CYANOBACTERIA FOR PHB PRODUCTION

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Before you act, listen.
Before you react, think.
Before you spend, earn.
Before you criticize, wait.
Before you pray, forgive.
Before you quit, try.

Ernest Hemingway

.....Se riesci a fare le cose bene,
.....cerca di farle meglio.
.....Sii audace, sii il primo, sii differente,
sii giusto.

Anita Roddick

.....Amare il proprio lavoro è la cosa
.....che si avvicina più concretamente alla felicità
sulla terra.

Rita Levi Montalcini

TABLE OF CONTENTS

RIASSUNTO

La disponibilità delle risorse fossili e la capacità dell'uomo a sfruttarle hanno fatto sì che i prodotti di plastica hanno pervaso la nostra vita sia a livello industriale e sia nella vita quotidiana. I prodotti sviluppati nel secolo scorso hanno trovato un grandissimo impiego nei più diversi campi applicativi, sostituendo con successo i materiali convenzionali fino ad allora usati. I polimeri di sintesi da fonti fossili sono stati nel secolo scorso i protagonisti di una vera e propria rivoluzione del sistema economico-produttivo e sociale. Non si può negare infatti che l'introduzione di tali materiali abbia dato il via ad un mercato di prodotti "usa e getta", che hanno contribuito enormemente al consolidarsi di uno stile di vita consumistico, con evidenti ripercussioni sull'ambiente. Infatti, i vantaggi costituiti dalle eccezionali proprietà di queste materie plastiche si sono scontrati con alcune problematiche legate al loro utilizzo sia sotto forma di prodotti di largo consumo, sia per il loro smaltimento.

Negli ultimi anni la ricerca scientifica ed industriale ha cercato strade alternative ai polimeri "classici", attraverso lo studio e la produzione delle bioplastiche. Esse sono matrici derivate principalmente da zuccheri e dai loro polimeri. Proprio grazie a questa loro derivazione, possono essere degradate facilmente e in tempi rapidi.

Il termine biodegradabile si riferisce alla proprietà di quelle sostanze che una volta rilasciate nell'ambiente vengono degradate, ovvero scomposte e assimilate a seguito dell'attività biologica dei batteri saprofiti, presenti nell'ecosistema naturale. Secondo l'European Bioplastics Associations, con il termine bioplastiche si intendono:

- polimeri con biodegradabilità approvata secondo norma EN 13432, includendo in tal caso sia i polimeri da fonte rinnovabile sia quelli di origine fossile;
- polimeri basati su materie prime rinnovabili (bio-based), includendo sia materiali biodegradabili che non.

Attualmente, secondo l'European Bioplastics Associations, le bioplastiche rappresentano circa l'1% dei 300 milioni di tonnellate di plastica che vengono prodotte ogni anno a livello mondiale. Il mercato delle bioplastiche è però un mercato in costante crescita (circa il 20% per anno), ed è previsto che la produzione mondiale delle bioplastiche possa quadruplicare: da 1.7 milioni di tonnellate nel 2014 a circa 7.8 milioni di tonnellate nel 2019.

Le principali famiglie di biopolimeri presenti oggi sul mercato derivano tutte da fonti rinnovabili e comprendono i polimeri derivati dall'amido, il poliestere derivato dall'acido polilattico (PLA) e quello derivato dai poli-idrossialcanoati (PHA) e i polimeri cellulosici. I poliidrossialcanoati (PHA), poliesteri prodotti naturalmente, stanno guadagnando attenzione nel mercato dei polimeri biodegradabili grazie alla loro elevata biodegradabilità e versatilità. La sfida consiste nella produzione di PHA che ha costi e prestazioni competitivi rispetto alla tradizionale produzione di polimeri a base fossile.

I PHA sono prodotti naturalmente da alcuni batteri all'interno della cellula e possono essere estratti o trasformati per diversi utilizzi. Tra i PHA, il poli-β-idrossibutirrato (PHB) è quello che ha riscontrato maggiore interesse per le sue caratteristiche chimico fisiche: la biocompatibilità, la biodegradabilità e la sua processabilità termoplastica. Queste caratteristiche suggeriscono che il PHB è un potenziale *building block* per la produzione di plastiche.

Scoperto e caratterizzato nel 1925 dal microbiologo francese Maurice Lemoigne, il PHB è prodotto da microrganismi (come *Ralstonia eutropha* o *Bacillus megaterium*) in risposta a condizioni di stress fisiologico. Il polimero è soprattutto un prodotto della assimilazione del carbonio da fonti quali il glucosio, amido o CO_2, ed è impiegato dai microrganismi come molecola di riserva da metabolizzare quando altre

fonti di energia non sono disponibili. Tra i microorganismi che producono PHB come metabolita secondario, grande importanza è stata data ai cianobatteri i quali essendo microorganismi fotosintetici, usano la luce del sole per convertire la CO_2 in prodotti di interesse come il PHB.

I cianobatteri, noti anche come alghe blu-verdi, sono batteri Gram negativi, autotrofi. I cianobatteri sono degli ottimi canditati per essere impiegati a livello industriale grazie a diverse caratteristiche di interesse. Essi infatti sono caratterizzati da un breve tempo di duplicazione. Per la crescita hanno bisogno di semplici nutrienti inorganici come il fosfato, nitrato, magnesio e calcio come macronutrienti, ferro, manganese, zinco, cobalto e rame come micronutrienti.

Da un punto di vista biotecnologico rappresentano delle vere e proprie "*cell factories*" da cui ottenere diversi tipi di prodotti di interesse. Sono in grado di accumulare una varietà di materiali di riserva come, cianoficine, polifosfati, polisaccaridi e PHB. Alcuni ceppi di cianobatteri possono anche essere utilizzati nel campo della biorimediation e per l'assimilazione dei metalli pesanti. I cianobatteri inoltre, producono diversi tipi di sostanze tra cui tossine, pigmenti che possono essere utilizzati come coloranti, vitamine e importanti biopolimeri.

Synechocystis PCC 6803 è considerato un ceppo modello tra i cianobatteri. *Synechocystis* PCC 6803 è il primo organismo fotosintetico di cui è stato sequenziato l'intero genoma. È un organismo naturalmente trasformabile, geneticamente e fisiologicamente ben caratterizzato. La sua crescita è abbastanza veloce (per il tempo di raddoppiamento della concentrazione cellulare sono sufficienti meno di sette ore) e non ha particolari esigenze nutrizionali.

Scopo della tesi. Lo scopo del lavoro svolto durante il DdR è stata la produzione di poliidrossibutirrato da colture fotoautrofica di cianobatteri. Il lavoro è stato svolto presso il Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale dell'Università degli Studi di Napoli Federico II e presso il Molecular Microbial Physiology Group of the Swammerdam Institute for Life Sciences in Universiteit van Amsterdam.

Le attività sono state articolate in tre linee: i) caratterizzazione macroscopica del processo di produzione di PHB in termini cinetici e di resa, utilizzando terreni di crescita caratterizzati da diverse concentrazioni di nitrati e selezione di ceppi di cianobatteri; ii) caratterizzazione del processo di produzione del PHB in accordo a modelli cinetici dettagliati; iii) utilizzo di tecniche di ingegneria genetica per incrementare la produzione di PHB da cianobatteri.

Processo di produzione del PHB: valutazione dell'effetto dei nitrati e selezione di ceppi di cianobatteri. Lo studio ha riguardato la valutazione delle cinetiche e delle rese cellulari e del PHB prodotto durante la crescita su terreni caratterizzati da una diversa concentrazione di nitrati: BG_{11} (concentrazione ottimale di nitrati); $BG_{1/2}$ (la concentrazione dei nitrati è metà di quella ottimale); $BG_{1/4}$ (la concentrazione dei nitrati è un quarto di quella ottimale) e BG_0 (privo di nitrati).

Le prove di ottimizzazione del mezzo di coltura per la produzione di PHB, sono state condotte con *Synechocystis* PCC6803. I risultati della fase preliminare di ottimizzazione del mezzo di crescita hanno mostrato che il terreno a concentrazione di nitrati pari alla metà rispetto a quella ottimale per la crescita era il terreno più promettente per la produzione di PHB. Infatti, si è ottenuta una frazione di PHB del 8%: circa 3.5 volte più alto rispetto a quello ottenuto alla concentrazione ottimale di nitrati (Figura 1 A).

Cinque ceppi di cianobatteri sono stati investigati utilizzando il terreno di coltura ottimizzato ($BG_{1/2}$) per selezionare il ceppo iper produttore di PHB: *Synechocystis* PCC6803, *Synechocystis aquatilis*, *Synechocystis fuscopigmentosa*, *Synechoccoccus nidulans* e *Chlorogloeopsis Fritshii* (Figura 1A).

Le prove di selezione dei ceppi hanno dimostrato che *Synechocystis* PCC6803 e S. *aquatilis* sono i migliori ceppi produttori di PHB. In entrambi i casi la frazione massima di PHB è di circa l'8% e la produttività massima è di 7 g/m^3giorno.

I risultati sono stati molto promettenti per supportare la crescita autotrofica di colture di cianobatteri al fine di produrre PHB utilizzando un substrato economico e abbonante come l'anidride carbonica. La produzione di PHB da colture autotrofiche può essere proposto quindi, come un processo eco-sostenibile in quanto unisce la produzione di un biopolimero alla cattura della CO_2 dall' ambiente.

***Modellazione cinetica della produzione del PHB in cianobatteri*.** AL fine di progettare ed ottimizzare efficacemente il processo di produzione di PHB da cianobatteri è necessario conoscere i micro e macro meccanismi alla base del processo stesso. L'ottimizzazione della produzione del PHB non è infatti un problema banale in quanto la produzione del PHB da microorganismi coinvolge diversi *pathway* metabolici. Un modello che descriva il processo, sembra essere la chiave per comprendere come massimizzare la produzione di PHB. In letteratura sono disponibili diversi modelli della crescita di microrganismi fotosintetici e della produzione di PHB da batteri non-fotosintetici. È stato infatti sviluppato un modello cinetico per la caratterizzazione del processo di produzione del PHB da cianobatteri.

A conoscenza dell'autore, non c' disponibilità di modelli di produzione di PHB da colture autotrofiche di cianobatteri. Di seguito sono riportati alcuni lavori che descrivono la crescita di organismi fotosintetici e la produzione di PHB da organismi non fotosintetici che sono stati presi in considerazione per lo sviluppo del modello matematico.

Kasiri *et al*., (2015) considerano il disaccoppiamento tra la cinetica di assimilazione dei nutrienti e la crescita cellulare. Essi propongono un modello dinamico non lineare per valutare le condizioni ottimali di CO_2, dei fosfati e dell' intensità luminosa per l' assimilazione della CO_2 e la crescita algale.

Il modello proposto da Kasiri *et al.,* (2015) è però un modello focalizzato solo sulla crescita algale e non prende in considerazione la produzione di metaboliti secondari. Kim *et al.*, (2015), propongono invece un modello cinetico multi-componente testando *Synechocystis* sp. PCC6803 in condizioni batch che permettono di estimare indipendentemente i parametri cinetici relativi a l'intensità luminosa e la concentrazione dei nutrienti inorganici.

Jeon *et al.*, (2014) propongono di integrare il modello del network metabolico con un modello semi-empirico (modello di Droop) per predire l'accumulazione simultanea di lipidi e biomassa nella microalga *Chlamydomonas reinhardtii*. Il modello proposto da Jeon et al., (2014), non include l'effetto dell' intensità luminosa sulla crescita microalgale.

Mozumder *et al*., (2015), descrivono la produzione autotrofica de PHB da *Cupriavidus necator*, usando un modello basato su un processo a due fasi: la crescita della biomassa seguita dalla fase di produzione del PHB. Faccin *et al*., (2012) hanno presentato un modello cinetico che descrive colture di *Bacillus megaterium* per la produzione di PHB. Khanna e Srivastava (2005), hanno proposto un modello strutturato e non segregativo per l' accumulazione di PHB in *Ralstonia eutropha* basato

su un' investigazione dettagliata sulla limitazione da substrato e inibizione da fonti di carbonio, azoto e fosforo.

I modelli proposti da Mozumder *et al*., Faccin *et al*., e Khanna & Srivastava, riguardano però la produzione di PHB non da microorganismi fotosintetici.

Un modello cinetico della produzione di PHB da *Synechocystis* PCC6803 da CO_2 è stato sviluppato utilizzando il simulatore biochimico COPASI. I dati misurati nel corso di prove di crescita di *Synechocystis* PCC6803 in un sistema dinamico di luci (ciclo luce/buio) variando la concentrazione iniziale dei nitrati presenti nel terreno di coltura, sono stati impiegati per la valutazione dei parametri cinetici e stechiometrici (Figura 1 B). Il modello è basato su bilanci di massa in condizioni transitorie riferiti a substrati, cellule in crescita (X), cellule produttrici di PHB (X_{PHB}), e a PHB. Sono stati considerati gli effetti di fattori significanti e dei nutrienti sulla crescita e produzione di metaboliti da cianobatteri. In particolare il modello proposto, include la velocità di utilizzazione di nitrati e fosfati, la velocità di produzione del PHB, la crescita cellulare e la velocità di lisi cellulare. La velocità specifica di formazione delle X_{PHB} (k_T), è stata considerate essere dipendente dalla concentrazione iniziale di nitrati.

È stata anche fatta un'analisi di sensitività per i parametri che influiscono sulla velocità di crescita delle cellule e la velocità di produzione del PHB. Il modello proposto ha dato risultati soddisfacenti per ognuno dei terreni investigati: il coefficiente di correlazione (r^2) della concentrazione dei metaboliti, calcolato confrontando esperimenti e simulazioni, è contenuto in un frange che varia tra 0.81 e 0.99.

Modifica genetica di cianobatteri per aumentare la produzione di PHB.

Durante il progetto di DdR, il ceppo *Synechocystis* PCC6803, è stato geneticamente modificato con lo scopo di incrementare la produzione di PHB da CO_2. Il pathway di formazione del PHB a partire dall' acetil-CoA coinvolge tre step catalitici. Il primo step consiste nella condensazione di due molecole di acetil-CoA in acetoacetil-CoA grazie all' azione della PHA beta-chetotialase (phaA). Questo primo step è seguito dalla riduzione dell'acetoacetil-CoA in (R)-3-idrossibutirril-CoA grazie all' azione della acetoacetil-CoA reduttasi (phaB). Infine il terzo step consiste nella condensazione del (R)-3-idrossibutirril-CoA in PHB grazie all' azione della PHA sintasi (phaC/phaE) (Khetkorn *et al.*, 2016).

La modifica del metabolismo è stata indirizzata alla riduzione della produzione di acetato mediante la delezione della fosfotransacetilasi (*pta*) e dell'acetil-CoA idrolasi (*ach*). Inoltre, la sovraespressione della fosfochetolasi (*xfpk*) è stata usata come strategia per incrementare la produzione di acetil-CoA e di conseguenza, del PHB (Figura 1 C).

Sette ceppi mutanti sono stati creati considerando il knock out di *pta* e di *ach* e la sovraespressione di *xfpk*, singoli o combinati tra loro. I ceppi ottenuti sono stati cresciuti in fotobioreattori a colonna, in condizioni di fotoautotrofia (ritmo circadiano: 18h di luce/6h di buio) in BG_{11} come terreno di crescita per verificare se le mutazioni prese in esame hanno comportato un incremento nella produzione di PHB.

I risultati hanno dimostrato che solo nei ceppi in cui è stato sovraespresso il gene codificante la fosfochetolasi (*xfpk),* c'è stato un incremento nella produzione del PHB rispetto al ceppo *wild type*. Questi ceppi sono poi stati ulteriormente testati nel terreno i cui la concentrazione iniziale di nitrati è la metà rispetto a quella ottimale ($BG_{1/2}$) per verificare se c'è un ulteriore aumento di PHB.

Il ceppo più promettente è stato quello in cui *pta* e *ach* sono stati soppressi e *xfpk* è stato sovraespresso. Infatti il PHB ha raggiunto una frazione di circa il 21% un valore che è 3.5 volte superiore rispetto al ceppo wild type.

