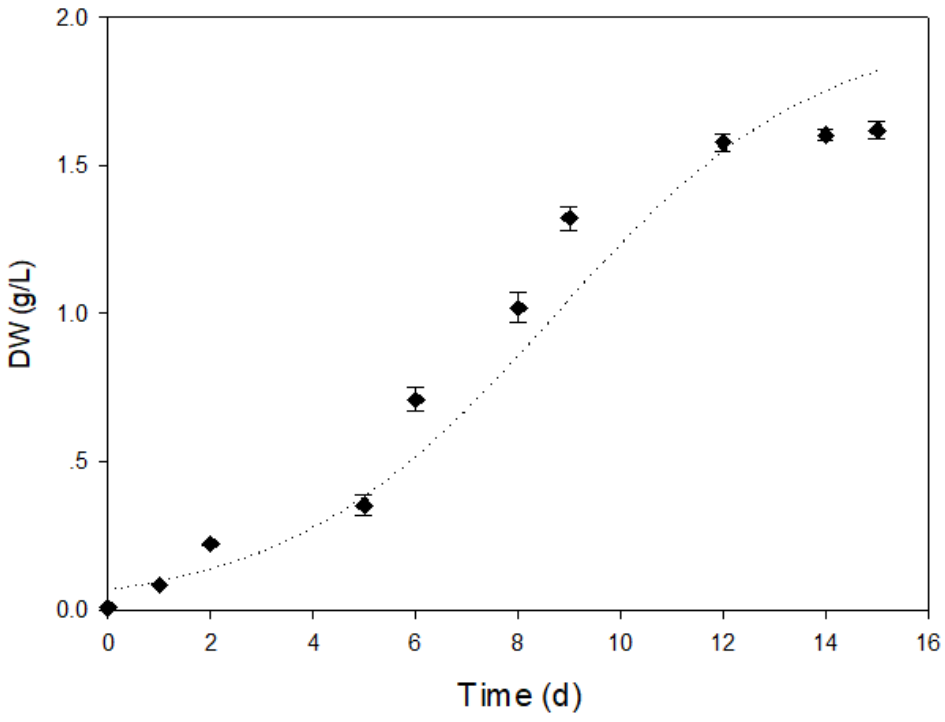

Figure 5.5. DW fitted using the kinetic model development, (♦) experimental and (-) theoretical. Error bars indicate the experimental deviation of DW

Table 5.12. Initial and fitted values for the kinetic parameters of the developed model

Kinetic Parameter	Initial value	Fitted value
μ (1/d)	0.94	0.96
K_{HCO3} (mg/L)	1.56	1.48
K_{HPO4} (mg/L)	10.32	7.22
K_{NO3} (mg/L)	27.72	31.76

The half-saturation constants estimated are in the range of other semi-saturation constants reported in the literature for different microalgae strains (Table 5.13).

Table 5.13. Nutrient half-saturation constants for different microalgae strains reported in the literature

Microalgae strain	Target nutrient	K (mg/L)	Reference
Desmodesmus sp	HCO_3^-	124.9	Eze et al., (2018)
Synechocystis sp. PCC 6803		1.476	This work
Chlorella vulgaris	NO_3^-	32	Aslan and Kapdan, (2006)
Nannochloropsis sp		30.0	Heo et al., (2018)
Synechocystis sp. PCC 6803		31.76	This work
Chlorella vulgaris	HPO_4^{2-}	10.5	Aslan and Kapdan, (2006)
Synechocystis sp. PCC 6803		7.22	This work

The K_{HCO3} determined in our model cannot be comparable to that reported in Eze et al. (2018) since our half-saturation constant varied with the pH in contrast to the other K_C which was fixed for overall carbon species, TIC. According to the values in Table 5.13., the *Desmodesmus sp.* growth is much more limited by HCO_3^- than *Synechocystis sp.* PCC 6803. In terms of NO_3^-, all the kinetic constants reported in Table 5.13 are in the range pf 30 to 32mg/L. The growth of both microalgae *Chlorella vulgaris* and *Nannochloropsis* and the cyanobacterium *Synechocystis sp*. PCC 6803

were similarly influenced by the NO_3^- concentration according to the reported values in Table 5.13. Regarding TIP, *Synechocystis sp.* PCC 6803 is more influenced by HPO_4^{2-} concentration than the microalga *Chlorella vulgaris.*

The pH was continuously measured during the second set of assays conducted in batch-mode (section 5.2, *materials and methods*). Figure 5.6 displays the speciation of inorganic carbon as a function of pH. The main species in solution during the second set of assays was HCO_3^- according to Figure 5.6. It is recommended to maintain the pH within the range 7.5-8.5 with an exhaustive control to ensure this range during the *Synechocystis sp.* PCC 6803 growth. When the pH is far away from this range, the cyanobacterium growth could be limited due to low concentration of HCO_3^- available in the PBR.

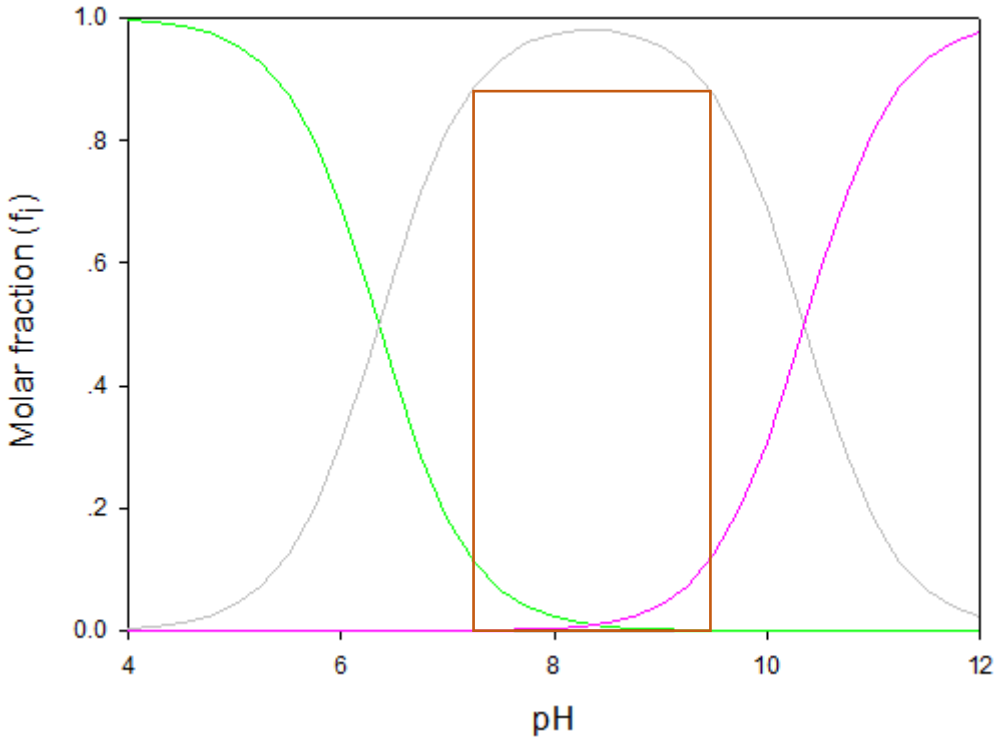

Figure 5.6. Speciation of TIC as a function of pH. Molar fraction of CO_2 (green), HCO_3^- (grey) and CO_3^{2-} (pink). Orange rectangle marks the pH maintained in the PBRs during the experiments performed.

5.3.5 Influence of initial TIP concentration over *Synechocystis sp.* PCC 6803 growth

Some assays were conducted according to the experimental conditions described in section 5.2.6.3 (*Synechocystis sp. PCC 6803 cultivation under P-deficiency*) to determine the influence of initial TIP concentration over the μ and DW obtained cultivating the cyanobacterium *Synechocystis sp.* PCC 6803. For these assays, the parameter fitted was μ_{OBS} which is the μ adjusted by the kinetic model developed. Semi-saturation constants remained constant for this simulation.

5.3.5.1 Assays conducted at 4mg/L of initial TIP concentration

The experimental values for TIP and DW and the fitted model predictions are represented in Figure 5.7.

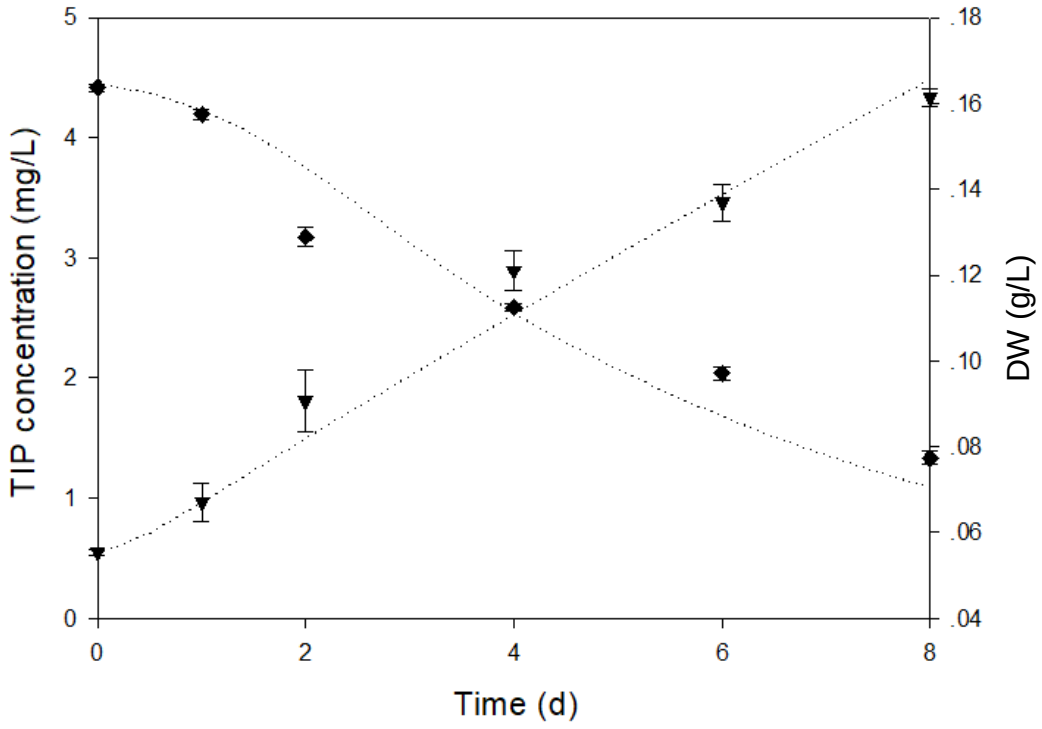

Figure 5.7. DW and TIP concentration over time. (♦) Experimental DW, (⋯) Fitted DW, (▼) Experimental TIP concentration and (⋯) Fitted TIP concentration

The model provides a good description of the experimental results according to the vales represented in Figure 5.7. The experimental TIP concentration varied from 4.44 to 1.33mg/L, while the model adjusted a TIP variation from 4.44 to 1.05mg/L. The experimental TIP consumption rate in this test was 0.38mg/L/d.

The TIP uptake rate fitted by the model was 0.41mg/L/d, which represented and error of 8.5%. Experimental DW increased during the assay from 0.055 to 0.161 corresponding to a BP of 0.013g/L/d. The DW profile fitted by the kinetic model varied from 0.055 to 0.167g/L achieving a BP of 0.014gDW/L/d. The difference between both BPs was $3.8 \cdot 10^{-3}$g/L/d, meaning an error of 3%.

5.3.5.2 Assays conducted at 8mg/L of initial TIP concentration

The experimental and adjusted profiles for TIP and DW obtained in this assay conducted at 8mg/L of initial TIP concentration are represented in Figure 5.8.

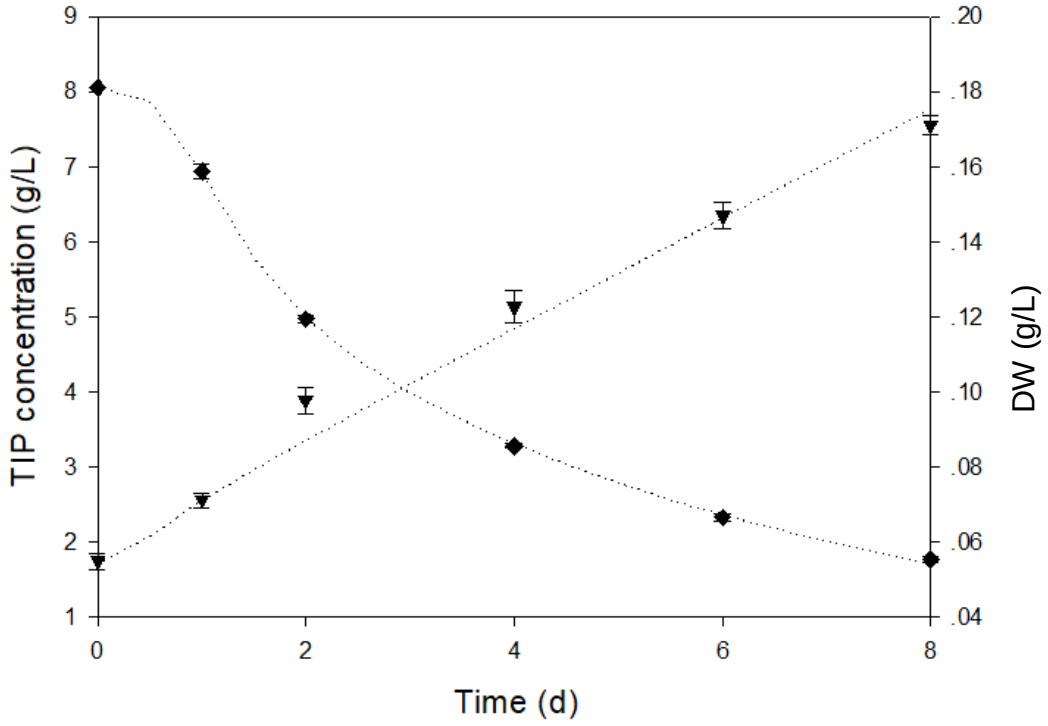

Figure 5.8. DW and TIP concentration over time. (♦) Experimental DW, (⋯) Fitted DW, (▼) Experimental TIP concentration and (⋯) Fitted TIP concentration

The model predicted accurately the experimental results according to the values represented in Figure 5.8. The experimental TIP concentration varied from 8.05 to 1.75mg/L. The TIP variation fitted by the kinetic model was from 8.05 to 1.72mg/L. The TIP uptake rate was 0.787 and 0.783mg/L/d, respectively for the experimental and modelled, corresponding to a deviation of only 0.5%. In terms of DW, the experimental results varied from 0.054 to 0.168g/L, while fitted DW concentration increased from 0.054 to 0.177g/L.

The BP in both scenarios was 0.014 and 0.014g/L/d, respectively for the experimental and fitted values. The difference between both BPs was $4.2 \cdot 10^{-4}$g/L/d corresponding to a deviation of 3%.

5.3.5.3 Assays conducted at 15mg/L of initial TIP concentration

The experimental and fitted profiles for TIP and DW obtained in the assay conducted at 15mg/L of initial TIP concentration are represented in Figure 5.9.

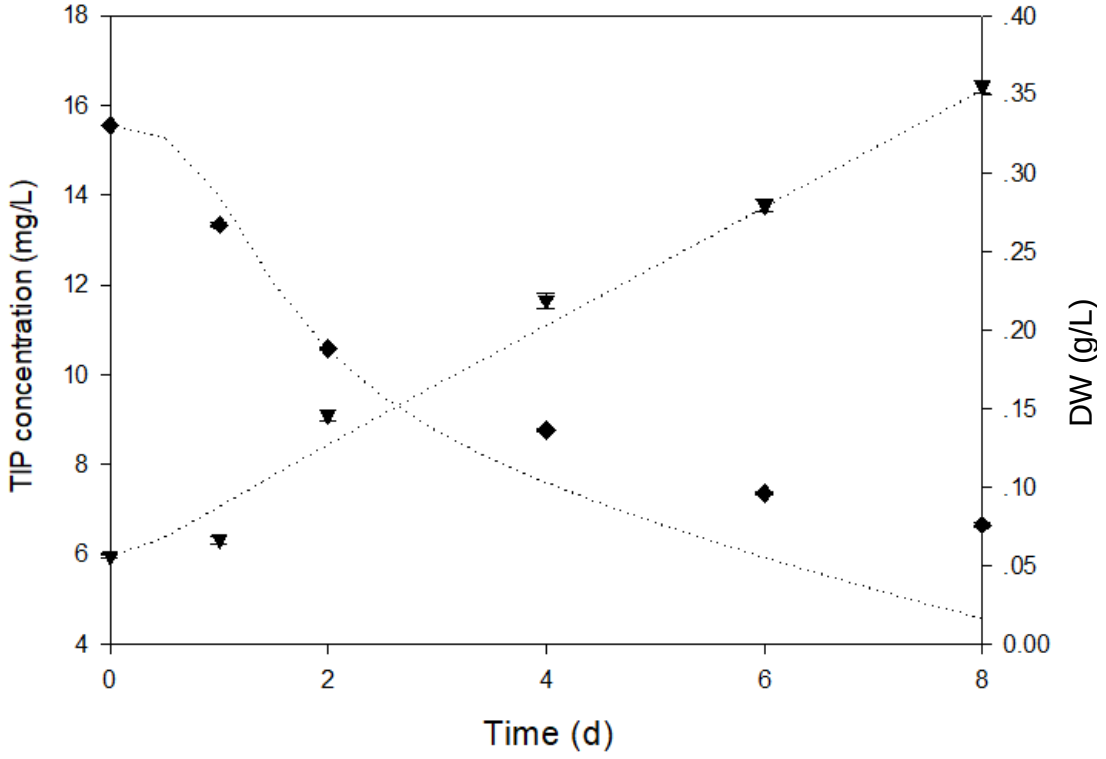

Figure 5.9. DW and TIP concentration over time. (♦) Experimental DW, (⋯) Fitted DW, (▼) Experimental TIP concentration and (⋯) Fitted TIP concentration

The model prediction described accurately these experimental results according to the results represented in Figure 5.9. The experimental TIP concentration decreased from 15.55 to 6.64mg/L while the model fitted a reduction from 15.55 to 4.55mg/L. The TIP consumption rate was 1.375 for the experimental assay and 1.08mg/L/d for the kinetic model. According to these results, there is a difference of 0.265mg/L/d between both values corresponding to a deviation of 25%. This deviation could be attributed to a precipitation of phosphate salts, mainly $Ca_3(PO_4)_2$ according to the pH achieved during the assay, 10.7.

DW increased experimentally from 0.055 to 0.359g/L/d, while the DW fitted using the kinetic model varied from 0.058 to 0.361g/L. The BPs were 0.038 for the experimental assay and 0.037g/L/d for the kinetic model, resulting in a difference of $1.25 \cdot 10^{-4}$g/L/d, corresponding to a deviation of 0.33%.

5.3.5.4 Comparison of the results obtained for all the assays conducted

Experimental and fitted μ, TIP uptake and BP obtained in the different assays conducted varying the initial TIP concentration are represented in the Table 5.14. The μ_{OBS} fitted increased according to the TIP concentration as is observed in Table 5.14. Regarding that the K_{HPO4} adjusted by the kinetic model proposed, 7.22mg/L, the assay conducted with an initial TIP concentration of 4 was run under P-limitation. The low BP this test could be attributed to the low TIP concentration available during the assay at 4mg/L of initial TIP concentration. BP obtained at 15mg/L of initial TIP concentration is 2.7 times higher than the obtained at 4 and 8 mg/L of initial TIP concentration. Hence, it confirms that the BP of the cyanobacterium *Synechocystis sp.* PCC 6803 is dependent on the nutrients availability in the liquid medium during its cultivation in PBRs.

Table 5.14. μ_{MAX}, TIP uptake and BP as a function of initial TIP concentration.

	Initial TIP 4mg/L	**Initial TIP 8mg/L**	**Initial TIP 15mg/L**
μ_{OBS}(1/d)	0.433	0.467	0.573
TIP uptake (mg/L/d)	0.389	0.787	1.110
BP (g/L/d)	0.013	0.014	0.038
τ	0.161	0.174	0.134

The μ can be compared with the obtained in the second set of experimental assays. In those assays maintaining a pH control, the μ obtained was 0.962(1/d) while in these tests without pH control, the μ obtained was 0.573(1/d). Therefore, the pH control conducted at the second set of experimental assays influenced positively the μ with an increase of 67% in comparison to assays conducted without pH regulation.