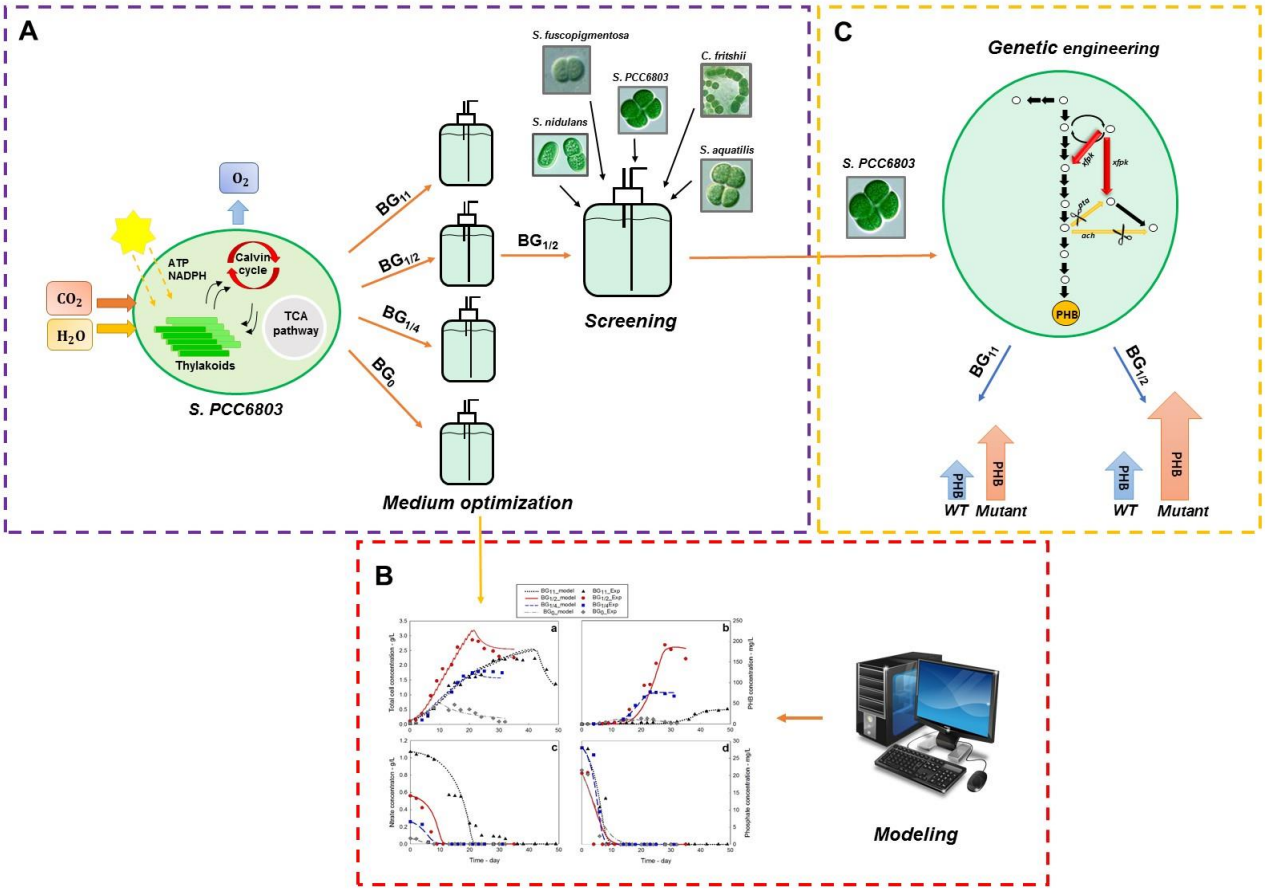

Figura 1 Schema riassuntivo del progetto di dottorato: A) ottimizzazione del mezzo di coltura e selezione dei ceppi; B) modellazione del metabolismo e crescita; C) ingegnerizzazione genetica per ottimizzare la produzione di PHB da cianobatteri.

SUMMARY

The study carried out during the present Ph.D. program aimed at investigating the PHB production process in autotrophic cultures of cyanobacteria. The work was carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale of the University of Naples 'Federico II' and at Molecular Microbial Physiology Group of the Swammerdam Institute for Life Sciences in Universiteit van Amsterdam. The activities were articulated according to three paths: i) the characterization of PHB production process as regards kinetics and yields using different growth media characterized by different nitrate concentration and screening cyanobacteria strains; ii) the characterization of the PHB production process by cyanobacteria according to kinetic models; iii) the development of genetic engineering approach on cyanobacteria to improve PHB production.

Selection of the optimal growth media to produce PHB and screening of cyanobacteria strains: characterization in terms of kinetics and yields.

The study was aimed at the assessment of both the kinetics and the yields of the cell growth and PHB produced during the growth on different growth media characterized by different nitrate concentrations: BG_{11} (optimal nitrate concentration), $BG_{1/2}$ (half of optimal nitrate concentration), $BG_{1/4}$ (one fourth of the optimal nitrate concentration) and BG_0 (nitrogen-starved conditions). Batch tests were focused on the preliminary characterization of the PHB production process from cyanobacteria with the aim of highlighting the relevant features of the process. *Synechocystis* PCC6803 was growth as model strains on the different culture media. The investigation was carried out in photobioreactors using CO_2 as carbon source. The best media for PHB production was $BG_{1/2}$ (half of optimal nitrate concentration).

Five cyanobacteria strains were screened using $BG_{1/2}$ medium, to select a high PHB producer: *Synechocystis* PCC6803, *Synechocystis aquatilis*, *Synechocystis fuscopigmentosa*, *Synecoccoccus nidulans* and *Chlorogloeopsis Fritshii*.

Characterization of the PHB production process: kinetic models.

An adequate kinetic model to describe the PHB production process is a key issue to address the conditions to maximize the PHB fraction in the cell. A kinetic model of PHB production by *Synechocystis* PCC6803 from CO_2 was proposed using the biochemical networks simulator COPASI. Data form growth tests under dynamic light system (light/dark cycle) carried out at different initial nitrate concentration were used to assess the kinetic and stoichiometric parameters of the proposed model.

Two types of cells were considered in the cultures: growing cells (X), which are able to grow, and PHB producing cells (X_{PHB}). The specific velocity (k_T) of X_{PHB} formation was considered dependent from the initial concentration of nitrate.

The proposed model includes the nitrate and phosphate utilization rate, the PHB production rate, the cell growth and lysis rate. The model adequately predicted the experimental data, indeed the square correlation coefficient of metabolite concentrations, calculated by comparing experiments and simulations, ranged between 0.81 and 0.99.

Genetic modified cyanobacteria strain to improve PHB production.

In this study *Synechocystis* sp. PCC6803 was genetically modified with aim to improve PHB production from CO_2. Seven mutant strains were created by single and combined mutations: deletion of phosphotransacetylase (*pta*); the deletion of acetyl-CoA hydrolase (*ach*); the overexpression of phosphoketolase (*xfpk*). The tests were carried out on BG_{11} medium. The mutant strain that gave best performance on BG_{11} medium was tested on optimized medium ($BG_{1/2}$ medium).

1. INTRODUCTION

The global chemical industry is playing a vital role for the development of new, innovative products and more efficient technologies. Companies may make their current manufacturing processes more efficient and may reorganize their research and development functions by focusing on environmentally-friendly technologies such as green chemistry (Iles & Martin, 2013). Since the 1990s, many companies have moved from pollution prevention to green chemistry as a more fundamental route for a sustainable production. Green chemistry was referred to chemical production aimed to reduce environmental and health hazards and to advance sustainability (Clark & Deswarte, 2008). Companies have focused on green chemistry process innovations that make conventional chemical production more efficient and less expensive (Eder, 2003; Radonjič & Tominc, 2007). According to this point of vires, a major challenge is to find routes to integrate sustainable innovation into the chemical industry's business models. The case of bioplastics illustrates how business models can play a central role in linking producers and customers through the development of new technologies and products.

1.1 Bioplastics: features and market

Plastics are materials widely used all over the world. Nowadays, plastics are indispensable as components of cars, home appliances, computer equipment, packaging and medical tool/devices. Although mechanical-technological features of plastics have worldwide been recognized, plastics have been vilified because they are environmentally unfriendly. Indeed, plastics are not biologically degradable and accumulate in the environment producing a large spectrum of pollutants (Sharma *et al.*, 2007). As a consequence, serious environmental concerns like safe disposal, solid waste management, and plastic waste incineration justify the production of materials like green polymers/biodegradable plastics/bioplastics utilizing renewable resources (Singh *et al.*, 2016).

Bioplastics have a history of approximately 150 years. The first artificial thermoplastic "celluloid" was produced from nitrocellulose in the 1860s. Since then, numerous new compounds were produced from renewable resources. The advent of the fossil-derived plastics caused the industrial failure of plastics derived from renewable resources: synthetic polymers have been successfully produced at industrial scale since the 1950s. The oil price shock at the end of the last millennium, and the environmental consciousness - matured during the last decades - have renewed the interest in bio-based chemistry and in particular in bio-based plastics (http://www.european-bioplastics.org/).

Biodegradable plastics are a class of bioplastics that are produced from biomasses (biobased materials) and are biodegradable: some microorganisms are able to convert them into simple, elemental, substances (e.g. water, carbon dioxide). The biodegradable plastics can be classified into four categories (reported in Table 1.1): photodegradable, semi-biodegradable, chemically synthesized, and polyhydroxyalkanoates (PHAs) (Singh *et al.*, 2016).

Nowadays, bioplastics are about one per cent of the about 300 million tonnes of plastic produced per year. However, the bioplastic market is growing by about 20 to 100 per cent per year to fulfil the rising demand and the request for more sophisticated materials, applications, and emerging products. According to the latest market data compiled by European Bioplastics, global production capacity of bioplastics is predicted to quadruple in the medium term: from about 2 million tonnes in 2014 to about 8 million tonnes in 2019 (http://www.european-bioplastics.org/).

Table 1.1: Classification of biodegradable plastics Types

Types of bioplastics	Characteristic features
Photodegradable bioplastics	The polymer contains groups that are sensitive to light.
Semi-biodegradable bioplastics	Composed by starch and conventional plastic fragments, where the starch acts as filler (polyethylene.) The starch that is incorporated in semi-degradable plastics can be attacked and degraded by microorganisms directly, thus releasing the remaining small polyethylene fragments for successive degradation.
Chemically synthesized bioplastics	Includes polyvinyl alcohol, polyethylene oxide, polyglycollic acid, poly(ε-caprolactone), and polylactic acid (PLA) that are vulnerable to enzymatic or microbial attack.
Polyhydroxyalkanoates bioplastics	They are 100% microbial origin biodegradable plastics composed of various hydroxyalkanoic acids (HAs) as monomer units.

1.2 Poly-β-hydroxybutyrate (PHB)

Polyhydroxyalkanoates (PHAs) are fundamental building blocks for biodegradable plastics. PHAs are microbial polyesters that may be produced by various microorganisms, cyanobacteria included. PHAs are among the most investigated biodegradable polymers in recent years, due to their chemical and physical features that include: non-toxicity, biocompatibility, and biodegradability.

PHA can be subdivided into three groups: short-chain-length-PHAs (SCL-PHAs), medium-chain-length-PHAs (MCL-PHAs) and long-chain-length-PHA (LCL-PHA) (Brandl *et al.*, 1988; Nomura *et al.*, 2005; Steinbüchel & Wiese, 1992). The polyhydroxybutyrate (PHB) (Figure 1.1) is the most common of the SCL-PHAs. PHBs have been characterized because of the interesting features: thermoplastic processability, hydrophobicity, complete biodegradability, and biocompatibility with optical purity (Braunegg *et al.*, 1998).

The production capacity of PHB is growing steadily in the last years. In particular, according to European-bioplastics (http://www.european-bioplastics.org/), the global production of PHB was about 34 ktonnes in 2014 and it is forecasted to grow at a 100 ktonnes in 2019.

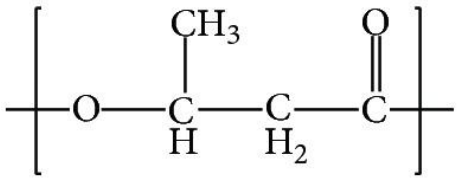

Figure 1.1: General structure of poly-β-hydroxybutyrate

1.3 Conventional production system of PHB

Commercial PHB production is based on the pure cultures of natural PHA producers, such as *Ralstonia eutropha*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Nocardia*, *Pseudomonas*, *Rhizobium* (Chandani *et al.*, 2014; Jiang *et al.*, 2011). Under optimal conditions, bacteria such as *Cupriavidus necator* H16 (previously known as *Ralstonia eutropha*) can produce PHB up to 80% of their cellular dry weight using fructose as the carbon source (Budde *et al.*, 2011a). In Table 1.2 reports an overview of bacterial

strains used to produce PHAs, the feedstock used as carbon sources, and the produced copolymers.

Table 1.2: Overview of bacterial strains used to produce PHB.

Bacterial strain	Carbon source	Polymer (s)	PHB %	References
Aeromonas hydrophila mutants	Lauric acid, oleic acid	MCL-PHA	64	(Han *et al.*, 2004)
Alcaligenes latus	Malt, soy waste, milk waste,	PHB	70	(Wong *et al.*, 2004)
Bacillus cereus	Glucose	PHB	9	(ŁAbuzek & Radecka, 2001)
Bacillus spp.	Glucose	PHB	67	(Thirumala *et al.*, 2010)
Burkholderia sacchari sp.	Glucose	PHB, PHBV	68	(Brämer *et al.*, 2001)
Burkholderia cepacia	bagasse	PHB	62	(Silva *et al.*, 2004)
Enterobacter aerogenes	wastewater	PHB	43	(Ceyhan & Ozdemir, 2011)
Caulobacter crescentus	glucose	PHB	18	(Qi & Rehm, 2001)
Escherichia coli mutants	Xylose	PHB	27	(Nikel *et al.*, 2006)
Halomonas boliviensis	Starch, hydolysate, maltose, maltotetraose and maltohexaose	PHB	56	(Quillaguamán *et al.*, 2005)
Legionella pneumophila	Nutrient broth	PHB	16	(James *et al.*, 1999)
Methylocystis sp.	Methane	PHB	51	(Wendlandt *et al.*, 2005)
Microlunatus phosphovorus	Glucose, acetate	PHB	30	(Akar *et al.*, 2006)
Pseudomonas aeruginosa	Agro-industrial oily wastes	MCL-PHAs	66	(Fernández *et al.*, 2005)
Pseudomonas hydrogenovora	Dairy whey	MCL-PHA	21	(Koller *et al.*, 2008)
Pseudomonas putida	Petrochemical plastic waste	PHB	30	(Goff *et al.*, 2007)
Rhodopseudomonas palustris	Acetate	PHB	34	(Mukhopadhyay *et al.*, 2005)
Cupriavidus necator	Corn syrup	PHB	30	(Daneshi *et al.*, 2010)
Azotobacter chroococcum H23	Waste water from olive oil mills	PHB	80	(Pozo *et al.*, 2002)
Ralstonia pickettii 61A6	Sugar cane liquor	PHB	10	(Bonatto *et al.*, 2004)
Pseudomonas fluorescens A2a5	Sugar cane liquor	PHB	70	(Jiang *et al.*, 2008)
Bacillus megaterium ATCC 6748	Corn steep liquor and molasses	PHB	43	(Chaijamrus & Udpuay, 2008)
Mixed Microbial Culture	Lactate	PHB	90	(Jiang *et al.*, 2011)

The high cost of the raw materials for PHB production by bacterial fermentation makes this process not economically competitive. Therefore, alternative low cost photosynthetic production system have been proposed (Mittendorf *et al.*, 1998; Poirier *et al.*, 1992). However, plant-based expression systems compete directly with subsistence crops for agricultural acreage. Moreover, further ethical concerns as the dissemination of transgenic plants is difficult to control, which has led to strict regulatory restrictions of transgenic plants in many countries (Hempel *et al.*, 2011).

Cyanobacteria, share all the advantages of photosynthetic microorganisms when compared to chemoheterotrophs. They are characterized by the potential to exploit sunlight energy and to convert CO_2 into product of interest such as PHB. The two main advantages of these systems are: i) they do not compete with the agro-food market for resources (Gopi *et al.*, 2014; Gupta *et al.*, 2013; Wijffels *et al.*, 2013); they contribute to capture and to use the CO_2 decreasing the pressure of the greenhouse gas release in the atmosphere.

1.4 Cyanobacteria and the PHB

Cyanobacteria are prokaryotic organisms. In the recent past, the interest for cyanobacteria increased remarkably because of their potential to contribute to the bio-based economy. Their photosynthetic metabolism can be addressed to synthesize valuable products from CO_2, sunlight and water, O_2 being the only bio product (Wijffels *et al.*, 2013). Cyanobacteria may carry out the oxygenic photosynthesis: to capture free energy from the (sun)light for the synthesis of ATP and NADPH (Gopi *et al.*, 2014).

The ancestors of cyanobacteria evolved oxygenic photosynthesis more than 3.2 billion years ago. They were responsible for the oxygen enrichment in the - originally anoxic - atmosphere of the earth. This event changed the life on earth forever and conditions became favourable for aerobic organisms. Oxygenic photosynthesis further evolved in the thylakoid membranes of the 'primordial cyanobacteria' (Blankenship, 2010).

A main reason for the evolutionary hardiness of cyanobacteria is their successful combination of effective metabolic pathways. They are among the few groups that can carry out oxygenic photosynthesis and respiration simultaneously in the same compartment. Moreover, many cyanobacterial species are able to fix nitrogen. Therefore, they can survive and prosper under a wide range of environmental conditions (Vermaas, 2001).

The combination of photosynthesis and respiration in a single compartment is a unique characteristic. Photosynthesis and respiration require electron transport pathways that are catalysed by protein complexes in membranes. Figure 1.2 illustrates the compartmentalization of the cyanobacterial cell.

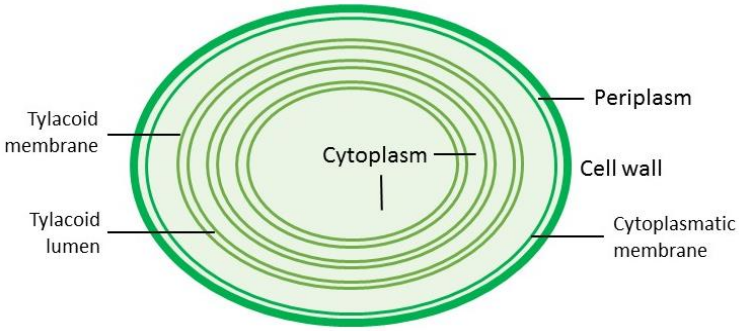

Figure 1.2: Membranes and compartments in a cyanobacterial cell (Vermaas, 2001).