The final pH measured for each assay conducted were 9.67, 9.89 and 10.65 for 4, 8 and 15mg/L of initial TIP concentration. The pH profile for each one of the tests conducted is represented in Figure 5.10.

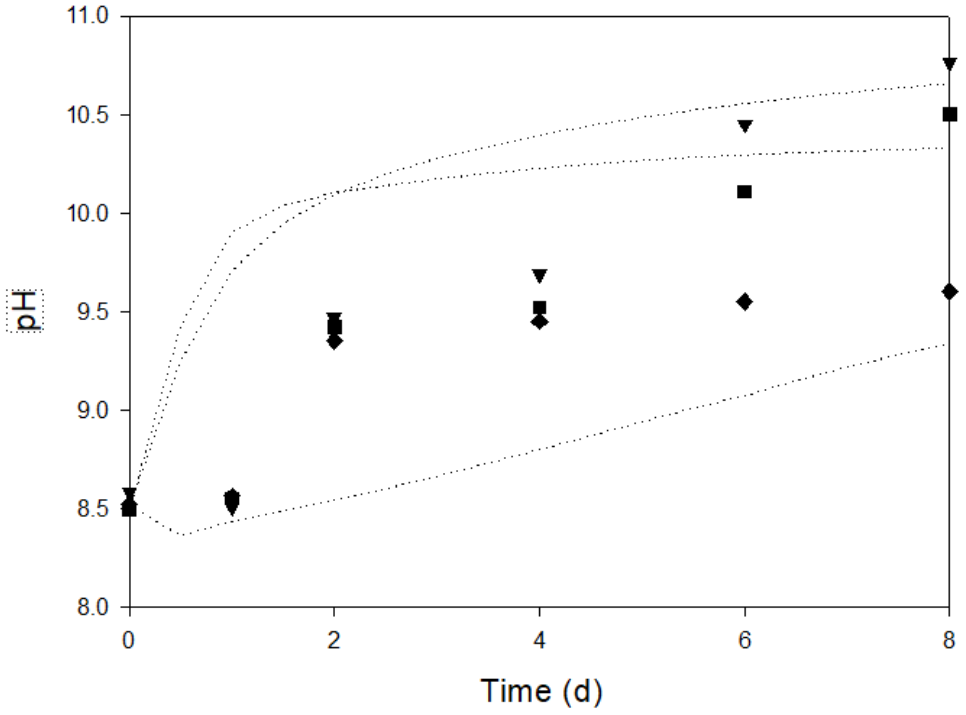

Figure 5.10. pH profile for assays conducted at different initial TIP concentrations. Experimental pH at 4mg/L (♦), 8mg/L (■), and 15mg/L of initial TIP concentration (▼); Fitted pH at 4mg/L (⋯), 8mg/L (⋯) and 15mg/L of initial TIP concentration (⋯).

The increasing of pH was directly related to the HCO_3^- consumption. When the HCO_3^- consumption is higher, the pH increases due to the loss of buffer capacity related to the equilibrium HCO_3^-/CO_3^{2-}. According to the final pH reached in these assays, the activity of the cyanobacterium is limited in P-deficiency due to the increasing of pH which reduce the HCO_3^- available for the cyanobacterium growth.

Therefore, it is suggested to maintain an adequate level of TIP concentration and an exhaustive control of the pH during the cyanobacterium *Synechocystis sp.* PCC 6803 cultivation in order to avoid growth limitation of the cyanobacterium due to a low availability of HCO_3^- and HPO_4^{2-} observed when the pH is far away from its optimal range.

5.3.6 Model exploitation

Some simulations were conducted changing the L/D cycles applied to the cyanobacterium aiming to describe the *Synechocystis sp.* PCC 6803 behaviour under these scenarios. According to the literature consulted, BP is dependent on LI and L/D applied to the system, for instance, we suggested to set a L/D cycles of 8:16h and 20:4h without pH control during the simulation with the objective to describe the response of the kinetic model developed in these situations.

5.3.6.1 Simulation conducted at L/D cycle of 20:4h

The pH and DW profile obtained by the kinetic model at 20:4h of L/D cycle applied to the cyanobacterium are represented in Figure 5.11.

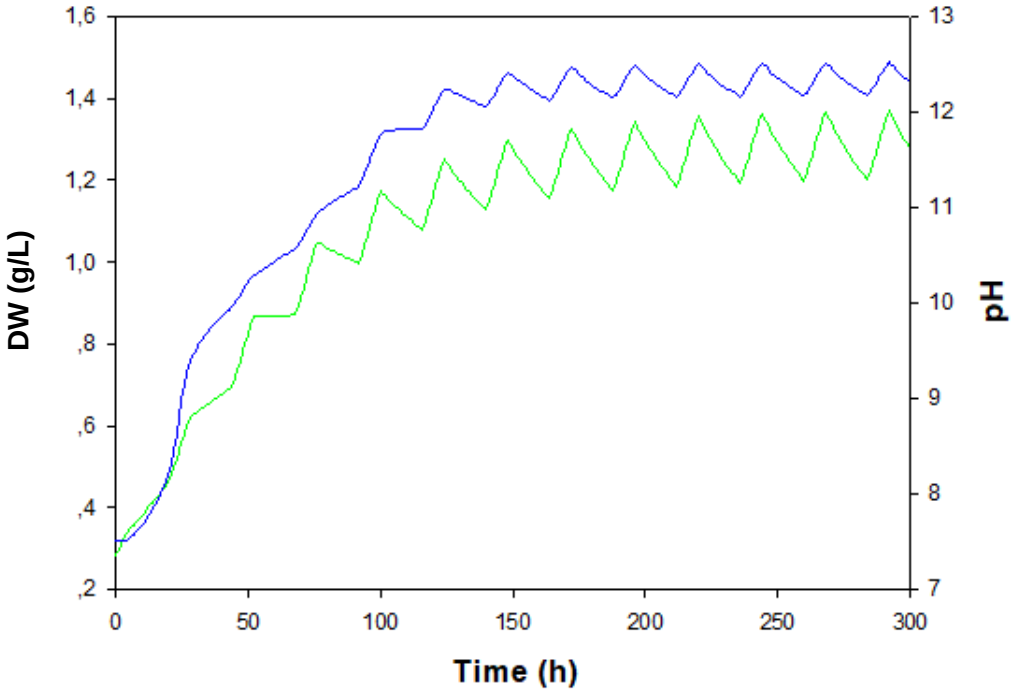

Figure 5.11. pH and DW profile at 20:4 of L/D cycle. DW (blue) and pH (green)

A quick-response at the beginning of the simulation was detected where the DW rapidly increased from 0.28 to 1.2g/L in the first 150h of simulation. It corresponded to a BP of 0.19g/L/d. After that, the DW increased from 1.2 to 1.3gDW/L during the rest of the simulation, achieving an overall BP in this period of 0.016g/L/d.

This low BP achieved at the second part of the simulation could be attributed to achieving the pseudo steady-state of the system where μ and μ_D are balanced.

In terms of pH, the system achieved the pseudo steady-state after 200 h, when the pH varied always from 12 to 13 for the rest of simulation. According to these results and considering the experimental results obtained at the experimentation stage, the CO_2 injection set at 2.5%v/v should be increased in a real scenario in order to maintain the pH around the optimal value of 8 in order to avoid the limitation growth of the cyanobacterium. TIC speciation simulated is represented in Figure 5.12.

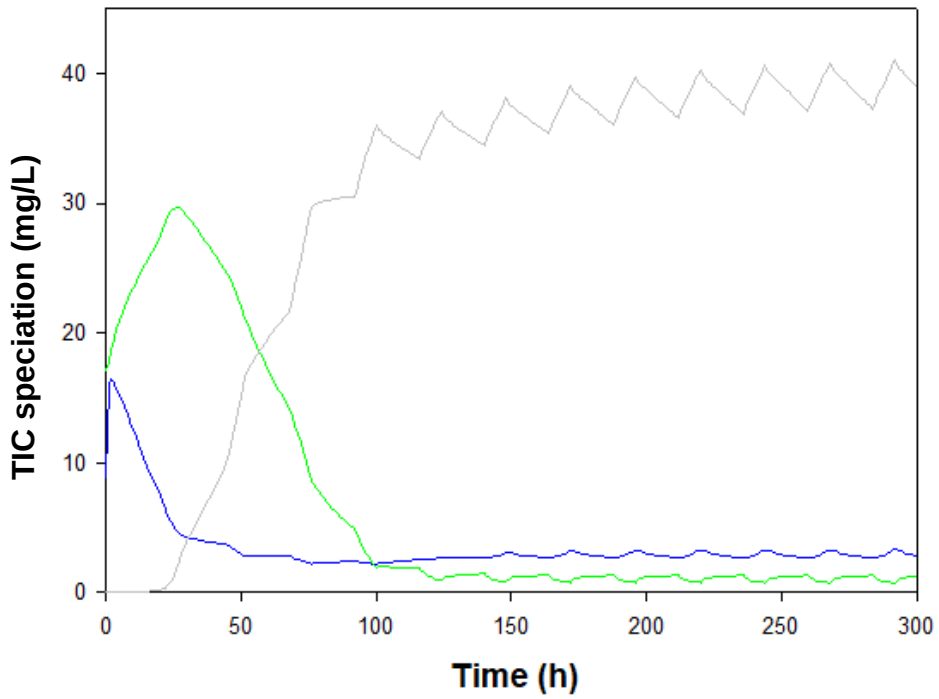

Figure 5.12. TIC profiles at simulation conducted at 20:4h of L/D cycle. HCO_3^- (green), CO_3^{2-} (grey) and CO_2 (blue).

The simulation conducted was under limiting HCO_3^- concentration according to the results represented in Figure 5.12. HCO_3^- at the pseudo steady-state varies from 1.3 to 0.75mg/L corresponding to a lower concentration than the half-saturation constant fitted by the kinetic model, 1.476mg/L. These results extracted from the simulation confirmed that pure CO_2 supply should be increased with the aim to operate the PBR far away from operational conditions which limit the cyanobacterium growth. TIP speciation is represented in Figure 5.13.