The thylakoid membrane, the internal membrane system that separates the cytoplasm from the lumen and that contains both photosynthetic and respiratory electron transport chains. The oxygenic photosynthesis (the conversion of CO_2 and water to sugars using the energy from sunlight) essentially is the reverse of respiration (conversion of sugars to CO_2 and water and releasing energy). The cytoplasmic membrane, separating the cytoplasm from the periplasm, contains a respiratory electron transport chain but not photosynthetic complexes in most cyanobacteria.

Therefore, photosynthetic electron transport in most cyanobacteria occurs in thylakoids, whereas respiratory electron flow takes place in both the thylakoid and cytoplasmic membrane systems. A schematic representation of the respiratory and photosynthetic electron transport chains in cyanobacterial thylakoid membranes is shown in Figure 1.3.

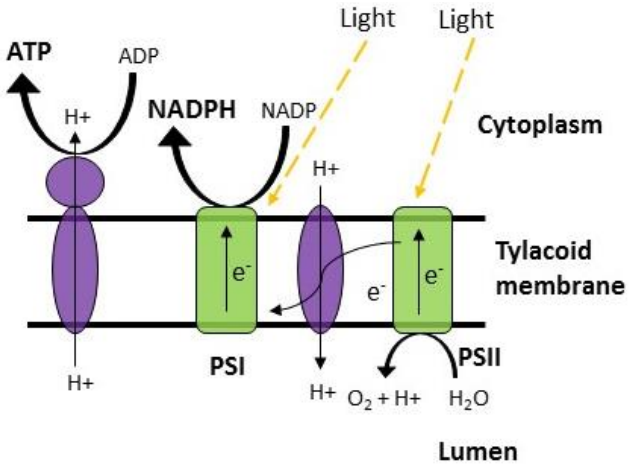

Figure 1.3: Schematic representation of the intersecting photosynthetic and respiratory electron transport pathways in thylakoid membranes of the cyanobacterium *Synechocystis* sp. PCC 6803

The phylum of cyanobacteria was initially divided according to the different morphology (Figure 1.4). The spectrum of cyanobacterium morphology includes: unicellular spherical shape; unicellular cylindrical shape; spiral forms. They can also form multicellular consortia. Filamentous strains form specialized cells, emerging through differentiation, into photosynthetic cells and nitrogen-fixing heterocysts.

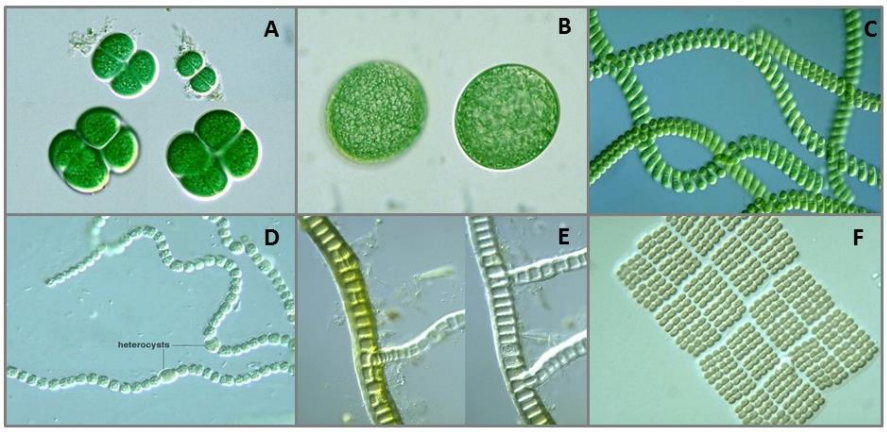

Figure 1.4: Morphologic spectrum of cyanobacteria (Rippka *et al.*, 1979). A): *Synechocystis* sp., B) *Cyanothece major*, C) *Spirulina sp*. D) *Nostoc sp*. E) *Fischerella major*, F) *Merismopedia glauca*.

The spectrum of potential applications of cyanobacteria or of their components is very large. Cyanobacteria have been a food and feed source. Strains of *Spirulina* are used as food supplement for their richness in vitamins and proteins.. Cyanobacteria are also effective as fertilizers. Nitrogen-fixing strains provide support for plant growth in rice fields (Abed *et al.*, 2009). Cyanobacteria also got some attention for the production of pharmaceuticals including anti-fungal, anti-bacterial and anti-cancer agents. Cyanophycin - a polymer built from aspartate and arginine - is considered a minor intracellular carbon and nitrogen storage compound of

cyanobacteria: it can replace polyacrylate (which is derived from propylene and thus from petroleum) in paints and surface coatings. Phycocyanin - a protein-pigment complex that cyanobacteria produce as part of their phycobilisomes - is used as a food colorant in cosmetics and in diagnostics (Rezanka & Dembitsky, 2006).
Some cyanobacterium strains naturally produce large amounts of polyhydroxyalkanoates (e.g. polyhydroxybutyrate) (Table 1.3). Cyanobacterial species may accumulate the homopolymer of PHB under photoautotrophic conditions. As shown in Table 1.3, Campbell *et al*., (1982), Suzuki *et al*., (1996) and Haase *et al*., (2012) produced 6%, 1% and 22%, respectively, of PHB under photoautotrophic conditions with cyanobacteria *Spirulina platensis, Synechococcus sp.* PCC 7942 and *Nostoc muscorum* respectively. PHB can be produced by cyanobacteria also in heterotrophic conditions using different carbon sources (eg. acetate, propionate, glucose, fructose). Table 1.3 reported some examples from literature. Stal *et al*., (1990), Takahashi *et al.* (1998), Panda *et al*., (2006) and Toh *et al*., (2008) produced PHB using acetate for growth of *Gloeothece* sp. PCC 6909, *Synechococcus* sp. PCC7942, *Synechocystis* sp. PCC 6803 and *Spirulina platensis* UMACC 161 respectively. Lama *et al.,* (1996) produced the PHB polymer from *Anabaena cylindrica* using propionate in growth media as carbon source while Samantaray & Mallick (2012) produced PHB from *Aulosira fertilissima* CCC 444 using fructose. PHB can be used as a specialized biomass component for storage of carbon and energy and is accumulated in a variety of microorganisms under conditions of nutrient imbalance (Wu *et al.*, 2001). Cyanobacteria can be considered as an alternative host system for the production of PHBs because they are photoautotrophic and require minimal nutrients. They need some simple inorganic salts such as phosphate, nitrate, magnesium and calcium, as macro-, and ferrous, manganese, zinc, cobalt, and copper as micronutrients for their growth and multiplication (Balaji *et al.*, 2013).

Synechocystis sp. PCC 6803 is the best-characterized species. It is a naturally transformable organism, genetically and physiologically well characterized. Its growth is quite fast (minimal doubling time less than seven hours) and it does not have specific nutritional demands (Angermayr *et al.*, 2009).

In many natural environments in which cyanobacteria live, the availability of phosphorus and combined nitrogen is growth-limiting. Therefore, microorganisms have evolved various mechanisms survive under these conditions (Hauf *et al.*, 2013a). Unicellular non-diazotrophic cyanobacteria (e.g. *Synechocystis* PCC6803) respond to nitrogen limitation by a process termed chlorosis (Gorl *et al.*, 1998). The light harvesting complexes are degraded and photosynthetic activity declines concomitant with degradation of thylakoid membranes (Figure 1.5) (Schwarz & Forchhammer, 2005). The response of cyanobacteria to nitrogen starvation largely depends on the NtcA (nitrogen control protein A) transcriptional regulator and PII-system. NtcA is a transcriptional activator and in some cases a repressor of a large number of genes mainly involved in nitrogen metabolism which induces alteration of gene expression as well as metabolic adaptation to altered nutrient availability (Llácer *et al.*, 2010; Schlebusch & Forchhammer, 2010). During nitrogen starvation, carbon polymers like glycogen (Schwarz & Forchhammer, 2005) and in some species PHB are accumulated (Panda *et al.*, 2006). The expression of the genes coding for precursor biosynthesis of PHB in *Synechocystis* sp. PCC 6803 is up-regulated upon nitrogen starvation (Schlebusch & Forchhammer, 2010).

Table 1.3: Literature comparison about accumulation of PHA in cyanobacteria

Cyanobacteria strain	Culture conditions	Polymer	Polymer content (%)	References
Arthrospira (Spirulina) platensis	Photoautotrophic	PHB	6	(Campbell *et al.*, 1982)
Gloeothece sp. PCC 6909	Acetate	PHB	9	(Stal *et al.*, 1990)
Anabaena cylindrica	Propionate	P(3HB-co-3HV)	2	(Lama *et al.*, 1996)
Recombinant *Synechococcus sp.* PCC 7942	CO_2	PHB	1	(Suzuki *et al*., 1996)
Recombinant Synechococcus sp. PCC7942	Acetate + nitrogen deficiency	PHB	26	(Takahashi *et al.*, 1998)
Nostoc muscorum	CO_2	PHB	22	(Haase *et al.*, 2012)
Synechocystis sp. PCC 6803	Phosphate-deficiency + gas exchange limitation +acetate	PHB	38	(Panda *et al.*, 2006)
Spirulina platensis UMACC 161	Acetate and CO_2	PHB	10	(Toh *et al.*, 2008)
Aulosira fertilissima	Citrate + acetate and K_2HPO_4	PHB	85	(Samantaray & Mallick, 2012)
Nostoc muscorum Agardh	Nitrogen deficiency + acetate + valerate	P(3HB-co-3HV)	60	(Bhati & Mallick, 2012)
Aulosira fertilissima CCC 444	Fructose + valerate	P(3HB-co-3HV)	77	(Samantaray & Mallick, 2014)

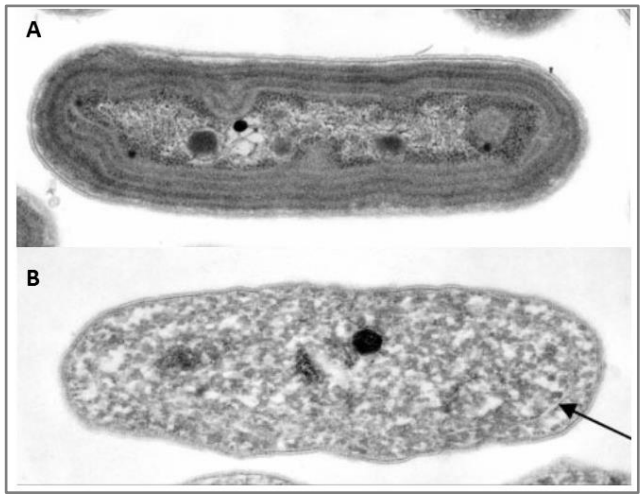

Figure 1.5: Morphological changes in cyanobacteria after nitrogen starvation (Schwarz & Forchhammer, 2005): A) optimal growth conditions; B) nitrate-deplete conditions. After chlorosis process activation there was a complete degradation of thylakoids.

1.5 Kinetics of PHB production

The potential of PHBs to replace conventional plastic materials justifies the increasing attention of researchers and industries involved in the blue biotechnology. The improvement of large-scale productivity and biochemical/genetic properties of producing strains requires mathematical modelling and process/strain optimization procedures (Luengo *et al.*, 2003).

Three class of PHB producer microorganisms may be identified:

a) Strains displaying strict separation between the biomass growth phase and the PHA production phase, typically induced by N or P limitation (prototype organisms: *Pseudomonas* sp. 2F, *Methylmonas extorquens*) (Braunegg *et al.*, 1998);

b) Strains that accumulate PHB already under balanced nutritional conditions and increase the fraction of PHB under the non-growth phase (usually induced by N or P limitation) (prototype organism: *Cupriavidus necator*) (Novak, 2015);

c) Strains displaying high PHB formation rates during growth phase without limitation of an essential growth component (Hartmann *et al.*, 2006; Koller *et al.*, 2010) (prototype organisms: *Azohydromonas lata* DSM 1122, or *Pseudomonas putida* GPo1 ATTC 29347).

Kinetic models used to describe PHB production can be classified in: formal kinetic models (based on enzyme and/or microbial kinetics); dynamic models (based on the time invariant characteristics of metabolic networks); metabolic models (based on the cellular metabolism); cybernetic models (based on optimal nature of microbial processes and on the metabolic regulation by the cybernetic framework which has to be discriminated from the pure kinetic ongoing); hybrid models (that combine at least two of the aforementioned types of model properties) (Novak, 2015).

A number of metabolic and bioreactor models have been proposed in literature for PHB production from bacteria.

The main investigations on PHB production modelling are reported hereinafter.

Mozumder *et al*., (2015) described the autotrophic PHB production in *Cupriavidus necator* by using a model based on a two-phase processes: biomass growth and subsequent PHB production. The aim was to simulate the two distinct phases to achieve a high PHB production rate and PHB content. Faccin et al. (2012) presented a model describing the culture kinetics of *Bacillus megaterium* for the production of P(3HB). Khanna and Srivastava (2015) proposed a structured unsegregated model for PHB accumulation in *Ralstonia eutropha* based on detailed investigations on substrate limitations and inhibitions by carbon, nitrogen, and phosphorus sources.

1.6 Genetically engineered cyanobacteria to produce PHB

Genetic engineering has made possible to enhance in photosynthetic microbes – cyanobacteria – the conversion of CO_2 into chemical commodities, (Ducat *et al.*, 2011; Wijffels *et al.*, 2013a). The development and application of engineered cyanobacteria are very promising to address renewable and carbon neutral processes for providing petroleum-based chemicals and alternative energy (Ruffing, 2011).

One of the primary concerns in genetic engineering is to efficiently transfer exogenous DNA into the host organism. Fortuitously, several cyanobacterial species are naturally competent, transporting DNA across the cell membrane by some undetermined mechanism. *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002 are naturally transformable. Therefore, these strains are among the most commonly investigated cyanobacteria: they are ideal hosts for genetic engineering efforts (Koksharova, 2002).

Cyanobacteria can be considered the cell factories of choice. The introduction of an exogenous biosynthetic pathway in a cyanobacterial cell may enhance the production of the specie of interest from CO_2. The new genetic material may encode information to provide a metabolic pathway to convert CO_2-derived carbon-based intermediates into almost any (carbon- based) product of interest, such as a specialty or commodity chemical, a biofuel, or a precursor of a bioplastic (Figure 1.6) (Angermayr *et al.*, 2015).

The products of interest expressed by cyanobacteria can be grouped in protein products and chemical products. Figure 1.7 shows the photosynthetic conversion of CO_2 and H_2O into high-value chemicals as ethanol (Deng & Coleman, 1999), ethylene (Ungerer *et al.*, 2012), 3-hydroxybutyrate (Wang *et al.*, 2013) and 2,3-butanediol (Oliver *et al.*, 2013), using genetically engineered cyanobacteria. As a rule, cyanobacteria strains must be genetically engineered with foreign genes to produce the desired high-value chemical or next- generation biofuel. Depending on the desired end-product, the production of the desired metabolite may ask for the addition of single genes or it may ask for including new pathways into the organism metabolism. The central carbon metabolism of cyanobacteria comprises the CBB cycle, glycolysis, the pentose-phosphate (PP) pathway and the TCA cycle. These paths form a very complex and interconnected network (Angermayr *et al.*, 2015).

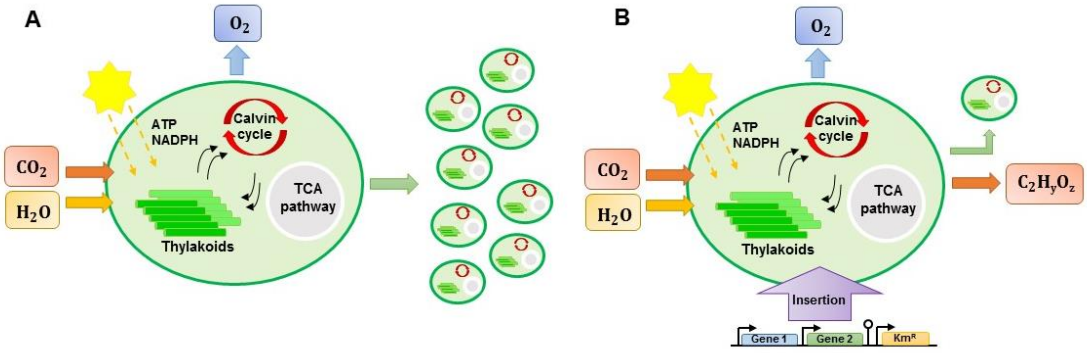

Figure 1.6: Schematic representation of a cyanobacterial cell factory. **(A)** A non-modified cyanobacterial cell is investing carbon (in form of CO_2) and energy sources into growth and the generation of biomass. **(B)** A modified cyanobacterial (here: through the introduction of an alternative biosynthetic pathway) cell factory is investing the majority of the incoming carbon and energy sources into a (excreted) product (here: carbon containing products), allowing only the replacement of dying cells and the maintenance of the cells to be fuelled by the native metabolism.

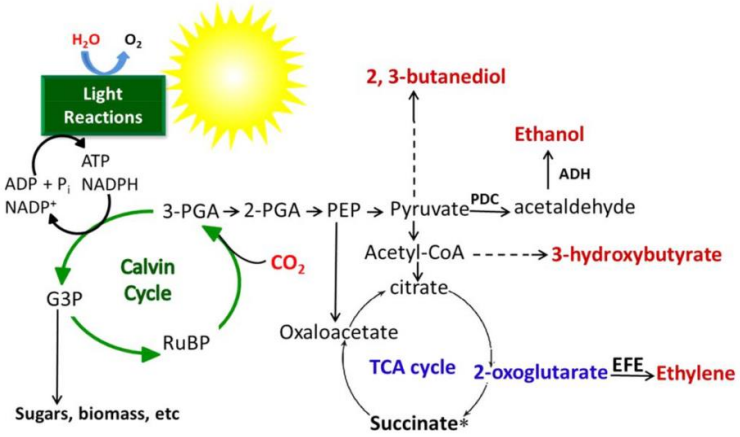

Figure 1.7: Photosynthetic conversion of CO_2 and H_2O to ethanol, ethylene, 3-hydroxybutyrate and 2,3-butanediol by genetically engineered cyanobacteria. (Johnson *et al.*, 2016)

Cyanobacteria may accumulate considerable amounts of PHB as product of the wild metabolism or as results of genetic engineering of strains by heterologous transformation with genes involved in the PHB pathway of *R. eutropha* (Miyake et al.,

2000). In many cyanobacterial cells, PHB is a native carbon-storing polymer generated via the polyhydroxyalkanoate (PHA) biosynthetic pathway. The pathway from acetyl-CoA to PHB involves three catalytic steps. The first step is the condensation of two molecules of acetyl-CoA into acetoacetyl-CoA by PHA- specific beta-ketothiolase (phaA); the second step is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (phaB); the third step is the (R)-3-hydroxybutyryl-CoA condensation into PHB by PHA synthase (phaC/phaE) (Khetkorn *et al.*, 2016) (Figure 1.8).

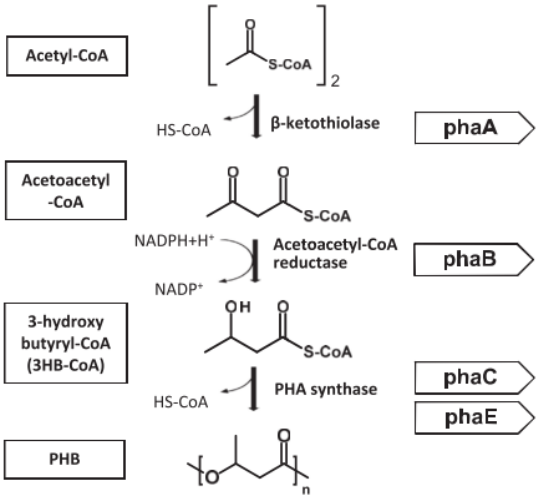

Figure 1.8: PHB biosynthetic pathway.

Many different genetic engineering approaches to increase PHB content in cyanobacteria have been described in literature with varying degrees of success. The several studies focused on the evaluation of PHB accumulation under mixotrophic growth conditions (Sudesh *et al.*, 2002a; Takahashi *et al.*, 1998; Wu *et al.*, 2002) in mutants of either *Synechocystis* or *Synechococcus* PCC7942 have regarded the supplement of acetate to the growth medium. Acetate can be converted into acetyl-CoA via mostly the activity of acetyl-CoA synthetase, and thereby support many metabolic pathways such as the TCA cycle for cell growth, glycogen synthesis and fatty acid metabolism relevant for biofuel production, and also PHB biosynthesis (Khetkorn *et al.*, 2016). The heterologous expression of PHA-synthesizing gene operon from the bacterium *R. eutropha* in *Synechocystis* sp. PCC 6803 increased the PHB content from 7% to 11% of dcw when grown in N-deprived medium supplemented with 10 mM acetate (Sudesh *et al.*, 2002). Similar results have already been reported for the cyanobacterium *Synechococcus* sp. PCC 7942 in which the entire *A. eutrophus* PHB-synthesizing machinery was express heterologously. These recombinant *Synechococcus* cells accumulated 25% PHB of dcw under N- deprivation with 10 mM acetate (Takahashi *et al.*, 1998). The PHB content of the phaAB strain obtained by Khetkorn et al. (Khetkorn *et al.*, 2016), increased to 35% of dcw in the presence of 4 mM acetate under nitrogen deprived condition when compared to about 13% PHB of dcw in the wild type under the same condition.

The values reported when PHB content was evaluated under photoautotrophic growth conditions using only CO_2 as carbon source were somewhat more modest (Hondo *et al.*, 2015a; Osanai *et al.*, 2013a). The overexpression of the native sigE gene integrated in the *Synechocystis* chromosome has increased the PHB content from 0.6% to 1.4% when grown in N-deprived medium. Hondo et al. (Hondo *et al.*, 2015a) transformed *Synechocystis* cells with the vector pAM461c harbouring a PHA

biosynthetic operon from *Microcystis aeruginosa* NIES-843 and reached a PHB content about 7% in N-deprived medium.

1.7 PHB recovery

The market penetration of PHB is still scarce due to its high production cost. The main reasons behind the economic disadvantages are the cost of the fermentation and of the recovery/purification technologies. The latter issue significantly affects the overall process economics. Several investigations have been carried out to reduce the PHB production cost by the use of effective and inexpensive carbon source and genetically engineered microorganisms. However, the real cost associated with PHB would only diminish with the development of a cheaper and environmentally friendly PHA recovery method (Kunasundari & Sudesh, 2011).

PHB is a lipid-like compound synthesized by many microorganisms as a form of storage material. PHB is accumulated in the form of granules in the bacterial cell cytoplasm. Figure 1.9 shows the morphology of PHB granules when observed using various microscopy techniques. Atomic force microscopy analysis has shown the presence of a protein mono-layer on the surface of PHB granules (Kunasundari & Sudesh, 2011; Sudesh *et al.*, 2002). The recovery of the PHB granules requires the rupture of the bacterial cell and the removal of the protein layer that wraps the PHB granules. Alternatively, the PHB has to be selectively dissolved in a suitable solvent.

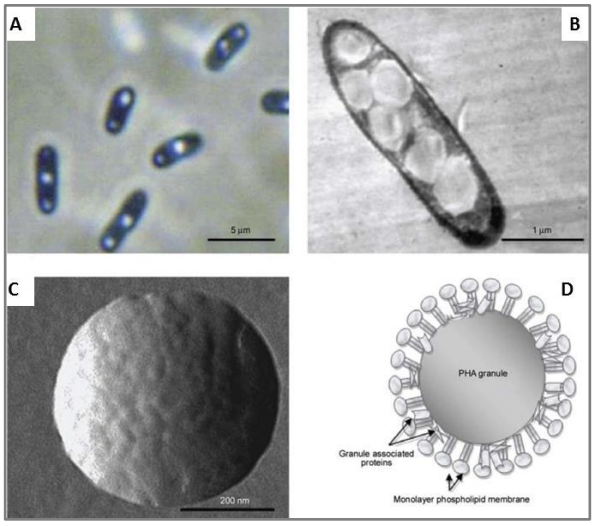

Figure 1.9: Morphology of PHA granules in the bacterial cells observed under (a) Phase contrast and (b) Transmission Electron Microscope. (c) Atomic force microscope deflection image showing the presence of globular particles on the granule surface. (d) A model representing the native PHA granule with a protein monolayer on the surface (Kunasundari & Sudesh, 2011).

The formation of PHB granules in the cells are currently under discussions. Three different models were developed based on theoretical considerations and on experimental data obtained with *Ralstonia eutropha* cells. The first model, the Micelle model (Figure 1.10 A) assumes that soluble (cytoplasmic) PHB synthase molecules (PhaC) starts to produce the hydrophobic PHB molecule if the concentration of the substrate (3-hydroxybutyryl-CoA) is sufficiently high (Gerngross *et al.*, 2001). The initial polymer chains of PHB formed aggregates characterized by hydrophobicity and low solubility in aqueous environment. These aggregates form the micelle structures in the cytoplasm of the cells. The consequence of the Micelle model is that the PHB granules can be randomly localized in the cell (Jendrossek & Pfeiffer, 2014; Stubbe *et al.*, 2005).

The second model, the Budding Model (Figure 1.10 B) assumes that the PHB synthase is located in the cytoplasmic membrane and that the formation of PHB chains is liberated into the bilayer of the membrane (Das *et al.*, 1997). The third model, the Scaffold Model (Figure 1.10 C) assumes that PHB synthase of nascent PHB granules is or becomes attached to a yet unknown scaffold molecule within the cell. In this case, subcellular localization of PHB granules would depend on the nature and localization of the scaffold of the PHB-accumulating cell (Jendrossek & Pfeiffer, 2014).

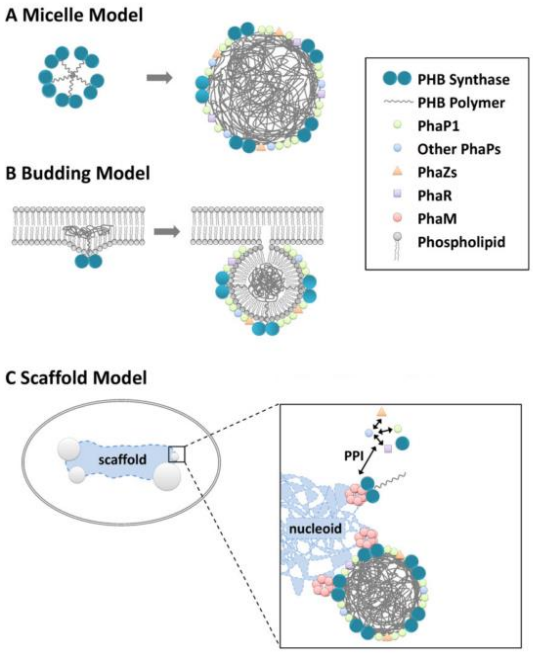

Figure 1.10: Models of PHB granules formation: A) Micelle model; B) Budding model; C) Scaffold model (Jendrossek & Pfeiffer, 2014).

Solvent extraction is the most extensively adopted method to recover PHB from the cell biomass. This method is also used routinely in the laboratory because of its simplicity and rapidity (Kunasundari & Sudesh, 2011). Two main steps are involved, first is the modification of cell membrane permeability thus allowing release and solubilization of PHB. This is then followed by non-solvent precipitation (Jacquel *et al.*, 2008). Extraction of PHA with solvents such as chlorinated hydrocarbons, i.e. chloroform, 1,2-dichloroethane (Ramsay *et al.*, 1994) or some cyclic carbonates like ethylene carbonate and 1,2-propylene carbonate is common. Precipitation of PHA is commonly induced by non-solvent such as methanol and ethanol (Ramsay *et al.*, 1994). Solvent extraction has undoubted advantages over the other extraction methods of PHA in terms of efficiency. This method is also able to remove bacterial endotoxin and causes negligible degradation to the polymers (Jacquel *et al.*, 2008). Therefore, it is possible to obtain very pure PHA with high molecular weights. Unfortunately, large-scale application of solvent extraction is generally viewed as a method that is not environmentally friendly (Table 1.5).

While solvent extraction techniques involve the solubilization of the PHB granules, digestion methods involve the solubilization of the cellular materials surrounding the PHB granules. Digestion methods are well established approaches developed as an alternative to solvent extraction.

Table 1.4 reported the information about the advantages and disadvantages of the main PHB recovery techniques.

Table 1.4: Comparison of the advantages and disadvantages of various PHA extraction methods (Kunasundari & Sudesh, 2011)

Recovery method	Advantages	Disadvantages
Solvent extraction	Removal of endotoxin; Useful for medical applications; High purity; Negligible/limited degradation to the polymer; Higher molecular weight;	Not environmentally friendly; Consumption of large volume of toxic and volatile solvents; Negligible/limited; High capital and operation cost; Difficulty in extracting PHA from solution containing more than 5% (w/v) P(3HB); Lengthy process; Native order of polymer chains in PHA granules might be disrupted.
Chemical Digestion (Surfactant)	Extracted PHA retains original molecular weight; Native order of polymer chains in PHA granules is retained.	Low purity of PHA; Treatment required to remove surfactant from waste-water.
Sodium hypochlorite	Higher purity of PHA can be obtained	Severe reduction in molecular weight of the extracted PHA
Sequential surfactant-hypochlorite	High quality of PHA; Rapid recovery and simple process; Retain native order of polymer chains in PHA granules; Lower operating cost compared to solvent extraction	Combined cost of surfactant and sodium hypochlorite; Wastewater treatment required to remove residual surfactant and sodium hypochlorite
Dispersion sodium hypochlorite and solvent extraction	High purity of PHA; Reduced viscosity of solvent phase due to digestion of non-polymer cellular material (NPCM) by sodium hypochlorite	Not environmentally friendly Consumption of large volume of toxic and volatile solvents Higher recovery cost
Surfactant-chelate	Convenient operation; High quality of product	Produce large volume of wastewater
Selective dissolution of NPCM by protons	Low operating cost; Higher recovery yield	Severe reduction in molecular weight if the process parameters are not controlled stringently
Enzymatic digestion	Mild operation conditions; Good recovery with good quality	Complex process High cost of enzymes
Bead mill	No chemicals used; Less contamination; Not susceptible to blockages; No micronization of PHA granules	Require several passes; Long processing time; Various process parameters have to be controlled precisely
High pressure homogenization	No chemicals used Less contamination	Severe micronization of PHA granules; Depends on both process and microbial physiological parameters; Possible for thermal degradation of desired products; Formation of fine cellular debris that interfere with downstream processing

1.8 Commercial status of PHB

The bioplastics are about one per cent of the about 300 million tonnes of plastic produced annually. However, as demand is rising and with more sophisticated materials, applications, and emerging products, the market is already growing by about 20 to 100 per cent per year. According to the latest market data compiled by European Bioplastics, global production capacity of bioplastics is predicted to quadruple in the medium term, from around 1.7 million tonnes in 2014 to approximately 7.8 million tonnes in 2019 (http://www.european-bioplastics.org/). In particular, as regards PHB, according to European-bioplastics (http://www.european-bioplastics.org/), the global production of poly-β-hydroxybutyrate was approximately 34 ktonnes in 2014 and it is forecasted to grow at a 100 ktonnes in 2019.

Many bioplastic production plants (Figure 1.11) are small compared to production facilities of conventional, petroleum-based plastics. For example, China's TianAn PHA plant has a capacity of approximately 2,000 metric tons per year —quite small by traditional standards. However, as bioplastics gain traction in various end-use sectors, a handful of producers have emerged as leaders in biopolymer production worldwide.

PHA bioplastic business is divided into North America, Europe, Asia-Pacific, and the rest of theworld (Singh *et al.*, 2016). To date, many companies are in the business of PHA bioplastics. e.g., Metabolix Inc. (USA), Shenzhen Ecomann Technology Co. Ltd. (China), Tianjin GreenBio Materials Co. Ltd. (China), Meredian Inc. (USA), Mango Materials (USA), Newlight (USA) and Biomer (Germany) (Singh *et al.*, 2016) (Table 1.5).

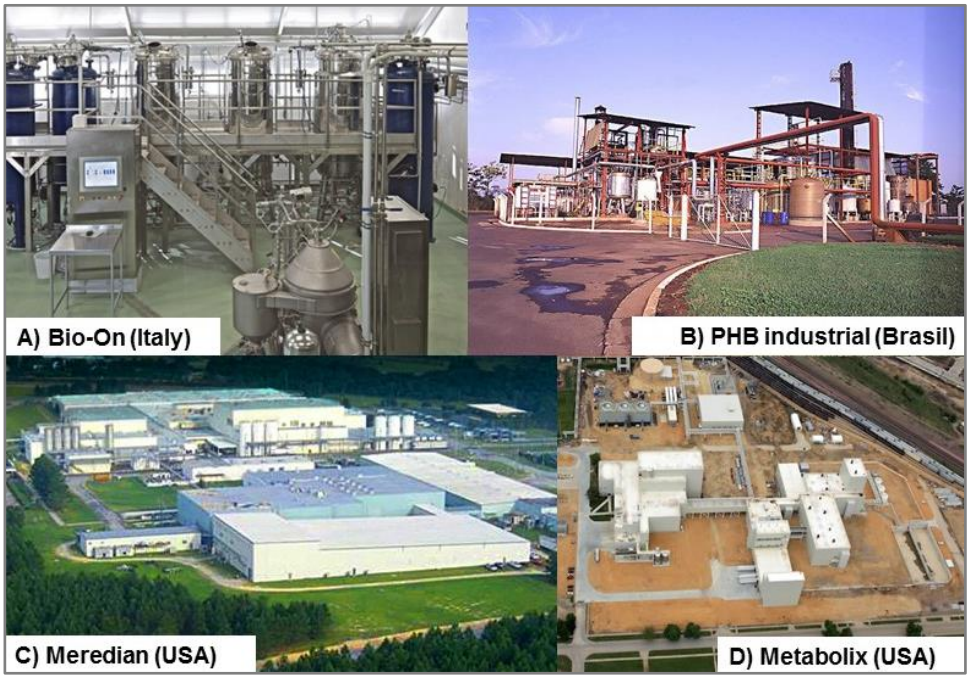

Figure 1.11: PHB production plants: A) Bio-on in Italy that produces Minerv; B) PHB industrial in Brasil that produces Biocycle; C) Meredian in the USA; D) Metabolix in the USA.