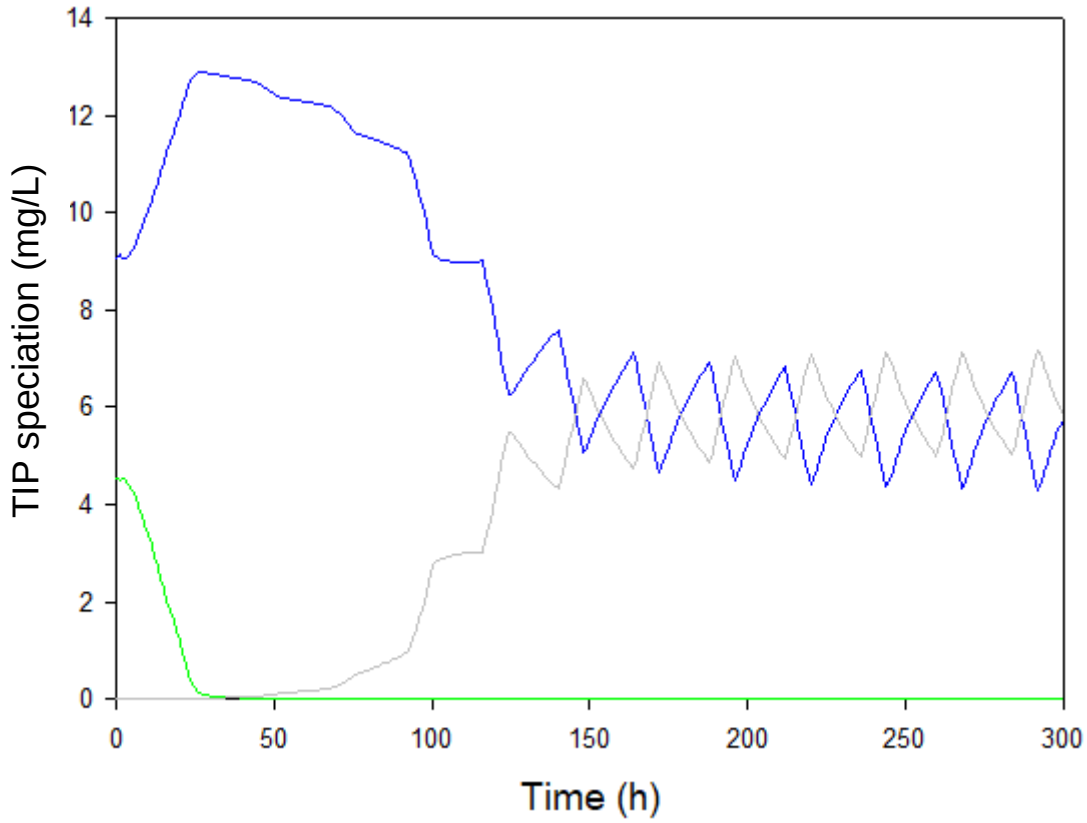

Figure 5.13. TIP speciation at the simulation conducted at 8:16h of L/D cycle. $H_2PO_4^-$ (green), PO_4^{3-} (grey) and HPO_4^{2-} (blue).

Cyanobacterium growth was not limited by TIP concentration due to the HPO_4^- concentration was always higher than the half-saturation constant determined of 7.22mg/L according to the results represented in Figure 5.13. Under this scenario, it would be advisable to control exhaustively the pH in real conditions in order to maintain an adequate HCO_3^- concentration which is the concentration affecting the cyanobacterium growth according to this simulation.

5.3.6.2 Simulation conducted at L/D cycle of 8:16h

The simulation was conducted by setting an L/D cycle at 8:16h as suggested above with the aim to describe the response of the kinetic model developed at this scenario. The profiles of DW and pH are shown in Figure 5.14

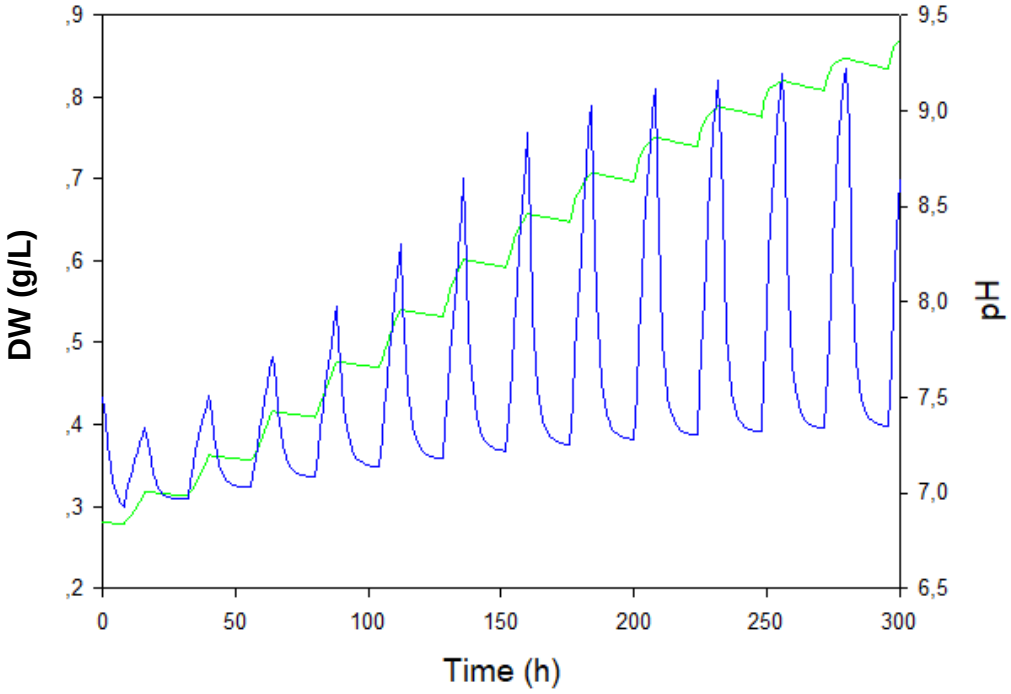

Figure 5.14. pH and DW profile at simulation conducted at 8:16h of L/D cycle. pH (blue) and DW (green)

The culture never reached the pseudo steady-state according to the results represented in Figure 5.14. As can be seen, the DW increased continuously without reaching a situation in which the μ and μ_D were balanced during the simulation. In addition, comparing the modelled results obtained with the simulation at L/D 20:4h, the final DW was reduced, 0.85g/L in front of 1.1g/L. Bearing in mind that in the 20:4h assay the culture reached the pseudo steady-state at 150 hours of test, it can be confirmed that a continuous illumination of the PBR would enhance the growth of *Synechocystis sp.* PCC 6803.

In terms of pH, we can observe in comparison to the results commented above, that around 250-270h a pseudo steady-state was achieved since the variation between 7.4 and 9.2 was maintained for two consecutive L/D cycles. TIC speciation modelled is represented in Figure 5.15.

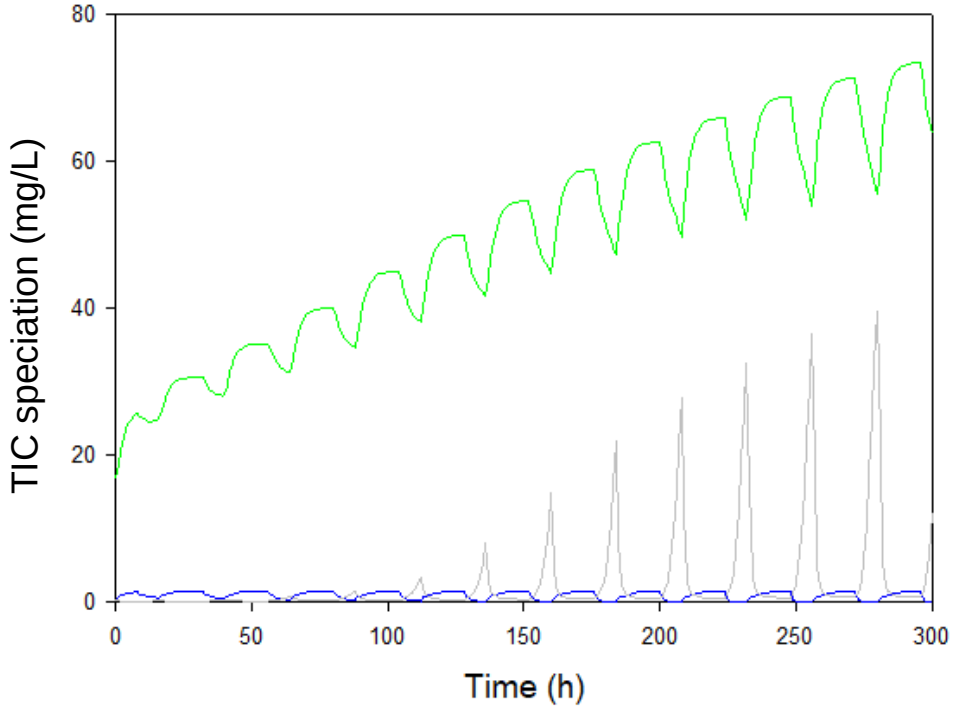

Figure 5.15. TIC speciation at 8:16h of L/D cycle. HCO_3^- (green), CO_3^{2-} (grey) and CO_2 (blue).

The simulation conducted was under non-limiting TIC concentration according to the modelled results represented in Figure 5.15. Although the pseudo stationary-state of the culture was never reached during the adjust, the concentration of HCO_3^- was always above the half-saturation constant of 1.47mg/L fitted by the kinetic model which limits the cyanobacterium growth. TIP speciation is represented in Figure 5.16.