The initial commercial plant for the production of P(3HB-co-3HV) co-polymer was established in the USA in 2006 when Metabolix and Archer Daniel Midland (ADM) formed a joint venture called Telles. With ADM, Metabolix became the largest PHA producer in the US. Unfortunately, this joint venture did not last longer and finally ended in 2012 (Roland-holst & Heft-neal, 2013).

In the U.S., Meridian, Inc. produces a PHA bioplastic from plant-based oils (fatty

acids) which are metabolized by bacteria in a fermentation tank. End uses for the resins include films, non- woven fabrics, and food-contact packaging. The company's facility, produces 15,000 tons of PHA per year. When built to full capacity, it will be able produce more than 300,000 tons of PHA per year (Roland-holst & Heft-neal, 2013; Singh *et al.*, 2016).

Mango Materials, (USA) a Redwood City startup company founded in 2010, is developing a PHB bioplastic using technology based on intellectual property licensed from Stanford University. The company seeks to convert waste methane from landfills and wastewater treatment facilities into plastic by feeding the gas to methane-eating bacteria, known as methanotrophs, which metabolize it through fermentation into PHB. The PHB is then extracted from the cell biomass and converted to bioplastic pellets, ready to be made into a plastic product (Roland-holst & Heft-neal, 2013).

The Dutch chemical company DSM announced a plan to invest in a PHA plant together with a Chinese biobased plastics company—Tianjin Green Bio-Science Co. The company is now producing PHA resin with an annual capacity of 10,000 metric tons.70 The Japanese company Kaneka planned to produce 50,000 metric tons annually of PHB in 2010 (Roland-holst & Heft-neal, 2013; Singh *et al.*, 2016).

Table 1.5: Global manufacturers of PHB/PHA thermoplastics (http://bioplasticsinfo.com/; Singh *et al.*, 2016).

Name of the company	Country	Production (kt year^{-1})	Brand name
Biomer	Germany	-	Biomer
Biomatera	Canada	-	Biomatera
Bio-On	Italy	10	Minerv
Kaneka	Singapore	10	Mirel
Tianjin Green Biosciences	China	10	Green Bio
Imperial Chemical Industries (ICI),	UK	0.3	-
Metabolix	USA	50	-
Meredian	USA	0.3	-
PHB Industrial	Brasil	0.1	Biocycle
Telles	USA	50	Mirel
TEPHA	USA	-	Tephaflex/ TephElas
Tinan	China	10	Enmat
Yikeman ShanDong	China	3	-
ZheJiang Tia An	China	2	-

2 AIM OF THESIS

The study carried out during the present Ph.D. program aimed at investigating the poly-β-hydroxybutyrate (PHB) production process by cyanobacteria in photoautotrophic conditions. The interest in this production system is twofold: to produce the building block of biodegradable plastics by using renewable resources (sunlight and CO_2); to contribute to the decrease of the CO_2 concentration in the atmosphere by capturing and using the CO_2.

The work was carried out at the Dipartimento di Ingegneria Chimica, dei Materiale e della Produzione Industriale and at the Dipartimento di Scienze Biologiche of the Università degli Studi di Napoli 'Federico II' and at Molecular Microbial Physiology Group of the Swammerdam Institute for Life Sciences in Universiteit van Amsterdam.

The activities were articulated along three lines reported hereinafter.

The PHB production process by adopting different growth media and testing different cyanobacteria strains: characterization in terms of kinetics and yields.

The study was aimed at the assessment of the kinetics and the yields of cell growth and PHB. The focus was on the strain screening and the optimal nitrate concentration to maximize the PHB production.

Five cyanobacteria strains were screened to select a high PHB producer: *Synechocystis* PCC6803, *Synechocystis aquatilis*, *Synechocystis fuscopigmentosa*, *Synecoccoccus nidulans* and *Chlorogloeopsis Fritshii*.

Tests were carried out at different nitrate concentrations: BG_{11} (optimal nitrate concentration), $BG_{1/2}$ (half of optimal nitrate concentration), $BG_{1/4}$ (one fourth of the optimal nitrate concentration), and BG_0 (nitrogen-starved conditions).

The investigation was carried out in photobioreactors using CO_2 as carbon source. Batch tests were focused on the preliminary characterization of the PHB production process from cyanobacteria with the aim of highlighting the relevant features of the process.

Characterization of the PHB production process: kinetic models.

The optimization of the production process of PHB is not a trivial task because the PHB production by microorganisms involves different metabolic pathways. However, an adequate model to describe the process is a key issue to address the conditions to maximize the PHB fraction in the cell. For an effective design, scale-up, and optimization of PHB production process, it is necessary to have insight into the mechanisms of the process.

A kinetic model of PHB production by *Synechocystis* PCC6803 from CO_2 was proposed using the biochemical networks simulator COPASI. Data from growth tests carried out in dynamic light systems (light/dark cycle) at different initial nitrate concentration were used to assess the kinetic and stoichiometric parameters of the proposed model.

Development of engineered cyanobacteria strain according the genetic engineering approach to improve PHB production.

Cyanobacteria can be considered the cell factories of choice. The genetic manipulation of pathway in a cyanobacterial cell may enhance the production of the specie of interest such as a specialty or commodity chemical, a biofuel, or a precursor of a bioplastic from CO_2 (Angermayr *et al.*, 2015).

In this study, *Synechocystis* sp. PCC6803 was genetically modified with aim to improve PHB production from CO_2. Seven mutant strains were produced by single and combined mutations: the deletion of phosphotransacetylase (*pta*); the deletion of and acetyl-CoA hydrolase (*ach*); the overexpression of phosphoketolase (*xfpk*).

3 CYANOBACTERIA GROWTH IN PHOTOBIOREACTORS AND PHB PRODUCTION

The activity focused on the cyanobacteria strain screening and the optimal nitrate concentration assessment to maximize the PHB production. The section 3.1 reports the results of preliminary tests carried out with *Synechocystis* PCC6803 on two different growth media: BG_{11} (medium with optimal nitrate concentration) and BG_0 (nitrate-free medium). The section 3.2 reports the results of medium optimization tests and cyanobacteria strain screening. The model strain (*Synechocystis* PCC6803) was used for the assessment of best nitrate concentration for PHB production. Indeed, tests were carried out with different media: BG_{11} (optimal nitrate concentration), $BG_{1/2}$ (half of optimal nitrate concentration), $BG_{1/4}$ (one fourth of the optimal nitrate concentration), and BG_0 (nitrogen-starved conditions). Five cyanobacteria strains were tested using the selected media ($BG_{1/2}$): *Synechocystis* PCC6803, *Synechocystis aquatilis*, *Synechocystis fuscopigmentosa*, *Synecoccoccus nidulans* and *Chlorogloeopsis Fritshii*. The section 3.3 reports the results of tests carried out to characterize the kinetics of *Synechocystis* PCC6803 in flat photobioreactors.

3.1 The Cyanobacterial Route to Produce Poly-β-hydroxybutyrate

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Abstract

The amount of plastics produced in the world and released in the environment has dramatically increased putting societies and the environment to hard test. The biodegradable plastics are a potential solution to reduce the environmental impact and the fossil resource exploitation.

Polyhydroxyalkanoates (PHAs) are widely adopted as building blocks for biodegradable plastic production and they may be produced by microorganisms. The poly-β-hydroxybutyrate (PHB) is the most widespread and thoroughly characterized PHA found in bacteria. In particular, cyanobacteria are potential host systems for the PHB production, as they may synthesize this polymer from CO_2.

This contribution reports a joint research between the University of Napoli and the University of Amsterdam on the feasibility of PHB production by *Synechocystis* PCC6803. Both BG_{11} (balanced conditions) and BG_0 (nitrogen-starved conditions) media were investigated to find the best growth conditions and optimal PHB production. Batch autotrophic cultures were carried out in inclined bubble column photobioreactors under 12h/12h light/dark cycles.

Cultures were characterized in terms of biomass, PHB content, pH and nitrate consumption. After cell-rupture with sonication the PHB concentration was assessed by means of GC, adopting a modified propanolysis method. The maximum PHB concentration was 4 mg/L and the maximum PHB productivity was 0.19 mg/(L*day). The PHB content of the cells is definitively higher in nitrogen-starved medium (1 %) than in balanced medium (0.25 %).

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Introduction

Plastics are among the materials widely used all over the world. Nowadays, plastics are indispensable as components of cars, home appliances, and computer equipment, in packaging and as medical tool/devices. Although mechanical-technological features of plastics have worldwide been recognized, plastics have been vilified because they are environmentally unfriendly. Indeed, plastics are not biologically degradable and accumulate in the environment producing mountains of pollutants (Sharma *et al.*, 2007).

Bioplastics have a history of approximately 150 years. The first artificial thermoplastic "celluloid" was invented in the 1860s. Since then, numerous new compounds were produced from renewable resources. The advent of the fossil-derived plastics caused the industrial failure of plastics derived from renewable resources: synthetic polymers have been successfully produced at industrial scale since the 1950s. The oil price shocks of the last decades of the last millennium, in combination with the environmental consciousness that arose during the last decades, have refuelled the interest in bio-based chemistry and in particular in bio-based plastics (http://www.european-bioplastics.org/). Biodegradable plastics are a class of bioplastics that are both produced from biomasses *(*biobased materials) and are biodegradable: microorganisms are able to convert them into simple, elemental, substances (e.g. water, carbon dioxide).

Polyhydroxyalkanoates (PHAs) are fundamental building blocks for biodegradable plastics. PHAs are microbial polyesters that may be produced by various microorganisms, cyanobacteria included. PHAs are among the most investigated biodegradable polymers in recent years, due to their chemical and physical features that include: non-toxicity, biocompatibility, and biodegradability. Particular attention has been paid to poly-β-hydroxybutyrates (PHBs) because of their interesting features: thermoplastic processing, hydrophobicity, complete biodegradability, and biocompatibility with optical purity (Braunegg *et al.*, 1998).

Cyanobacteria – or blue-green algae - are prokaryotic organisms. They may carry out oxygenic photosynthesis under photoautotrophic conditions and are characterized by a short duplication time (Gopi *et al.*, 2014). Cyanobacteria can be considered as an alternative host system for the production of PHBs because they are photoautotrophic and require minimal nutrients. They need some simple inorganic salts such as phosphate, nitrate, magnesium and calcium, as macro-, and ferrous, manganese, zinc, cobalt, and copper as micronutrients for their growth and multiplication (Balaji & Gopi, 2012). Cyanobacterial species may accumulate the homopolymer of PHB under photoautotrophic conditions. Indeed, PHB functions as a specialized reserve material for carbon- and energy supply: storage accumulated in a variety of microorganisms under conditions of nutrient imbalance (Wu *et al.*, 2001).

Synechocystis PCC6803 is an extensively investigated cyanobacterium. It is a naturally transformable organism, genetically and physiologically well characterized. Its growth is quite fast (minimal doubling time less than seven hours) and it does not have specific nutritional demands (Angermayr *et al.*, 2009). Figure 3.1.1 shows its most important metabolic pathways.

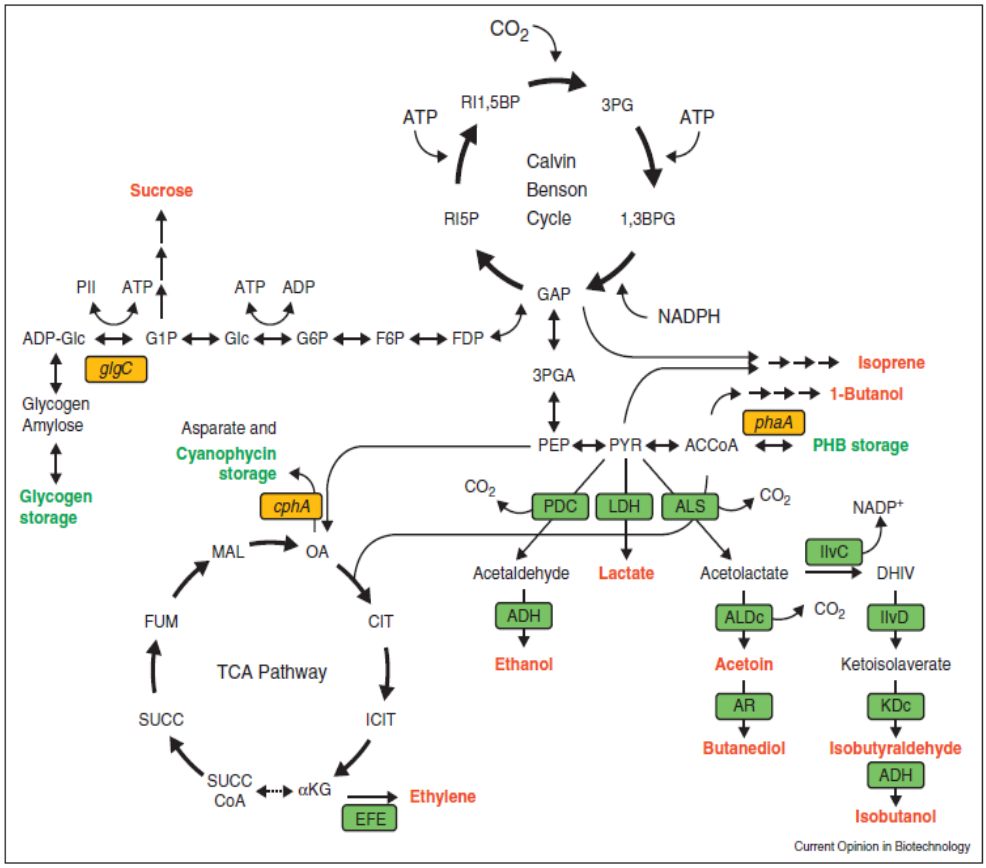

Figure 3.1.1: *Synechocystis* PCC6803 intermediary metabolism (Wijffels *et al.*, 2013a)

Present contribution reports some results of a joint study between the University of Study of Napoli Federico II and the University of Amsterdam on PHBs production by means of autotrophic cultures of cyanobacteria. The strain *Synechocystis sp.* PCC6803 was selected as the model organism for these studies. The production of PHBs was investigated in two media (i.e. with and without added nitrate). Cultures were grown in inclined bubble column photobioreactors with alternating dark/light cycles.

Materials and method

Microorganism and medium

Synechocystis sp. PCC6803 was provided by the University of Amsterdam. Cyanobacteria were grown under photoautotrophic conditions in two growth media: BG_{11} and BG_0 medium (Table 3.1.1). The difference between the two media is in the nitrate content: BG_0 does not contain added nitrate.

***Table 3.1.1**: BG_{11} and BG_0 composition (g/L)*

	BG_{11}	BG_0
$NaNO_3$	1.5	0
K_2HPO_4	0.04	0.04
$MgSO_4$	0.075	0.075
$CaCl_2*2H_2O$	0.036	0.036
Citric acid	0.006	0.006
Ammonium ferric citrate	0.006	0.006
$Na_2EDTA*2H_2O$	0.001	0.001
$NaCO_3$	0.02	0.02
HBO_3	2.86	2.86
$Mn\ Cl_2 * 4H_2O$	1.81	1.81
$ZnSO_4 * 7H_2O$	0.22	0.22
$Na_2MoO_4 *2\ H_2O$	0.39	0.39
$Co(NO_3)_2$	0.05	0.05

Physico-chemical methods

A fraction of the sampled cell broth was centrifuged at 5,000 rpm for 15 min. Nitrate concentration and pH were measured in the supernatant with ion selective electrodes (Hanna Instruments) and a Mettler Toledo pH meter, respectively.

The biomass (i.e. cyanobacteria) concentration was measured with a spectrophotometer Specord 50 – Analytic Jena at wavelength of 730 nm. Analysis of the dry cell weight was carried out by filtering 10 mL aliquots of culture on a Whatman filter paper, Grade 1. Each filter was dried at 60°C until the weight approached a constant value. The dry weight of each filter was subtracted from that of the cyanobacteria-containing filter, to obtain the dry cell weight of the cyanobacteria. The OD values were converted to biomass concentration via an appropriate calibration line between OD and dry cell weight, showing the conversion factor.

Assay of PHBs

The PHB assays required cell rupture, which was carried out by means of sonication. A 50 mL sample was sonicated with a Sonics Vibracell VCX 500 for 30 min (10 s on/ 10 s off), amplitude 21%, and energy 90 kJ. The sample is cooled in ice during sonication to prevent overheating.
Sonicated samples were centrifuged in glass tubes at 3,000 rpm for 1 h and the resulting pellets were dried overnight at 40 °C.

PHB was quantified using a slight modification of the gas chromatographic method of Riis and Mai (1988) (Riis & Mai, 1988). Two mL of 1,2-Dichloroethane (DCE), 2 mL n-Propanol, containing hydrochloric acid (HCl) (1 volume concentrated HCl + 4 volume n-Propanol), and 200 µL internal standard (2.0 g benzoic acid in 50 ml n-Propanol) were added to the dried pellet. The mix was incubated for 4 h in a water bath at 85 °C with intermittent shaking. After cooling to room temperature, 4 mL water were added and the mixture shaken for an additional 20-30 s. The heavier DCE-Propanol phase was collected and injected directly into a gas chromatograph. Quantitative evaluation was based on peak areas of hydroxy-butyric acid and benzoic acid (Nagamani & Mahmood, 2011).