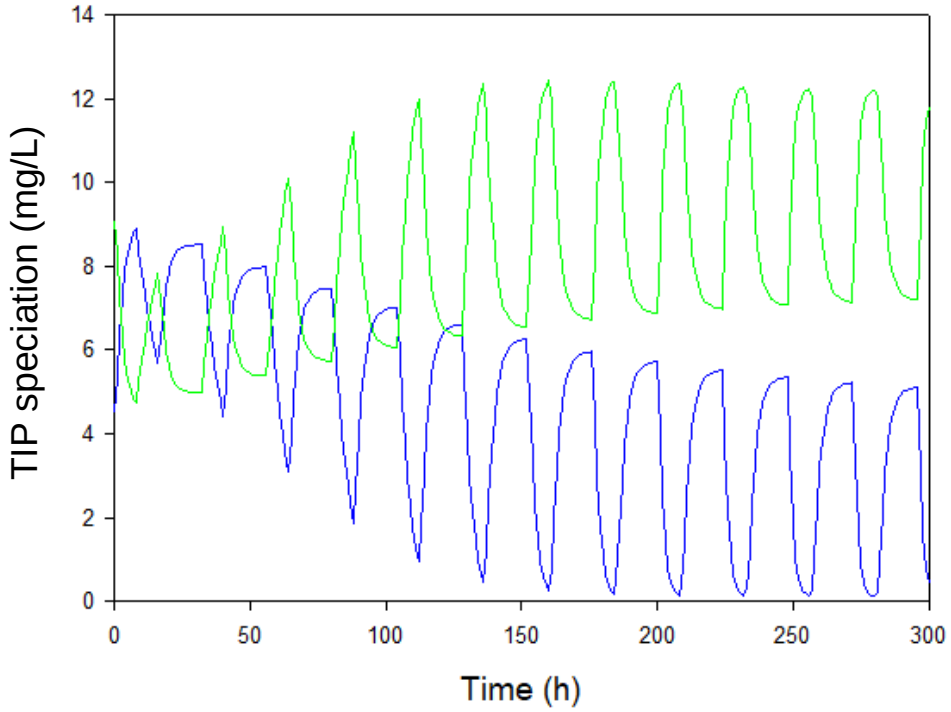

Figure 5.16. TIP speciation at the simulation conducted at 8:16h of L/D cycle. HPO_4^{2-} (green) H_2PO4^- (blue)

The simulation conducted was under non-limiting TIP concentration according to the modelled values represented in Figure 5.16. The HPO_4^{2-} concentration was at least 1.4 times higher than the half-saturation constant fitted by the kinetic model which is has been demonstrated that influences the cyanobacterium growth according to the assays conducted in P-deficiency conditions.

5.4 Conclusions

A multicomponent kinetic model was developed with the aim to understand better the cyanobacterium *Synechocystis sp.* PCC 6803 behaviour during its cultivation in an efficient PBR. LI, speciation of inorganic nutrients, TIC and TIP depending of the culture pH were considered during the kinetic model development.

1st and 2nd series of assays in batch mode served to adjust several parameters of the kinetic model proposed such as μ = 0.962 (1/d), semi-saturation constant for TIC (K_{HCO3}) K_{HCO3} = 1.476mg/L, semi-saturation constant for inorganic nitrogen (K_{NO3}) K_{NO3} = 31.76mg/L and semi-saturation constant for TIP (K_{HPO4}) K_{HPO4} = 7.22mg/L. Values obtained for all of these parameters fitted are in the same range than other kinetic models reported in the literature. Moreover, the kinetic model developed in our investigation described accurately the experimental results achieved during the experimental procedures conducted.

Some assays were conducted by changing the initial TIP concentration with the aim to describe the *Synechocystis sp.* PCC behaviour when it is cultivated under P-limitation. Experimental and fitted values determined that the cyanobacterium growth is limited when TIP concentration at the beginning of the assay is lower than the semi-saturation constant K_P. Moreover, it was also detected that the pH which was not controlled during these assays does not increase similarly to the other tests conducted where the initial TIP concentration was not reduced from the BG-11 medium.

Simulations by changing the L/D cycle applied to the culture were conducted with the aim to describe the response of the kinetic model developed during the *Synechocystis sp.* PCC 6803 cultivation at these scenarios. L/D cycle 20:4h reached the pseudo steady-state under limited conditions of TIC at 150h of simulation while the simulation conducted with L/D cycle of 8:16 did not reached the pseudo steady-state after 300h of simulation. According to the simulations conducted, it is recommended an exhaustive control of the pH in order to maintain the pH within 7.5-8.5 where the HCO_3^- is optimal for the cyanobacterium growth. pH far away from this optimal (20:4 L/D cycle modelled) induced the limitation of the growth due to the reduction of HCO_3^- availability.

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6 Energy recovery through anaerobic digestion

Abstract

The present research estimates, for the first time, the biogas production potential (BPP) for *Synechocystis* PCC 6803, 169.23±2 mL/gVS. Different thermal pretreatments such as microwaving and ultrasonication were tested in order to enhance the BPP (microwaving reached 315.61±0.48mL·gVS^{-1} while ultrasonication achieved 299.53±0.9 mL/gVS). Co-digestion with olive mill wastewater to increase the initial C/N ratio resulted in a BPP of 209.0±0.7 mL/gVS. A combination of both methodologies, i.e. microwaving and co-digestion, led to the highest BPP obtained (398.7±0.5 mL/gVS). Finally, a modified Gompertz equation was fitted to the experimental results achieved for a better understanding of the results obtained.

6.1 Introduction

Anaerobic digestion (AD) is the most common used technique for producing biogas from various organic wastes and wastewaters. Biogas, a mixture of mainly methane and carbon dioxide, can be used to produce electricity or heat, or it can be upgraded to biomethane, a potential automotive fuel to replace conventional natural gas (Prajapati et al., 2014).

AD of microalgae species as a feedstock for biogas production has recently been studied due to their large area yields compared to conventional crops (Angelidaki et al., 2018), e.g. with AD of *Chlorella spp*., *Chroococcus spp*. and mesophilic/thermophilic digestion of *Scenedesmus obliquus* and *Phaeodactylum tricornutum* (Prajapati et al., 2014, 2013; Zamalloa et al., 2012). The biogas production from *Scenedesmus sp*. in a continuous anaerobic reactor has also been reported recently (Tartakovsky et al., 2013). The biogas production potential (BPP) for different microalgae reported in the literature is represented in table 6.1.

Table 6.1. BPP for *Chlorella sp., Scenedesmus sp., A. platensis, S.oblicuus, D.salina* and *Chroococus* reported in the literature

Microalgae Strain	HRT (d)	Conditions	BPP (mL/gVS)	Reference
Chlorella sp. & Scenedesmus sp.	10	Mesophilic	130-200	(Chen et al., 2011)
A. platensis	32	Mesophilic	481	(Mussgnug et al., 2010)
S. obliquus	32	Mesophilic	287	(Mussgnug et al., 2010)
D. salina	32	Mesophilic	505	(Mussgnug et al., 2010)
S. obliquus	22	Mesophilic	240	(Zamalloa et al., 2012)
Chroococcus	30	Mesophilic	400-490	(Prajapati et al., 2013)

Mussgnug et al., (2010) reported the BBP for several microalgal strains. According to the values represented in Table 6.1., *Dunaliella salina* achieved the high biogas production, 505mL/gVS; while *Scenedesmus sp.* shown a low biogas production, 240mL/gVS in their investigation. Moreover, the BPP potential for *Chlorella sp.* and *Scenedesmus sp.* reported in Chen et al., (2011) is also lower in comparison to the BPP obtained with *D. Salina*.

6.1.1 Parameters affecting AD of microalgae

Textbook knowledge states that there are two main factors affecting the anaerobic digestibility of microalgal biomass: the carbon-to-nitrogen (C/N) ratio and the resistance of the cell wall.

6.1.1.1 Carbon-to-nitrogen (C/N) ratio

the C/N ratio is an essential parameter for an efficient performance and the BPP depends on this ratio (Alzate et al., 2012; Angelidaki et al., 2018; Ometto et al., 2014). Moreover, ammonia inhibition occurs when C/N ratio is below to 25-30 (Rétfalvi et al., 2016) due to the free ammonia produced during the acidogenesis step of the AD process. Table 6.2. shows typical C/N ratio values for microalgal biomass, which are far away from the optimal value recommended (i.e. 25-30).

Table 6.2. C/N ratio of different microalgae strains which have been used in AD process reported in the literature

Microalgal strain	**C/N Ratio**	**Reference**
Taihu blue algae	6	(Hiltunen et al., 2013)
C. vulgaris	5	(Rétfalvi et al., 2016)
Chlorella sp.	6.43	(Zhang et al., 2019)
Chroococcus spp.	7.44	(Prajapati et al., 2013)
Synechocystis sp PCC 6803	4.13	(Panda et al., 2006)

For instance, the conventional C/N for microalgae species is in the range 4.3-5.33 but *Synechocystis sp.* PCC 6803, the cyanobacteria strain selected in our investigation, has a C/N ratio of 4.13. A common option is to add a co-substrate rich in carbon to increase the C/N ratio (Calicioglu and Demirer, 2016; Passos et al., 2014; Rétfalvi et al., 2016; Zhang et al., 2019).

6.1.1.2 Cell wall resistance of microalgae

The cell wall is also responsible for the low BPP observed when digesting certain microalgal biomass. Mussgnug et al. (2010) reported the BPP of diverse microalgae as function of their cell wall composition. *Dunaliella salina* (which does not have cell wall) achieved the highest BPP (505 mL/gVS) whereas *Scenedesmus obliquus* (which contains hemi-cellulosic compound in its cell) achieved the lowest biogas production (287mL/gVS). Different pretreatments have been put forward to overcome this limitation (Calicioglu and Demirer, 2016; Ometto et al., 2014; Passos et al., 2014, 2013). Among them, thermal pretreatments has been proved as the most effective method since they are can disrupt the cell wall. Calicioglu and Demirer, (2016) reported an increase of the BPP for *Chlorella vulgaris* from 223 to 408 mL/gVS after autoclaving the microalgal biomass for 1h at 121ºC. Passos et al., (2013) microwaved the microalgae biomass and enhanced the biogas productivity in the range 25 to 75% due to different energy steps were investigated.

6.1.2 Kinetic modelling of AD process

An understanding of AD kinetics is important for investigating biogas production yields and conducting energy analyses. Due to the complexity of the AD processes, several simplified models have been developed to estimate biogas yields from anaerobic digestion of microalgae biomass (Martín Juárez et al., 2018; Wang et al., 2017; Zhen et al., 2016). Adopting the correct model that produce reliable prediction results is not trivial.

By using the first-order kinetic model, the hydrolysis rate of particulate organic matter is fixed as the limiting step, while in modified Gompertz model, lag-phase is the main limiting step during the AD process. Both models are represented in the following Eq. (1)-(2).

First-Order Model $$BPP = M \cdot (1 - \exp(-K_H \cdot t)) \quad (1)$$

Modified Gompertz Model $$BPP = M \cdot exp\left(-exp\left[\frac{K_M \cdot e}{M} \cdot (\lambda - t) + 1\right]\right) \quad (2)$$

Where BPP is the biogas production potential, M (mL/gVS) is the maximum biogas potential, K_H (1/d) is the hydrolysis constant of organic matter, K_M (mL/gVS/d) is the maximum biogas production rate and λ (d) is the lag phase. The most widely used model for AD processes is modified Gompertz model which is used to predict biogas from energy crops, microalgae biomass or thermally treated co-digestions.

6.2 Objectives

The aim of this investigation was to determine, for the first time, the BPP of *Synechocystis sp.* PCC 6803 and to compare it with literature reports on different microalgal strains. Moreover, this work also aimed at enhancing the BPP of *Synechocystis sp.* PCC 6803 by (i) applying thermal pretreatments (i.e. microwaving and ultrasonication) and (ii) adding a co-digestion substrate (olive mill wastewater, OMWW). Finally, a kinetic model for the prediction of the BPP of *Synechocystis* sp. PCC 6803 was fitted to the experimental for a better understanding and comparison of the results obtained.

6.3 Materials and Methods

6.3.1 PHA extraction from *Synechocystis sp.* PCC 6803

Regarding the goal of Oli-PHA project, the organic target product, PHA, must be extracted from the cyanobacterium *Synechocystis sp. PCC 6803* prior to the use of the remaining biomass in the AD process.

Cells were harvested from the PBR and kept for 2h inside a refrigerator (4ºC) prior to the PHA extraction from the cells. Cyanobacteria solutions were concentrated by centrifugation (8000rpm, 10min), washed with osmotic water and the resulting biomass was suspended in methanol overnight at 4ºC for the pigments and chlorophyll removal. The pellet obtained after the centrifugation was dried at 60ºC in an oven for 1h and PHB was extracted in hot chloroform. PHA was precipitated from the chloroform solution into chilled methanol.

The methanol-chloroform mixture was decanted and the precipitated PHA was separated by centrifugation (4000rpm, 5min). Then, the polymer was re-dissolved in chloroform. After the evaporation of chloroform, remaining dry PHA was obtained as (Costa et al., 2018).

6.3.2 Biomass powder preparation

The aqueous phase after the PHA extraction was concentrated by centrifugation (4000rpm, 5min) and the remaining chloroform was separated from the microalgae biomass by decantation.

Biomass was washed with osmotic water and concentrated again by centrifugation (4000 rpm and 5 min) three times to ensure the complete chloroform removal. After the purification operation, the biomass was washed twice time with osmotic water before the lyophilization step. Then, the biomass was kept in Falcon tubes (50mL) at -82ºC prior to its use in the AD tests.

6.3.3 Microwaving and ultrasonication treatments

Microwaving and ultrasonication pretreatments used to enhance the biogas production potential of the microalgal biomass were conducted according to the procedures described before. (*see Chapter 3, Thermal treatments in AD tests*)

6.3.4 Anaerobic digestion (AD) assays

The anaerobic sludge used was collected at the wastewater treatment plant of Sabadell (Spain). AD of microalgal biomass was conducted by triplicate in batch mode for 21 days in a glass bottles of 0.250L at 37ºC (mesophilic conditions) with a methodology adapted from Mussgnug et al. (2010). Oxygen was removed from digesters by purging the headspace with pure nitrogen and the bottles were closed with rubber seals and aluminium caps. Each bottle was inoculated with 0.15mL of anaerobic sludge and 4g of lyophilized microalgae biomass achieving a substrate-to-inoculum ratio (S/I) of 1:1. For each bottle, a final volume of liquid fraction of 0.160L was set, allowing 35% of the total volume for biogas production. To provide enough buffer capacity during the AD process, 5g/L of $CaCO_3$ was added to the mixture and the pH was adjusted to 7.

Bottles containing only anaerobic sludge were run as blanks for the endogenous BPP quantification. Control experiments with a readily biodegradable substrate (cellulose) were run to determine the activity of the anaerobic sludge. The volume of biogas produced was calculated by measuring the pressure of the headspace. The BPP was calculated by subtracting the blank production from the amount of biogas measured in each sample.

6.3.5 Analytical methods

6.3.5.1 Total and volatile solids determination

Total solids (TS) representing the total amount of mater in a liquor sample and volatile solids (VS) representing the volatile organic fraction in TS were measured according to the procedures described before. (*See chapter 3, Total solids (TS) and volatile solids (VS) determination*).

6.3.5.2 BPP determination

Daily biogas production during the anaerobic digestion assays were measured according to the standard procedures described before. (*See Chapter 3, 3.2.4 Biogas production potential (BPP) determination*).

6.3.5.3 Olive mill wastewater (OMWW) physic-chemical properties determination

This investigation was conducted in parallel to a research project focused on olive mill wastewater (OMWW) re-utilisation wherein *Synechocystis sp.* PCC 6803 was cultured under mixotrophic conditions in order to increase their internal PHA

accumulation. During oil processing, olives are crushed and mixed with water in the adequate mills.

The oil is separated from the rest of the wastewater and from the solid waste. In the Mediterranean countries, where 97 percent of the world's olive oil is produced, olive mills generate annually almost 8 billion gallons of OMWW, which need to be treated.

OMWW is a dark, turbid and acidic effluent (pH 4.5-5.5) with an excessive high organic load. It also includes high levels of phytotoxic inhibitor compounds such as phenols. OMWW could be used as co-substrate for anaerobic digestion provided that the phenol content is removed. Phenol separation process consisted of OMWW ultrafiltration and a membrane contactor with sodium hydroxide. Once the phenols from the OMWW liquor were extracted, the resulting wastewater could be used as a co-substrate for *Synechocystis sp.* PCC 6803 anaerobic digestion.

Physic-chemical characterization of the OMWW was conducted according to conventional standards (ASTM, standard procedures). Electrical conductivity and pH were analysed in a 1:10 (w/v) water-soluble extract. Total nitrogen and total organic carbon were determined by automatic microanalysis.

Macro- and micro-elements were brought into solution by acidic digestion (25mL of OMWW digested with HNO_3 and HCl), then analysed by an atomic absorption spectrophotometer. The measurements were run in triplicated to normalize the determinations. The physic-chemical characteristics of OMWW are presented in Table 6.3.

Table 6.3. OMWW physic-chemical parameters characterization

Parameters	**OMW**
pH	4.75
Electrical conductivity (mS/cm)	18.72
TOC (g/L)	28.64
Total nitrogen (mg/L)	1214.26
Potassium (mg/L)	64.36
Magnesium (mg/L)	325.12
Sodium (mg/L)	290.71
C/N ratio	23.59

6.4 Results and discussion

6.4.1 BPP for *Synechocystis sp.* PCC 6803

Figure 6.1 shows the biogas production of *Synechocystis sp*. PCC 6803 during 21 days for untreated biomass and thermal treated biomass by microwaving and ultrasonication process. As can be observed, *Synechocystis sp.* PCC 6803 showed a good BPP (i.e.169.23 ± 2 mL/gVS).

The BPP was compared with results reported with other similar strains (Table 6.4). The BPP of *Synechocystis sp.* PCC 6803 obtained was in the same range than other microalgae digested anaerobically. Mussgnug et al., (2010) reported a high BPP of 287mL/gVS with *Scenedesmus sp.* as a substrate. This value is 1.6 times higher than our BPP. The C/N ratio for *Scenedesmus sp.* is higher than the C/N ratio for *Synechocystis sp.* PCC 6803 (i.e. 4.13). A lower C/N ratio could limit the anaerobic digestibility of biomass due to the ammonia production during the AD assays.

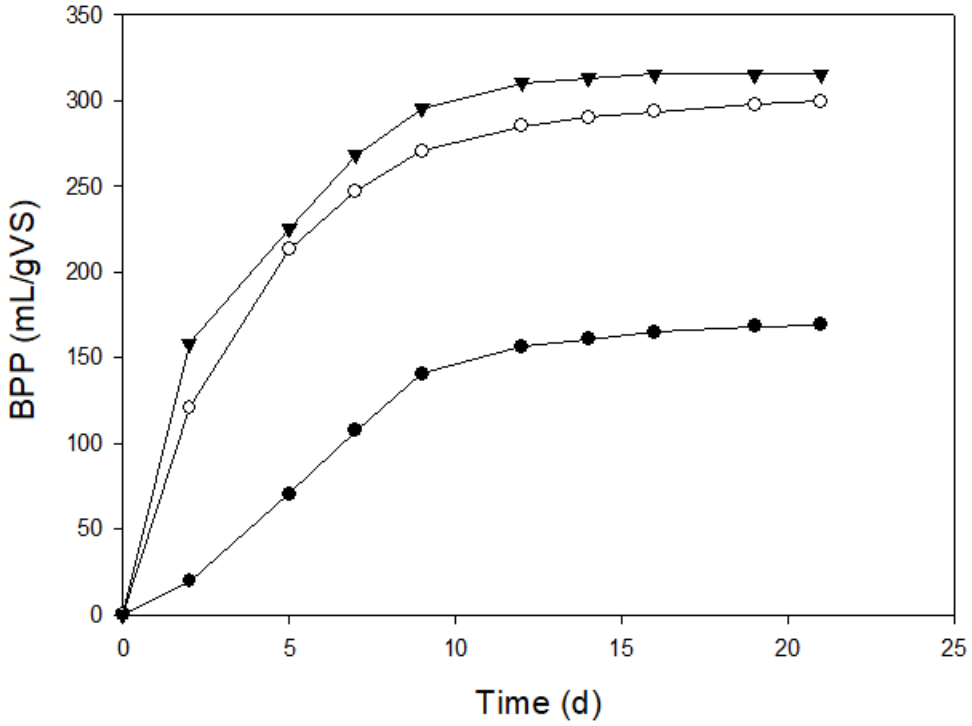

Figure 6.1. BPP for (●) untreated *Synechocystis sp.* PCC 6803 biomass, (○) with ultrasound pretreatment and (▼) with microwave pretreatment

Table 6.4. Biogas production potential for some microalgae biomass digested anaerobically

Strain	AD process (d)	BPP (mL/gVS)	Reference
Scenedesmus sp.	30	287	(Mussgnug et al., 2010)
S. obliquus	22	240	(Zamalloa et al., 2012)
S. maxima	16	90-150	(Samson and Leudy, 1982)
Chlorella sp.	10	90-140	(Yen and Brune, 2007)
Chlorella vulgaris sp.	16-28	150-240	(Ras et al., 2011)
Synechocystis sp. PCC6803	21	169.2	This work

6.4.2 Thermal treatments effect over BPP

Figure 6.1 also shows the biogas production of the thermally treated biomass. The use of pretreatments to disrupt the cell walls facilitates the assimilation by the anaerobic sludge and, thus, it increases the BPP.

Among the different methodologies proposed, microwaving and ultrasonication seemed the best choice to enhance the biogas productivity (Passos et al., 2013; Rétfalvi et al., 2016). Thus, both pre-treatments were tested in order to assess the potential enhancement of BPP.