An Agilent 6890 gas chromatograph was fitted with an automatic injector and a flame ionization detector. The injection split ratio was 100:1 with a helium flow of 0.9 mL/min through the 25 m Poraplot Q capillary column. The injector port temperature was 120 °C and increased by 8 °C per min to a final temperature of 210 °C. Injections

of 5 µL were made and the retention times for the PHB and benzoic acid were 4.1 and 7.0 min respectively (Aremu *et al.*, 2010).

PHB calibration was carried out by dissolving 200 mg of PHB standard (Sigma Aldrich) in warm DCE. After cooling at room temperature, the mixture was made up to 10 mL. Volumes of 200, 400, 600 and 800 µL of this solution were treated as outlined above. The relationship between the peak-area quotient and the quantity of PHB was observed to be linear.

The concentration of PHB measured during the tests was referred to the unit of volume of the culture.

Apparatus

Pre-cultures were grown in 250 mL Erlenmeyer flasks housed in a climatic chamber (Gibertini) at 28 °C. The chamber was equipped with daylight fluorescent Philips lamps (TLD 30W/55) set at 150 $\mu Em^{-2}s^{-1}$ for 24/24 h.

Inclined bubble column photobioreactors (ID: 5 cm; volume: 800 mL) were adopted for cyanobacterial growth. The photobioreactors where housed in climate chamber (Solar Neon) at 28 °C, equipped with lamp. Gas stream was sparged through the bottom of the photobioreactors by means of multiple-orifice (1 mm ID) Teflon tube. A hydrophobic filter (0.2 µm) sterilized the gas flow inlet. A gas mixing device (M2M engineering) provided the selected concentration of CO_2 in the gas stream fed to the photobioreactors, by mixing air and pure carbon dioxide from a pressurized vessel. Gas flow rate was set at 4 vvm. The CO_2 concentration in the air stream was set at 2% (v/v). The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations (Gargano *et al.*, 2013).

Procedures

The cyanobacteria were transferred from a Petri plate into 250 mL Erlenmeyer flasks containing 50 mL medium. After about two weeks the pre-cultures were inoculated into photobioreactors: 10% (v/v) of actively growing pre-cultures.

Cultures in inclined column photobioreactors were grown in light/dark cycles: 12 h light at 150 $\mu Em^{-2}s^{-1}$, 12 h dark. The cultures were sampled every 48 h. The samples were characterized with respect to biomass content, pH and PHB concentration.

Measured data were processed to assess the PHB concentration in the biomass according to the relationship:

$$\omega_{PHB} = \frac{PHB}{X} \quad (1)$$

where X is the cyanobacterial concentration, and PHB the poly-β-hydroxybutyrate concentration.

Results

Figure 3.1.2 reports the time resolved data regarding two autotrophic cultures of *Synechocystis sp.* PCC6803. Data reported in the figures are: the cell concentration, the PHB-cell concentration assessed according to eq (1), and the PHB concentration. The runs refer to a culture carried out with a balanced-medium (BG_{11}) (Figure 2 A and C) and to a culture carried out with a nitrogen-deplete medium (BG_0) (Figure 2 B and D). The growth in the balanced medium was quite linear during the investigated growth period. The typical S-shaped curve cell-concentration vs. time was observed for the culture in nitrogen-less medium to highlight the gradual decrease of key substrate.

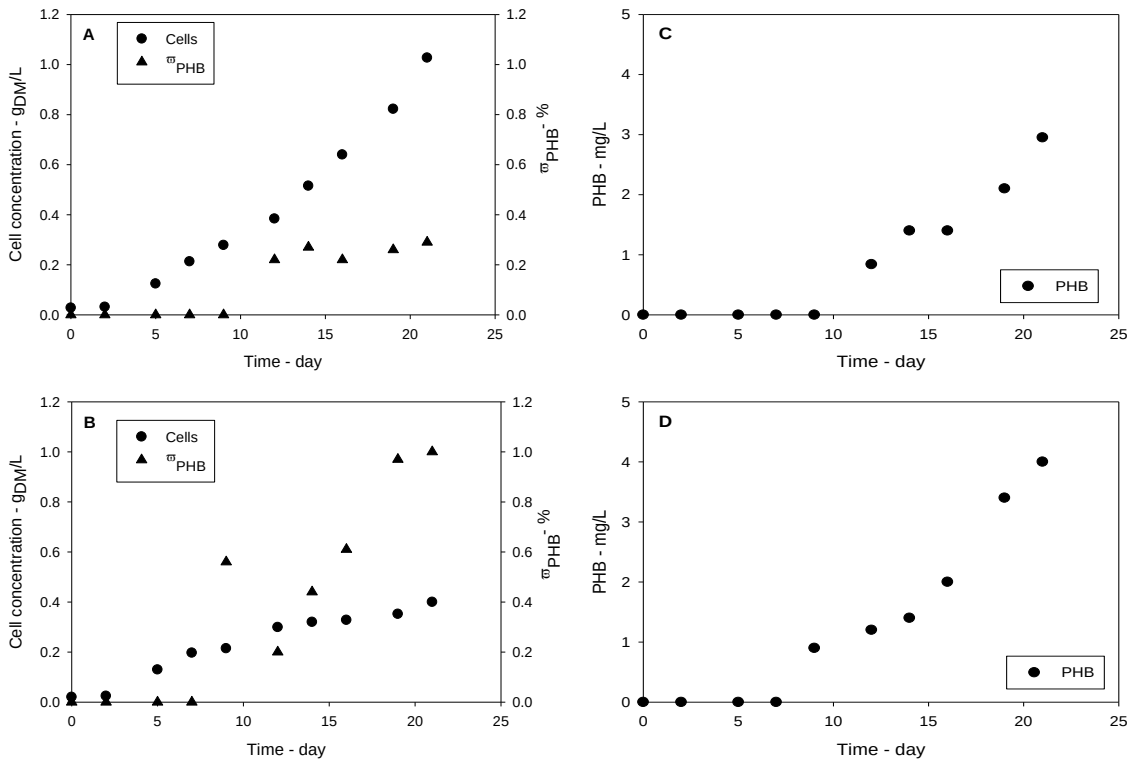

Figure 3.1.2: Data measured during the growth of *Synechocystis* PCC6803: cell concentration, PHB concentration, PHB-cell composition (ω_{PHB}). A & C): balanced medium; B & D) nitrogen-deplete medium.

Figure 3.1.3 shows the pictures of the cultures at day 21. The blue-green cultures typically of the balanced cultures turned into yellow-green for nitrogen-depleted culture. The cell colour changed from blue-green to yellow-green because under nitrogen-starvation conditions the phycobiliproteins are degraded. Indeed, phycobiliproteins are nitrogen storage compounds with deep blue colour in cyanobacteria (Allen, 1984). The amino acids released by the phycobiliprotein breakdown can be further used to synthesize new cellular materials to sustain cell growth.

Figure 3.1.3: Pictures of *Synechocystis* sp. PCC6803 cultures in balanced medium (BG_{11}) and in medium lacking added nitrate (BG_0). Cultures were grown for 21 days.

Figure 3.1.4 shows the PHB accumulated at the bottom of vials (white region). The samples were from nitrate free cultures.

The analysis of the data of PHB concentration vs. time assessed for both cultures (Figure 3.1.2 B and D) highlights that nitrogen deficiency was favourable to PHB synthesis. Indeed, the PHB concentration at the end of the 21 day culture was about 4 mg/L for the nitrogen-starved conditions and just 2.95 mg/L for the balanced cultures. Moreover, the PHB concentration is almost constant at about 0.25 % during the balanced-medium cultures and it gradually increases up to 1 % in the nitrogen-starved culture.

The different PHB final concentrations are in agreement with the results reported in the literature. In particular, the higher accumulation of PHB under nitrogen-starvation conditions may be interpreted taking into account the absence of nitrogen into the medium while the photosynthetic activity fixes CO_2. Since the cells cannot synthesize protein, the carbon/energy is accumulated by cells also as PHB (Smith, 1982). As a result of the flux re-direct under nitrogen-starved conditions, it is interesting to note that the PHB yield is higher for the nitrogen-starved culture than that for the balanced medium culture.

The PHB productivity – assessed as the final concentration divided for the culture time – is 0.19 mg/(L*d).

Figure 3.1.4: PHB pellet after sonication: white region (cells were grown in BG_0).

The comparison of the final concentration of PHB in the cell and of the daily PHB productivity with data reported in the literature for autotrophic cultures points out that present results are promising. Although the final concentration of PHB in the cell and the PHB productivity measured for heterotrophic-mixed cultures (Jiang *et al.*, 2011) are larger than those found in the present investigation, it should be noted that the feedstock used for the present investigation (CO_2) does not compete with any food/feed resource.

Final remarks

The production of PHB by means of autotrophically-grown *Synechocystis sp.* PCC6803 was successfully carried out. The cyanobacteria were able to produce PHB just from CO_2. The level of PHB production is enhanced under nitrogen-starvation conditions. In nitrogen-starved cultures about 4 mg/L of PHB was produced, as compared to about 25 % of this amount in nitrogen-replete cultures.

Although the PHB production was quite low with respect to data reported in the literature, the results are promising, be it that it is necessary to adopt a strategy to improve the PHB final concentration and the PHB productivity.

Acknowledgements

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3.2 Photoautotrophic production of poly-β-hydroxybutyrate (PHB) from cyanobacteria: nitrate effects and screening of strains

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Abstract

The polyhydroxybutyrate (PHB) production by means of in photoautotrophic cultures is an eco-sustainable potential process for bioplastics. Indeed, the biosynthesis of PHB by cyanobacteria meets a twofold target: the use of CO_2 as feedstock alternative to the fossil resource used in the conventional processes; the contribution to the reduction of the CO_2 concentration in the atmosphere. This contribution reports results of a study regarding the optimal growth conditions for cyanobacteria to produce polyhydroxybutyrate (PHB) in photoautotrophic conditions, using a light/dark strategy for cyanobacteria growth.

Synechocystis PCC6803 was selected as the model organism for optimization of the medium composition with respect to PHB production. Four culture media, characterized by different nitrate concentrations, were investigated under batch conditions: BG_{11} (optimal nitrate concentration), $BG_{1/2}$ (half of optimal nitrate concentration), $BG_{1/4}$ (one fourth of the optimal nitrate concentration) and BG_0 (nitrogen-starved conditions). $BG_{1/2}$ proofed to be the best medium to optimize the PHB production in terms of PHB content (8% dcw) and PHB maximal productivity (7g/m^{3}d).

Five strains of cyanobacteria were then compared to select the best strain to produce PHB. *Synechocystis* PCC6803 and *Synechocystis aquatilis* proved to be the best strains for PHB production. A PHB fraction and maximal productivity of about 8% and 7g/m^{3}d were obtained for both strains. The production performance was promising when considering the cheap substrate (CO_2) used.

Keywords: cyanobacteria; nitrogen-starvation; photobioreactor; polyhydroxybutyrate (PHB); screening; strain.

Introduction

Modern society continuously asks for plastic items inexpensive, versatile, and eco sustainable. Indeed, the plastic worldwide demand - more than 300 million tons of plastic per year –has included the plastics among the key player of the today's economy (http://www.european-bioplastics.org/). Petroleum-based plastics are a major constituent of world plastic consumption. Although mechanical-technological features of plastics have worldwide been recognized, plastics have been vilified because they are environmentally unfriendly. In particular, the fossil-based plastics have several environmental disadvantages: they accumulate in the environment because they are typically non-degradable; a large amount of CO_2 is released in the environment during the production and the conventional waste disposal processes. Alternative process of producing plastics in large quantities that are both economically and environmental friendly have recently gained considerable attention (Bhati *et al.*, 2010). Roughly 85% of plastics could technically be substituted with biobased plastics (http://www.european-bioplastics.org/): plastics produced by renewable resources. The use of renewable resources contributes to the reduction of the CO_2 concentration in the atmosphere. The reduction is still more marked if the CO_2 is captured from gas streams produced by anthropic activities

The shift from traditional plastics to biodegradable plastics can potentially reduce the total plastic waste by up to 20% (Dias *et al.*, 2006). A large fraction of plastic products may be made by biopolymers belonging to the polyhydroxyalkanoates (PHAs) class. Indeed, PHA based products have a wide spectrum of potential applications: packaging, medical uses, agricultural uses, and in carbon nano tubes (Akaraonye *et al.*, 2010). In particular, Poly(3-hydroxybutyrate) (PHB) received a special attention for its interesting features: thermoplastic processability, hydrophobicity, complete biodegradability and biocompatibility (Braunegg *et al.*, 1998). According to European-bioplastics, the global production of poly-β-hydroxybutyrate (PHB), was approximately 34 ktonnes in 2014 and is forecasted to grow to a 100 ktonnes in 2019.

PHB is a biopolymer that exhibits thermal properties and a tensile strength equivalent to those of petroleum-based plastics (Verlinden *et al.*, 2007). Moreover, PHB is also biocompatible to various human tissues, and so has been used as a biomaterial for tissue engineering. Currently, PHB is commercially synthesized by heterotrophic bacteria using costly organic compounds. Indeed under optimal conditions, bacteria such as *Ralstonia eutropha* H16 can produce up to 80% of PHB of cellular dry weight (Hempel *et al.*, 2011; Pötter *et al.*, 2004). Nevertheless, costs for PHB production by bacterial fermentation are still very high (high costs of conventional substrate) which brought plants into focus as photosynthesis low-cost production system (Mittendorf *et al.*, 1998; Poirier *et al.*, 1992a). Indeed, autotrophic cultures use CO_2 as carbon source and light as energy source. Therefore, autothrophic cultures could be a promising system using solar energy to generate reducing equivalents and incorporate atmospheric CO_2 into organic molecules (Atsumi *et al.*, 2009a). As a matter of fact, the PHB can be produced by some photoautotrophic cyanobacteria using abundant solar energy and CO_2 – in particular that presents in gas streams produced by anthropic activities - as the sole carbon substrate (Gopi *et al.*, 2014a; Hai *et al.*, 2001; De Philippis *et al.*, 1992; Wijffels *et al.*, 2013a).

The tuning of substrates concentration in the cyanobacteria culture media can induce drastic shifts of the central carbon metabolism in non diazotrophic cyanobacteria such as *Synechocystis*. In particular, under nitrogen depletion

conditions, Anfelt et al. (2015) have proved that the carbon flux is directed to storage compounds such as PHBs.

This paper reports the results of a preliminary study aimed at the optimization of the cyanobacterial growth media to increase the PHB production in photobioreactor by tuning the nitrate concentration. *Synechocystis* PCC6803 was selected as the model organism for the optimization of the media culture tests. Five cyanobacteria strains were screened to select a high PHB producer.

Materials and methods

Microorganism and medium

Synechocystis PCC6803 (used for culture medium optimization) was provided by the Laboratory of Microbiology of University of Amsterdam. *Synechocystis aquatilis*, *Synechocystis fuscopigmentosa*, *Synecoccoccus nidulans* and *Chlorogloeopsis fritshii* (selected after a literature review focused on high PHB production strains) were investigated during the screening investigation and were provided by ACUF collection (www.acuf.net).

S. aquatilis cells are characterized by rounded shape with a very thin mucilaginous sheath around the cells. The thylakoid membranes are located in a peripheral position. Cell division occurs by two perpendicular planes of division in successive generations (Korelusova *et al.*, 2009).

The strain *S*. PPC6803 is frequently used as a model organism in a wide spectrum of biochemical studies. The *S*. PPC6803 cells are characterized by morphological features like to those of *S. aquatilis* cells. Indeed, *S*. PPC6803 is often considered as the reference strains of *S. aquatilis* (Komárek *et al.*, 2014). *S. fuscopigmentosa* Kováčik is characterized by morphological features like those of *S. aquatilis* too. However, *S. fuscopigmentosa* Kováčik is characterized by the extracellular excretion of a brown pigment.

The *S. nidulans* cells are cylindrical rod-shaped, often bent at one or both ends, without mucilage and are characterized by homogeneous cell content. Thylakoids are generally in a peripheral position, located around the cell wall. Cells divide symmetrically by binary fission in a plane producing two equal cells.

C. fritshii cells are cylindrical and forming regular clusters of cells and small filaments, about 3 µm in diameter, rarely formed by more than 10 cells. Thylakoids are generally oriented parallel to the cell wall. Cells reproduce by endospores that originate clumps or filaments. Heterocyst-like cells form about 5% total cell population in heterotrophic cultures under nitrogen starvation (Whitton & Peat, 1967).

For culture medium optimization tests, *S.* PCC6803 was grown under photoautotrophic conditions in four growth media: BG_0, $BG_{1/4}$, $BG_{1/2}$ and BG_{11}. The composition of the four media is reported in Table 3.2.1. The four media differ between each other with respect to the nitrate content: BG_{11} contained the optimal concentration of nitrate for cyanobacterial growth according to Waterbury et al. (Waterbury, 1981); BG_0 did not contain nitrate; $BG_{1/2}$ contained half the optimal nitrate concentration; and $BG_{1/4}$ contained one fourth of the optimal nitrate concentration.

The screening tests were carried out under photoautotrophic conditions using the medium selected from the optimization tests ($BG_{1/2}$).

Table 3.2.1. Composition (g/L) of the investigated media.