Figure 6.1 shows that both pretreatments enhanced the BPP of *Synechocystis sp. PCC 6803*: microwaving achieved 315.6±0.5 mL/gVS while ultrasonication reached 299.5±0.9 mL/gVS, which represents an enhancement of 87 and 77% with respect to the untreated biomass, respectively.

An energy and monetary balance are required to state if this procedure would be feasible at industrial scale. The energy balance of the anaerobic treatment of cyanobacterial biomass was calculated to get an insight of the feasibility of its industrial scale implementation.

The energy consumed (E_C) during the pretreatment process was calculated as the energy required to raise the cyanobacteria powder temperature (T_C) to the pretreatment temperature (T_P) Eq. (3). This was considered as the main energy consumption for all the applied treatment. T^C was defined as 20ºC (i.e. room temperature) and T_P was defined as 85ºC or 73ºC (microwaving and ultrasound, respectively).

$$E_C = \rho \cdot \gamma \cdot (T_P - T_C)/VS \quad (3)$$

where the cyanobacteria specific density (ρ) and specific heat (γ) were assumed to be those of water since the pretreatment processes were conducted by suspending cyanobacteria into water to reduce the thermal damage over organic matter. Therefore, ρ was fixed at 1g/mL and γ was $4.18 \cdot 10^{-3}$ J/g/ºC, respectively. E_C was divided by the volatile solids content for all assays to normalise the results.

E_P (kJ/kgVS) was calculated as Eq. (4) as the energy per litre of biogas (γ_B) multiplied by the overall biogas (q_B) produced at the end of the tests. The energy contained in each litre of biogas produced has been reported as 26.6 kJ/L (Strömberg et al., 2014).

$$E_P = q_B \cdot \gamma_B \quad (4)$$

The energy balance resulting from these calculations is summarized Table 6.5.for untreated and pre-treated biomass of *Synechocystis sp.* PCC 6803.

Table 6.5 Energy balance for the conducted test with different thermal treatments of biomass

Treatment	**E_C (kJ/kgVS)**	**E_P (kJ/kgVS)**	**Net Energy balance (kJ/kgVS)**
Biomass	0	$7.16 \cdot 10^3$	$7.16 \cdot 10^3$
Microwave	$1.11 \cdot 10^3$	$8.39 \cdot 10^3$	$7.28 \cdot 10^5$
Ultrasonication	$9.71 \cdot 10^2$	$7.96 \cdot 10^3$	$6.98 \cdot 10^3$

According to the results represented, the energy balance for microwaving pre-treatment is satisfactory since the E_P is higher than E_C resulting in favourable net energy balance. However, the increase of E_P with an ultrasound pre-treatment does not compensate the E_C, resulting in an unfavourable net energy balance for this thermal treatment.

Although the energy balance is enhanced on the microwaving step, the increase when compared to the untreated scenario is only about $1.4 \cdot 10^2$ kJ/kg^{-1}VS which is lower than expected. This low increase cast doubts on the possibility of investing and upscaling this process at full-scale. For a more realistic energy assessment, a continuous process should be considered.

6.4.3 Co-digestion effect over BPP

Co-digestion assay was performed by replacing 50mL of initial anaerobic sludge by 50mL of OMWW reaching the same volume of liquid than other assays conducted. This mixture achieved a C/N ratio of 13. Figure 6.2 represents the BPP when the substrate was amended with phenol-free OMWW. According to the experimental results, the OMWW utilization enhanced BPP since the C/N ratio was increased. The productivity achieved at the end of the assay was 209.0±0.7 mL/gVS, an enhancement in BPP of 23.5% was achieved in the co-digestion assay. Several investigations have been conducted using a carbon-rich substrate in combination with microalgae biomass to increase the C/N ratio enhancing the biogas production of the mixture. Some of previous investigations are represented in the Table 6.6.

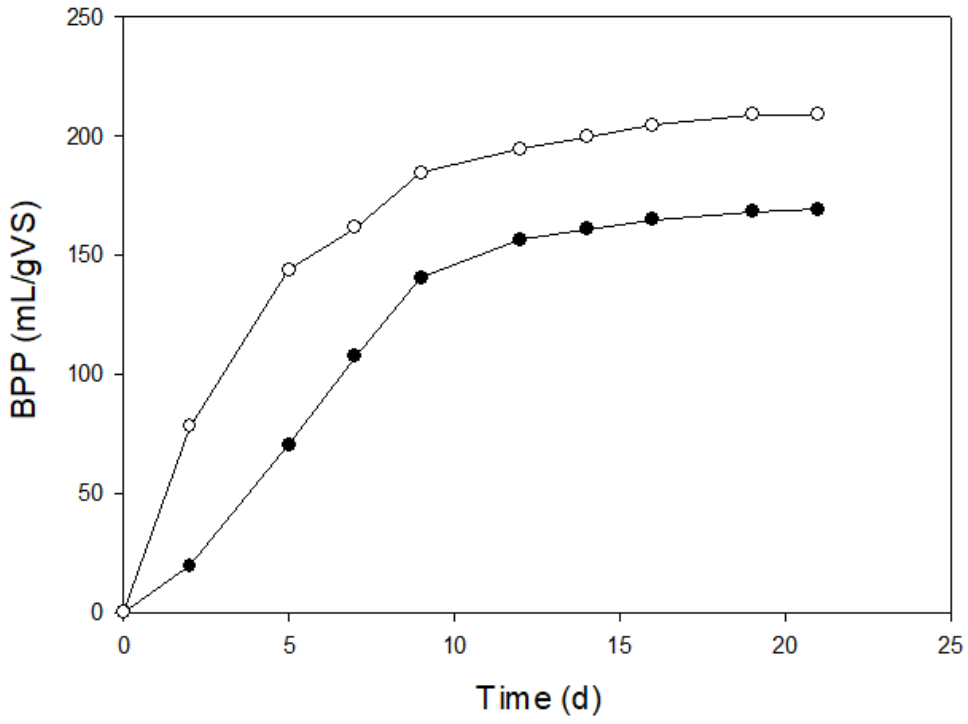

Figure 6.3. BPP for (●) untreated *Synechocystis sp.* PCC 6803 biomass and (○) with OMWW co-digestion

Table 6.6. BPP for co-digestion assays conducted reported in the literature

Mixture	C/N ratio	BPP (mL/gVS)	Reference
Chlorella vulgaris + potato waste	40.78	340	Zhang et al., (2019)
Chlorella vulgaris + maize silage	16	253	Rétfalvi et al., (2016)
Chlorella sp + swine manure	25	261	Dogan and deminer, 2016
Chlorella sp +wastewater	10.3	431	Wang et al., 2015
Scenedesmus sp + Cellulose	various	450	Bohutskyi et al., 2018
Synechocystis sp PCC 6803 + OMWW	13	209.0±0.7	Our investigation

Although OMWW increased the C/N ratio in our investigation, the BPP achieved is lower than other biogas productions achieved in co-digestion assays as is reported in the previous Table 6.6.

Zhang et al. (2019) reported an increase of methane yield by adding potato processing waste to *Chlorella sp*. biomass: the C/N increased from 6.43 to 40.78 and, the BPP increased from 158 to 340mL/gVS. Rétfalvi et al. (2016) used cooking oil, maize silage and mill residue as a co-substrate for the AD of *Chlorella vulgaris*.

The C/N ratio increased from 5 to 477, 16 and 12, respectively and the methane yield increased from 0.38 L CH_4/LVS to 1.56, 1.19 and 1.16 L CH_4/LVS, respectively. In opposite, OMWW added in our investigation only enhanced 3 times the C/N ratio, reaching a lower C/N lower than the recommended of 25-30 (Calicioglu and Demirer, 2016; Passos et al., 2014; Rétfalvi et al., 2016; Zhang et al., 2019).

Hence, the poor biogas production achieved could be attributed to this low C/N ratio used in our investigation. For future purposes, the carbon-rich substrate added to *Synechocystis sp.* PCC 6803 should be varied in order to reach at least a C/N ratio of 25 to enhance properly the biogas production potential of the mixture.

6.4.4 Co-digestion and microwaving thermal treatment effect over BPP

Finally, an experiment was conducted with both improvements (i.e. co-digestion and microwaving). BPP for this test is represented in Figure 6.3.

This experiment produced the highest BPP of this work (397.7 ±0.5 ml/g VS) and showed again the great BPP enhancement of the use of microwave. The use of both approaches led to a BBP enhancement of 245 %.

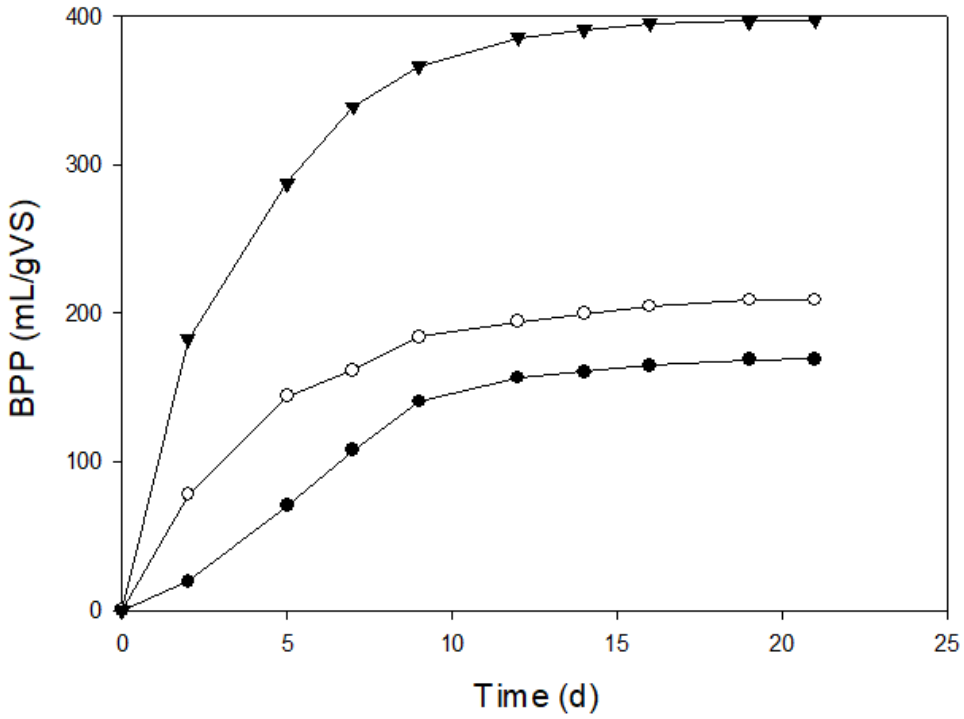

Figure 6.3. BPP for (●) untreated *Synechocystis sp. PCC 6803* biomass, (○) with co-digestion and (▼) with co-digestion and microwave pretreatment

6.4.5 Kinetic model validation for the AD process

The present investigation is the first research using *Synechocystis* sp. PCC 6803 as substrate for AD process and, thus a conventional AD kinetic model was fitted to the experimental results to gain knowledge on the process. Different kinetic models for AD process have been reported (Alzate et al., 2012; Brulé et al., 2014; Veluchamy and Kalamdhad, 2017), but according to experimental results, the proper model used in the literature when lag-phase is detected during the AD assays is represented by modified Gompertz equation. Experimental predictions obtained applying the modified Gompertz model are represented in Figure 6.4.