	BG_0	$BG_{1/4}$	$BG_{1/2}$	BG_{11}
$NaNO_3$	0	0.375	0.75	1.5
K_2HPO_4	0.04			
$MgSO_4$	0.075			
$CaCl_2*2H_2O$	0.036			
Citric acid	0.006			
Ammonium ferric citrate	0.006			
$Na_2EDTA*2H_2O$	0.001			
$NaCO_3$	0.02			
HBO_3	2.86			
$Mn\ Cl_2 * 4H_2O$	1.81			
$ZnSO_4 * 7H_2O$	0.22			
$Na_2MoO_4 *2\ H_2O$	0.39			
$Co(NO_3)_2$	0.05			

Analytical

The pH and the cell concentration (X) were measured in the culture sampled from the photobioreactors. The sample was centrifuged at 4,890 g for 15 min. The liquid phase was characterized in terms of concentration of nitrate and phosphate. The solid phase was processed to assess the PHB content.

The biomass (i.e. cyanobacteria) concentration was measured with a spectrophotometer Specord 50 – Analytic Jena at wavelength of 730 nm. Analysis of the dry cell weight was carried out by filtering 10 mL aliquots of culture by means of a Whatman filter paper, Grade 1. Each filter was dried at 85°C until the weight approached a constant value. The dry weight of each filter was subtracted from that of the cyanobacteria-filter to yield the dry cell weight of the cyanobacteria. The OD values were converted to biomass concentration via an appropriate calibration procedure pairing OD and dry cell weight. The conversion factor from OD to cell concentration was: 1OD=0.23 g_{DM}/L.

pH was measured with a Mettler Toledo pH meter.

The nitrate concentration was determined using a modified method proposed by Collos et al. (Collos *et al.*, 1999). The method was based on ultraviolet absorption spectrometry. The absorbance of the liquid phase recovered after the centrifugation was measured at a single wavelength (220 nm) supplementing 20 µL of HCl 1M to 1mL of sample.

The phosphate concentration was measured according to the molybdate colorimetric test for ortho-phosphate. The colorimetric test was based on the monitoring the redox state the antimonyl-phosphomolybdate complex produced during the reaction among ammonium molybdate, potassium antimonyl tartrate and ortho-Phosphate. This complex is reduced to an intense blue-colored (molybdenum blue) complex by ascorbic acid (Pierzynski & Simis, 2000). The preparation of the reagents required the mixing of: 250 mL of 4.5 M H_2SO_4; 45 mL of ammonium molybdate solution 95 g/L; 5 mL of potassium antimonyl tartrate solution 32.5 g/L; 50 mL of ascorbic acid solution 70 g/L. 30 µL of reagent and 30 µL of ascorbic acid were added to 960 µL of diluted sample. The absorption time-evolution of each sample was measured by a spectrophotometer at 704 nm.

Cell rupture was required to release PHB granules. A procedure based on that proposed by (Schlebusch & Forchhammer, 2010) was used for the cell rupture. Biomass (10 mL of culture at 1 OD) was harvested by centrifugation (20 min, 4,890g, 4°C). The cell pellets were dried overnight at 85°C. The dry pellets were boiled in 1 mL

of H_2SO_4 96%for 40 min, diluted with 9 mL of 0.007 M H_2SO_4, and filtered by means of a polyvinylidene difluoride membrane. Quantitative analysis of PHB content in the solution produced from the chemical processing of the cells was carried out by means of HPLC (Agilent 1100 system) equipped with a multi-wave detector set at 210 nm. The separation column was Aminex HPX-87H (300x7.8 mm). Crotonic acid was used as the external standard for HPLC analysis. Indeed, PHB was converted in crotonic acid after treatment with sulphuric acid (Karr *et al.*, 1983a).

The presence of PHB in cells was visualized by means of the Nile red staining method. 200 µL of samples with cyanobacteria were supplemented with 50 µL of Nile red dye (from a stock with 1mg/mL in DMSO) and incubated for 10 minutes at room temperature. The suspension was rinsed with double distilled water. Slides with stained cyanobacterial cells were prepared according to the wet mount method and observed at fluorescent microscope settings with the excitation at 465 nm. The PHB granules were visible as red dots in the cells.

Apparatus

Pre-cultures were grown in 250 mL Erlenmeyer flasks housed in a climatic chamber (Gibertini) at 28 °C. The chamber was equipped with daylight fluorescent Philips lamps (TLD 30W/55) set at 150 $\mu Em^{-2}s^{-1}$ for 24/24 h.

Inclined bubble column photobioreactors (volume: 800 mL) were adopted for cyanobacterial growth. The photobioreactors were housed in a climate chamber (Solar Neon) at 28 °C. The chamber was equipped with fluorescent lamps (Philips TLD 30 W/55). A gas stream was sparged at the bottom of the photobioreactors by means of multiple-orifice (1 mm ID) Teflon tube. A hydrophobic filter (0.2 µm) sterilized the gas stream fed to the photobioreactors. The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations (Olivieri *et al.*, 2013). A gas mixing device (M2M engineering) provided the selected concentration of CO_2 in the gas stream fed to the photobioreactors by mixing air and pure carbon dioxide from a pressurized vessel. The CO_2 concentration in the air stream was set at 2% (v/v). Gas flow rate was set at 4 vvm.

Procedures

The cyanobacteria were transferred from a Petri plate into 250 mL Erlenmeyer flasks containing 50 mL medium. After about two weeks the pre-cultures were inoculated into photobioreactors: 10% (v/v) of actively growing pre-cultures. Each test was carried out in biological triplicate and the mean values are reported as results. The error was typically lower than 5%.

Cultures in inclined column photobioreactors were grown under light/dark cycles: 16 h light at 150 $\mu Em^{-2}s^{-1}$, plus 8 h dark. The cultures were sampled every 48 h. Samples were characterized in terms of pH and concentration of biomass, nitrate, phosphate and PHB concentration.

Measured data were processed to assess the PHB fraction (ω_{PHB}) and PHB productivity. ω_{PHB} was calculated according to (Eq. 1):

$$\omega_{\mathrm{PHB}} = \frac{\mathrm{PHB}}{\mathrm{X}} \cdot 100 \qquad (1)$$

where X is the cyanobacterial cell concentration, and PHB the poly-β-hydroxybutyrate concentration.

The PHB productivity ($\mathrm{P}_{\mathrm{PHB}}$) – production for volume unit - was calculated according to Eq. 2:

$$\mathrm{P}_{\mathrm{PHB}} = \frac{\mathrm{PHB}|_t}{\mathrm{t}} \qquad (2)$$

where $\mathrm{PHB}|_t$ is the poly-β-hydroxybutyrate concentration measured at the instant t.

Results and discussion

Nitrogen optimal composition

Figure 3.2.1 reports the time resolved data regarding autotrophic cultures of *S*. PCC6803 using the four culture media. In particular, Figure 3.2.1(a), (b), (c) and (d) reports biomass concentration, PHB fraction, PHB productivity and nitrate concentration measured during the growth using the four tested media, respectively. The typical four phases of the cell growth may be observed in Figure 3.2.1(a):

- a lag phase: cell concentration was almost constant and PHB was not produced;
- an exponential phase: cell concentration increased sharply and the PHB concentration increased remarkably. The onset of the exponential phase is marked by the beginning of the nitrogen starvation (marked by the dashed lines in Figure 3.2.1 - the vertical label indicate the medium);
- a stationary phase: the cell concentration was almost constant and the PHB production was active.

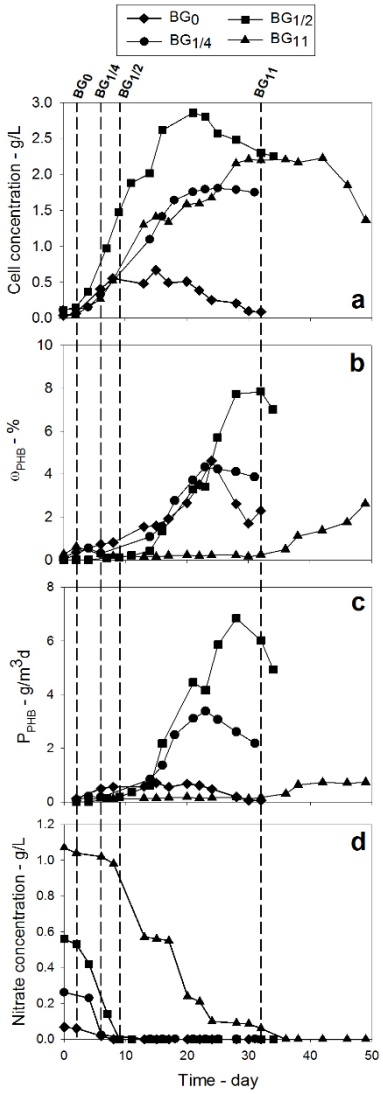

Figure 3.2.1: Data measured during the culture of *Synechocystis* PCC6803 in the four investigated media (BG_0, $BG_{1/4}$, $BG_{1/2}$, BG_{11}): A) cell concentration; B) PHB-fraction; C) PHB productivity; D) nitrate concentration. Reported data are the average of biological triplicate test and the error was typically lower than 5%.. Filled diamond (♦) BG_0, filled circle (●) $BG_{1/4}$, filled square (■) $BG_{1/2}$, filled triangle (▲) BG_{11}.Vertical lines mark the nitrogen starvation

The analysis of Figure 3.2.1(a) suggests that cyanobacteria continued to grow even though nitrogen starvation occurred. This observation may be explained by taking into account the spectrum of sources available for growth of the microorganism: extracellular substrates are consumed under nutrient replete condition; stored intracellular nutrients are consumed under nutrient depletion conditions. In particular, (Palabhanvi *et al.*, 2014) reported that the stored intracellular nutrients are present in three forms: (i) structural form of nutrient, SFN (structural protein, DNA, rRNA, etc.); (ii) readily utilizable nutrient, RUN (inorganic phosphate, nitrate, ammonia etc.); and (iii) non-readily utilizable nutrient, Non-RUN (some protein, mRNA, polyphosphate bodies etc.). Therefore, it is expected that investigated cyanobacteria used intracellular nitrogen during the large part of the exponential growth.

The analysis of Figure 3.2.1(a) also suggests that *Synechocystis* PCC6803 grew faster in $BG_{1/2}$ medium than in BG_{11} medium. This finding could be explained taking into account that the uptake of nitrate can be described by Haldane model (Mozumder *et al.*, 2015). Therefore, the growth rate under the nitrate concentration in BG_{11} could be low because the nitrate concentration is larger than that characteristic of the maximum uptake rate (probably close to $BG_{1/2}$ nitrate concentration).

The comparison of the PHB fraction of cells measured during the culture carried out in the four media may be done by analysing data reported in Figure 3.2.1(b). This data analysis points out that $BG_{1/2}$ was the best compromise between cell growth and PHB accumulation. Indeed, the maximum cell concentration does not change markedly for the cultures carried out in $BG_{1/2}$ and BG_{11} media but the cellular PHB accumulation was high and early in $BG_{1/2}$ growth medium.

The Figure 3.2.1(c) reports a comparison of the PHB productivity measured during the culture carried out in the four media. This data analysis reveals that the PHB productivity was characterized by a maximum for all the tested media. In particular, the maximum value of PHB productivity was found using $BG_{1/2}$ media as growth medium. The analysis of Figure 3.2.1(b) and 3.2.1(c) pointed out that the instant at which the maximum PHB fraction was measured was different from that at which PHB productivity was measured. A choice between the two targets must be made on the basis of post-processing operations: economic and technical analysis is required.

The best results were measured for the culture carried out in the $BG_{1/2}$ medium: PHB content of about 8% and maximum PHB productivity of about 7 g/m^3d.

The high accumulation of PHB under nitrogen-starvation conditions may be interpreted taking into account the absence of nitrogen into the medium while the photosynthesis was still active and fixed CO_2. Indeed, the cells cannot synthesize proteins and they accumulate the carbon/energy as PHB (Smith, 1982). As a result of the flux re-direction under nitrogen-starved conditions, it is interesting to note that $BG_{1/2}$ medium was better than BG_{11} because in this medium nitrogen starvation was reached in a shorter period of time. Moreover $BG_{1/2}$ medium was better than BG_0 medium because in BG_0 medium PHB was produced from the beginning of the test but, the cells concentration remained very low. With $BG_{1/4}$ medium the PHB fraction reached during cyanobacteria growth, was higher than PHB fraction reached with BG_0 and BG_{11}, but lower than PHB fraction reached using $BG_{1/2}$ medium.

Table 3.2.2 reports a comparison of data regarding the PHB production by cyanobacteria reported in the literature. The main observations are reported hereinafter:

Table 3.2.2: literature comparison

Ref	Strain	Carbon source	Culture condition	Reactor	Light intensity (µmol photon m^{-2} s^{-1})	Type of polymer	Polymer productivity (mg/L*day)	Polymer fraction (% dcw)	Polymer concentration (mg/L)
Bhati and Mallick (2015)	*Nostoc muscorum* Agardh	Glucose	Nitrogen depletion	flask	75 (14h light/10h dark)	P(3HB-co-3HV)	109.7	78	438.9
			Phosphorous depletion			P(3HB-co-3HV)	98	71	393
Coelho et al. (2015)	*Spirulina sp.* LEB 18	sodium bicarbonate	0.05 g/L sodium nitrate	flask	59.2, (12h light/12 h dark)	PHB	nr	30.7	nr
			0.5 g/L potassium phosphate						
Samantaray and Mallick (2014)	*Aulosira fertilissima* CCC 444	Fructose + Valerate	Phosphorous depletion	flask	nr	P(3HB-co-3HV)	38	77	537.5
Panda and Mallick (2007)	*Synechocystis* sp. PCC 6803	Fructose + Acetate	Nitrogen depletion	flask	75 (14h light/10h dark	PHB	nr	38	nr
Haase et al (2012)	*Nostoc muscorum*	CO_2	Phosphorous depletion	flask	85 (continuous light)	PHB	nr	21.5	nr
Kaewbai-Ngam et al. (2016)	*Calothrix scytonemicola* TISTR 8095	atmospheric CO_2	Nitrogen depletion	flask	50 (continuous light)	PHB	nr	25.4	356
Wu et al. (2000)	*Synechocystis* sp. PCC 6803	CO_2	Nitrogen depletion	flask	4000 lux (continuous light)	PHB	nr	4.1	27
This work	*Synechocystis* sp. PCC 6803	CO_2 (2%)	Nitrate concentration is the half of the optimal concentration	photobioreactor	150 (18h light/6h dark)	PHB	**7**	**8**	**180**

- The maximum PHB productivity and/or concentration are obtained under heterotrophic growth conditions (Bhati & Mallick, 2015; Panda & Mallick, 2007; Samantaray & Mallick, 2014). The data are very interesting. However, the PHB production requires the supplying of the carbon source.
- Coelho *et al.*, (2015), Haase *et al.*, (2012) and Kaewbai-ngam *et al.*, (2016) reported interesting results for autotrophic growth cultures carried out in flasks. They cultivated *Nostoc muscorum*, *Calothrix scytonemicola* and *Spirulina* sp. LEB 18, respectively. However, these strains are bifilm former strains (Di Pippo *et al.*, 2013) and their performance may strongly reduce when cultivated in photobioreactors;
- *Synechocystis* PCC6803 was characterized by high PHB productivity under heterotrophic conditions (Panda *et al.*, 2006). However, the performance drastically reduced when growth under autotrophic conditions (Wu *et al.*, 2001). The PHB fraction reached by Wu et al. (Wu *et al.*, 2001) was half the value reported in this work. This could be explained by the different conditions used for cyanobacteria growth. Indeed Wu et al. (Wu *et al.*, 2001) used a continuous light illumination while in this work a circadian rhythm (18h light/6h dark) for cyanobacteria growth was used. Greater PHB accumulation in cultures grown in light-dark cycles than those grown under continuous light agrees with the findings of other scientists (Ansari & Fatma 2016), which hypothesized that dark periods are obligatory for PHB accumulation in phototrophic cultures.

The reported results and comparison with the literature proved that the selected medium composition was optimal for PHB production under autotrophic conditions and it was used in the screening of the strains.

Strain screening

Pure cultures of *S.* PCC6803, *S. aquatilis*, *S. fuscopigmentosa*, *S. nidulans*, and *C. fritshii* were investigated for the PHB production screening. In particular, PHB accumulation was assessed by microscopic observations of Nile red stained cell method. The five strains were characterized by remarkable PHB accumulation: PHB inclusions were highlighted as bright orange intracellular granules (Figure 3.2.2).

The colorimetric assay did not allow to assess the PHB fraction but it was used just to point out the presence of PHB in the cells.