Figure 6.4 shows the model fit for the first test, without any pre-treatments or any co-digestion agent. The modified Gompertz model fitted accurately the experimental BPP obtained (r^2 = 0.994). The use of the modified Gompertz equation to predict the BPP has been already reported (Table 6.7). The predicted maximum biogas production rate K_M of 20.89mL/gVS/d in our study is in the same range that those ones reported in the literature. Moreover, the lag phase (λ) predicted, 1.53, is quite lower than that reported by Zhang et al., (2019). It means that the anaerobic sludge used in our investigation required less time to assimilate the organic matter and had a quick-response to the substrate inoculated producing biogas.

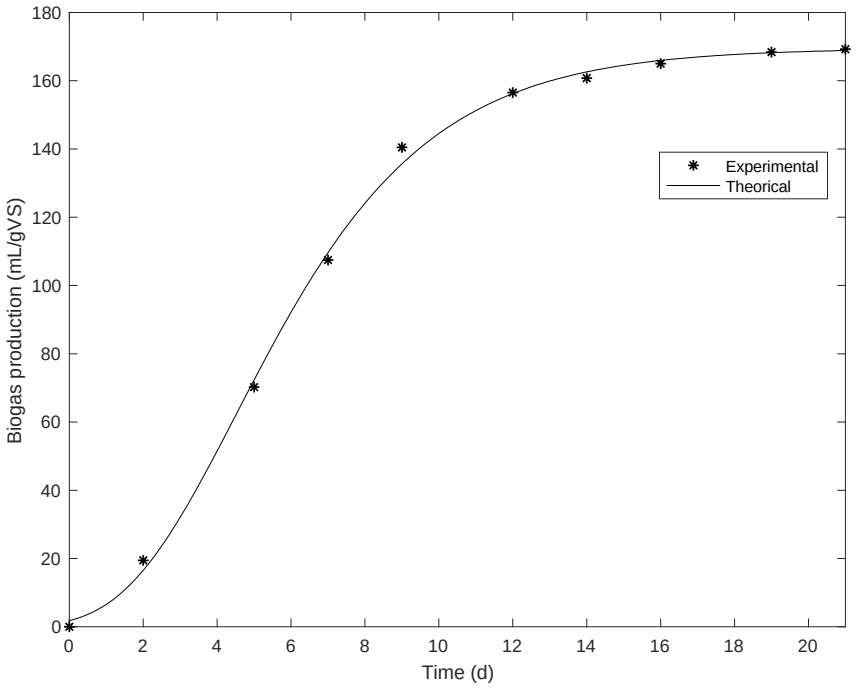

Figure 6.4. Prediction of the BPP for the mono-digestion by modified Gompertz kinetic model

Table 6.7. Kinetic parameters determined by using modified Gompertz equation

Microalgae	**K_M (mL/gVS/d)**	λ (d)	**Reference**
Scenedesmus	26 – 47.4	0.53 – 6.36	Bohutskyi et al., 2018
Chlorella vulgaris	15.8 – 40.9	6.85 – 7.47	Zhang et al., 2019
Synechocystis sp PCC 6803	20.89	1.53	Our investigation

The modified Gompertz equation was also fit to the experimental BPP profiles obtained in thermal pre-treated assays and co-digested test. The kinetic parameters obtained for these assays are displayed in Table 6.8.

Table 6.8. Kinetic parameters calculation for treated and co-digested biomass by using modified Gompertz equation

	M **(mL/gVS)**	**K_M** **(mL/gVS/d)**	**λ** **(d)**
Biomass	169.52	20.89	1.53
Microwaving	311.59	92.51	0.33
Ultrasound	299.08	95.96	0.70
Co-digestion	201.5	58.63	0.60

There exists a clear relation between M and K_M according to the results obtained. For the cases where the production is highly enhanced (microwaving and ultrasonication) the productivity is increased from 20.89 to 95mL/gVS/d representing an increasing of 4 times in comparison to mono-digestion process. Moreover, for the co-digestion assay, M is two times higher than the reached in the mono-digestion test.

On the other hand, lag-phase was reduced when pretreatments were applied indicating that the anaerobic sludge required less time to assimilate the organic matter, probably due to the cell wall disruption. The model relies on two main assumptions, which can affect its range of validity: (a) the maximum productivity initial boundary condition must be similar to the final biogas yield to get an accurate prediction, (b) the K_M and λ boundary conditions should not affect to the final biogas yield obtained in the model results.

Hence, prior knowledge of M can be useful to mitigate potential errors in parameter estimation. An accurate parameter estimation of the three kinetic parameters requires high frequency and good quality data. The kinetic parameters provided in this work would be useful for future research in view of designing energy recovery processes for *Synechocystis sp* PCC 6803.

6.5 Conclusions

The BPP for the cyanobacterium *Synechocystis sp. PCC 6803* was estimated for the first time and promising biogas yields were obtained according to the experimental results reported in our investigation. All thermal treatments, microwaving and ultrasonication, and co-digestion conditions enhanced the initial biogas production from 23.5 to 87% when are compared to the assay with the untreated biomass.

The net energy balance was favourable for microwaving treatment but unsatisfactory for ultrasonication pretreatment. Only in microwaving pretreatment, the energy produced as a consequence of the process application was higher than the energy consumed during the treatment process. Although the energy balance is favourable for microwaving treatment, the net energy determined is not enough to introduce this procedure at full-scale AD industry.

Adding a cos-substrate with a high C/N ration resulted in an enhanced biogas production of 23.5%. This confirms a potential new way to re-use a hard-to-disposal waste: the OMWW. Although the BPP is enhanced by adding OMWW as a co-substrate, the C/N ratio was still lower (13) than the recommended by several authors (25) to conduct an AD process properly. For future purposes is required to modify the carbon-rich substrate in order to enhance the C/N ratio until at least to that one recommended. A final assay using co-digestion and microwaving thermal treatment was conducted to determine both combination effects over the BPP. The use of both approaches led to a BBP enhancement of 245 %.

A modified Gompertz equation was used to fit the experimental results of biogas production for all the anaerobic digestion assays conducted in our investigation and to determine the kinetic parameters describing their behaviour. All kinetic parameters determined are in accordance to that other reported by different investigations conducted previously to our investigation. The same calculations were conducted for all the assays realized showing a clear relation between the lag-phase calculated and the pretreatments applied in the microalgae biomass.

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7 General conclusions

The present thesis has three main conclusions according to the research carried out and can be summarized as follows.

There are several factors that influence the cultivation of microalgae. Light has a strong influence on the metabolism of microalgae. An efficient LI and quality of light supplied to the system, and adequate L/D cycles allow the microalgae the proper conduction of their metabolism. Maintaining an efficient level of light transmission to the cells is also crucial to achieve efficient productivity of biomass and high value organic compounds in microalgae culture. The cultivation strategy also influences the BP of microalgae. Although the organic carbon source has demonstrated increased lipid production in the *Chlorella vulgaris* culture, it is necessary to find experimental conditions that improve the production of biomass and lipids. In addition, the source of organic carbon is expensive compared to photoautotrophic cultivation using CO_2. Accordingly, finding a balance between biomass productivity and associated costs is a challenge that must be taken into account when designing a PBR. Mixing must also be taken into account during the design phase of a PBR. Insufficient mixing in combination with high levels of LI could result in photo-inhibited cell growth. Conversely, insufficient mixing accompanied by low levels of LI could result in photo-limited growth of microalgae. All these parameters must be taken into account during the design phase of a PBR. Sometimes it can be an engineering challenge.

The development of the kinetic model made it possible to describe the behaviour of cyanobacterium *Synechocystis* sp. PCC 6803 during culture. The batch tests carried out in the experimentation phase were used to determine the initial value of the kinetic parameters to be adjusted during the simulation phase. The half-saturation constants determined by the kinetic model showed that the growth of cyanobacteria is highly dependent on the initial concentration of inorganic phosphorus. Some tests changing the initial concentration of phosphorus served to confirm the reduction of cyanobacterial growth under P limitation according to the experimental and predicted values obtained. Some simulations carried out in different L/D cycles made it possible to describe the behaviour of *Synechocystis* sp. PCC 6803 under different light availability. Simulations

performed at 20:4h of the L/D cycle confirmed that the system reached steady state after 4.5 days of cultivation.

The AD tests carried out allowed the determination of the biogas production potential of the depleted biomass of *Synechocystis* sp. PCC 6803 to be described for the first time. The BPP achieved was in the same range as other microalgae biomasses previously investigated and reported in the literature. Thermal treatments were tested to determine the improvement produced over the BPP of *Synechocystis* sp. PCC6803. Although both treatments improved the BPP, the energy balance performed was unsatisfactory for ultrasound pretreatment. In addition, the difference between the energy produced anaerobically and that consumed in the microwave treatment was not sufficient to consider the application of this treatment on a large scale. Some OMWW tests were carried out with the aim of describing the improvement of the BPP by changing the C/N ratio of the substrate. Co-digestion of both (exhausted biomass and OMWW) showed an improvement of about 25% compared to the monodigestion test. The improvement in co-digestion could be increased if the OMWW co-substrate is replaced in future research by another residue with a higher C/N ratio than OMWW. A modified Gompertz equation was used to describe the behaviour of biogas production during the AD tests performed. All the kinetic parameters determined by the model are in agreement with others previously reported in the literature using microalgae biomass to obtain energy by anaerobically digesting it.