Figure 3.2.3 reports data measured during cultures the screened strains of cyanobacteria using the $BG_{1/2}$ medium: *S*. PCC6803, *S. aquatilis*, *S. nidulans* and *C. fritshii.*

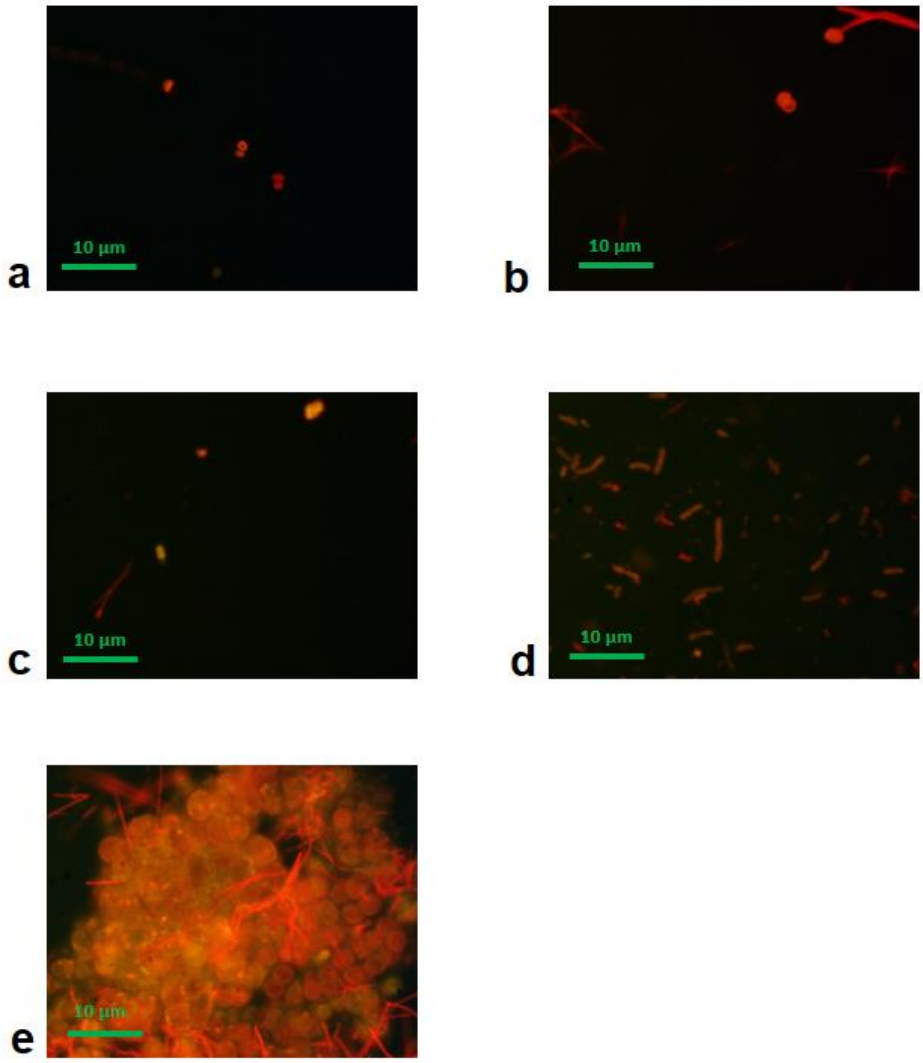

Figure 3.2.2: Microscope observation of Nile red stained cells of cyanobacteria: A) *Synechocystis* sp. PCC6803; B) *S. fuscopigmentosa*; C) *S. aquatilis*; D) *S. nidulans*; E) *C. fritshii*. Bright orange intracellular granules highlighted PHB inclusions.

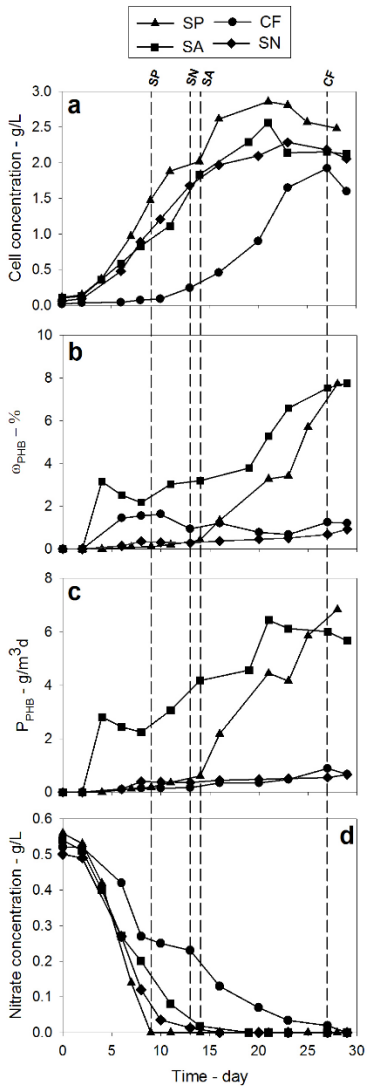

Figure 3.2.3: Data measured during the growth of the four screened cyanobacteria strains (*S. PCC6803, S. aquatilis, C. Fritshii, S. nidulans,*) using $BG_{1/2}$ medium: A) cell concentration; B) PHB-fraction; C) PHB productivity; D) nitrate concentration. Reported data are the average of biological triplicate test and the error was typically lower than 5%. Filled diamond (♦) SN, filled circle (●) CF, filled square (■) SA, filled triangle (▲) SP. Vertical lines mark the nitrogen starvation

Data regarding the culture of *S. fuscopigmentosa* were not reported because PHB was not produced during the test. Cyanobacteria cell concentration was reported in Figure 3.2.3 (a), PHB content, PHB productivity and nitrate concentration were reported in Figure 3.2.4 (b), (c) and (d), respectively. The beginning of the nitrogen starvation was marked by vertical dashed lines (the vertical label above the vertical lines indicate the strain).

Except for *C. fritshii*, all the screened cyanobacteria strains continued to grow even though nitrogen starvation occurred (Figure 3.2.3 a), according to the results pointed out during the medium optimization tests. The time resolved plot of the cell-concentration was quite similar for *S.* PCC6803, *S. aquatilis*, and *S. nidulans*. The maximum cell concentration of *S.* PCC6803 was about 30% larger than that of *S. aquatilis*, and *S. nidulans*. *C. fritshii* was characterized by a longer lag phase and by lysis as nitrogen starvation occurred.

S. PCC6803 and *S. aquatilis* were the best strains for PHB production. PHB content and maximum daily productivity were more or less constant for the two strains:

about 7.7 % and 6.8 g/m^{3}d for *S. PCC6803* and about 7.75% and 6.4 g/m^{3}d for *S. aquatilis*.

The analysis of Figure 3.2.3 (b) and (c) suggests that *S.* PCC6803 and *S. aquatilis* were characterized by the highest PHB content and productivity among the investigated strains. The main differences between these strains were:

i) *S. aquatilis* produced PHB since the beginning of the growth. PHB production continued even after nitrogen starvation;

ii) *S.*PCC6803 began to produce PHB at the nitrogen starvation onset.

iii) *S. aquatilis* reached the maximum value of the PHB productivity in a period shorter than that required by *S*. PCC6803.

Conclusions

The biosynthesis of PHB in autotrophic cultures of cyanobacteria was successfully carried out: it was enhanced under nitrogen-starvation conditions.

The medium optimization for PHB production was investigated with reference to *S*. PCC6803 (model strain). The optimal nitrogen concentration was half the optimal concentration assessed for cell growth. Under the optimized conditions the maximum PHB-cell fraction and the maximum PHB productivity were: 8 % and 7 g/m^{3}d, respectively.

The screening tests pointed out that *S*. PCC6803 and *S. aquatilis* were the best strains for PHB production. PHB fraction and productivity of about 8% and 7 g/m^{3}d were obtained for both strains. The results are very promising to support the autotrophic cyanobacteria cultures as a production route for PHB from very cheap and abundant substrate, the carbon dioxide.

The production of PHB by means of autotrophic cultures may be proposed as an eco-sustainable production process because it couples the production of bio-polymers with the capture of the CO_2 from gas streams produced by anthropic activities.

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The grant PRIN "Innovative downstream processing of conversion of algal biomass for the production of jet fuel and green diesel" from Ministreto dell'Università, della Ricerca is acknolowdged. The support of Mr. Saverio Niglio in performing tests is kindly acknowledged.

Abbreviations:

BG_{11}, medium with the optimal nitrate concentration; **BG_0**, medium without nitrate; **$BG_{1/2}$**, medium in which nitrate concentration was the half of optimal concentration; **$BG_{1/4}$**, medium in which nitrate concentration was one fourth of the optimal concentration; **CF**, *Chlorogloeopsis fritshii;* **PHB**, poly-β-hydroxybutyrate; **SP**, *Synechocystis* PCC6803; **SA**, *Synechocystis aquatilis*; **SF**, *Synechocystis fuscopgmentosa*; **SN**, *Synechococcus nidulans*.

Nomenclature

P_{PHB}	[g/m^{3}d]	PHB productivity
t	[day]	time
ω_{PHB}	[% w/w]	PHB fraction
X	[g_{DM}/L]	cyanobacteria cell concentration

3.3 Growth kinetics characterisation of *Synechocystis* PCC6803 in flat photobioreactors

Introduction

Cyanobacteria are photosynthetic microorganisms that offer extremely interesting industrial potential. Illumination has a major influence on cyanobacteria growth. Indeed, sunlight provides all the energy required to support metabolism, but, if present in excess, it can damage cells (photoinibition). To respond to excess light, photosynthetic organisms evolved physiological mechanisms which causes a reduction in light use efficiency and they must be minimized to reach an optimal productivity (Sforza *et al*., 2012).

Photobioreactors and open ponds are the systems used for growing phototrophic microorganisms such as cyanobacteria and microalgae. These microorganisms are able to convert light and CO_2 to chemical energy stored as organic carbon sources (e.g. carbohydrates and lipids), one of the most effective ways to produce biomass from sunlight (Olivieri *et al*., 2013).

The growth of cyanobacteria in photobioreactors causes the increase of optical density. As a consequence light distribution in the photobioreactors is not homogeneous. Indeed the cells on the surface, directly exposed to light, absorb most of the available radiation. Instead, the cells in the dark zone of the photobioreactors receive only a small fraction of radiation, which may be limiting for the growth. A solution to avoid the reduction of the efficiency is the use of flat photobioreactors characterized by thin depth. A reduction of light path could be beneficial for cell growth but thin reactors are unlikely to be economically sustainable on a large-scale (Sforza *et al*., 2014).

Flat configuration was investigated to characterize the growth kinetics of *Synechocystis* PCC6803 using LED technology as light source. LED technology has several advantages: (i) marked cost reduction due to higher energy efficiency than common light sources (halogen lamps and fluorescence tubes); (ii) emission in very narrow wavelength spectra appropriate for microalgal pigments; (iii) light and small devices to fit any PBR configuration; (iv) very long life-expectancy; and (v) low heating-up of irradiated cultures (Olivieri *et al*., 2013). Two levels of CO_2 concentration in gas stream were investigated: 0.035%v (Air) and 2%v.

Materials and Methods

Microorganism and medium

Synechocystis sp. PCC6803 was investigated for kinetic assessment. Cyanobacteria were grown under photoautotrophic conditions in BG_{11} media.

Apparatus and operating conditions

A gas mixing device (M2M engineering) provided the gas stream to fed at the photobioreactors at the selected concentration of CO_2. The air stream was mixed with a pure carbon dioxide stream from a pressurized vessel. The CO_2 concentration was set at 0.035 %v (air) and 2%v.

The temperature of precultures and of cultures were set at 28°C.

Precultures

Pre-cultures were grown in 250 mL Erlenmeyer flasks housed in a climatic chamber (Gibertini). They were used to inoculate photobioreactors. The chamber was equipped with daylight fluorescent Philips lamps (TLD 30W/55) set at 150 $\mu E/m^2s$ for 24/24 h.

Flat photobioreactor for kinetic growth assessmement

Flat configuration of photobioreactor (FPBR) was used for kinetic evaluation of cyanobacteria growth. FPBR was a parallelepiped (80 cm x 20 cm x 3 mm) with a working volume of 0.2 L (Figure 3.3.1). The head of the photobioreactors was equipped with three ports for gas inlet/outlet and culture sampling. FPBRs where housed in climate chambers equipped with LED panels and were placed at 20 cm from the light source. FPBRs were sterilized with Steril-C solution for 30 min. Gas was sparged at the bottom of the column by means of a multiple-orifice (1 mm ID) Teflon pipe.

Physico-chemical methods

The pH and the cell concentration were measured in the culture sampled from the photobioreactors. The sample was then centrifuged at 5,000 rpm for 15 min. The liquid phase was characterized in terms of concentration of nitrate and phosphate. The solid phase was processed to assess the PHB content.

Experimental procedures

Cyanobacteria were transferred from a Petri plate into 100 mL Erlenmeyer flasks containing 50 mL medium. The two-week pre-cultures were inoculated into photobioreactors: 10% (v/v) of actively growing pre-cultures.

Tests were carried out without any control of the pH under 24/24 h continuous constant irradiance. The irradiance ranged between 5 and 80 $\mu E/m^2s$. The cultures were sampled two time each day during the lag phase and each hour during the exponential phase. The samples were characterized in terms of biomass content. Each test condition was carried out in biological triplicate and the mean values are reported, typically with a variation of < 5 %.

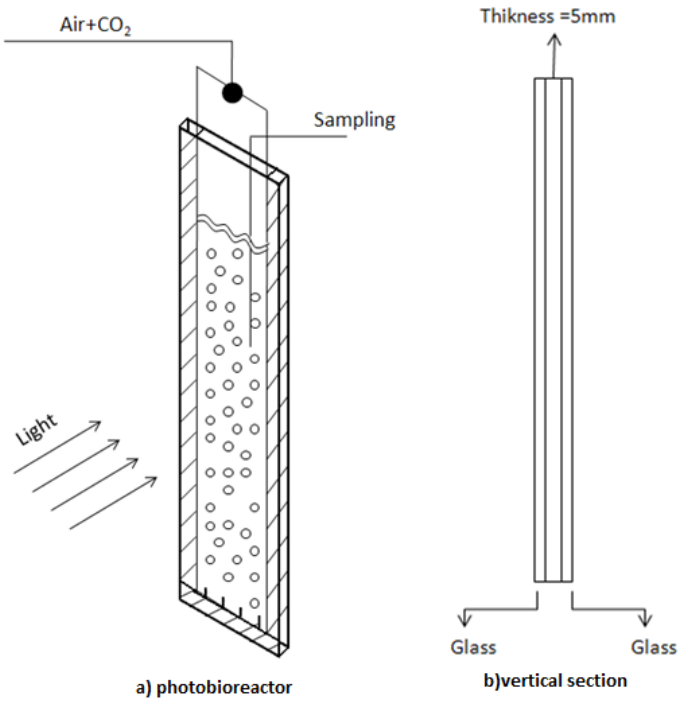

Figure 3.3.1: flat photobioreactor configuration (FPBR)

Theoretical Framework

The aim of the tests was to assess the relationship between growth rate (μ) and light intensity (I). According to the studies available in the literature, the growth rate (μ) of autotrophic cells depends on different variables (Eq. 1):

$$\frac{1}{X}\frac{dX}{dt} = \mu = \mu(X, C_{CO_2}, C_{O_2}, T, I, C_P, C_N, C_S, \dots) \quad \text{(Eq. 1)}$$

Tests were carried out keeping constant all variables except for the light intensity that was changed in the range 5 - 80 μE/m^{2}s. Therefore, the Eq. 2 yields:

$$\mu = \mu(I) \quad \text{(Eq. 2)}$$

The assessment of μ at a set value of I required that the cultures was at constant irradiance during all the test. Since the irradiance of the deep region of the photobioreaector progressively decreased as the time passed, the growth was carried out having care that I changed of less than 20% with respect to the nominal value. According to this observation, the cell growth was carried out until the cell concentration reached a critical value at which the light intensity decay (I-I_0) across the photobioreactor was equal to the 40% of the incident light (I_0) (Eq. 3):

$$\Delta I \leq 0.4\, I_0 \quad \text{(Eq. 3)}$$

This X critical value was assessed by means of the Lambert-Beer law (Eq. 4):

$$A = \varepsilon X \delta \quad \text{(Eq. 4)}$$

where A is the absorbance, ε the extinction coefficient and δ the light path. According to Eq. 4, the critical absorbance value was assessed to be 0.7 OD.

The growth rate was calculated by the linear regression of the straight line ln X/X_0 vs. time, where X is the current optical density and X_0 is the optical density at the beginning of the exponential phase. The regression procedure was extended up to the X critical value. The assessed growth rate was associated to 0.8 I_o.

Results

Cell growth rate

The effect of different illumination on *S.* PCC6803 growth rate was studied by varying light intensity between 5 and 80 μE/m^{2}s. Two CO_2 levels were investigated: 0.035 %v (CO_2 concentration in air), and 2 %v. The measured growth rate (μ) vs. light intensity (I) is reported in Figure 3.3.2 for both CO_2 conditions. The analysis of Figure 3.3.2 points out that:

- a minimum value of light intensity under which no growth was observed. This value was 5 μE/s m for both conditions;
- at light intensity between 5 and 80 μE/m^{2}s the specific growth rate increased with light intensity for both CO_2 conditions. Therefore, no photoinhibition effect for growth was experienced in the investigated range. Further experiments at high light intensity (80-300 μE/m^{2}s) are necessary to study the photoinhibition phenomenon;

- for each value of the light intensity in the investigated range, the growth rate increased with the CO_2 concentration;

On the basis of the aforesaid observations, a modified hyperbolic correlation (Eq. 5) was used to fit the experimental data (Figure 3.3.2 theoretical line):

$$\mu = \mu_{max} \frac{I - I^*}{K_m + I - I^*} \qquad \text{(Eq. 5)}$$

where μ_{max} is the maximum growth rate, I^* the minimum light intensity under which no growth was observed, $(K_m + I^*)$ the light intensity at which μ is half its maximum value (μ_{max}).

Table 3.3.1 reports K_m, μ_{max}, and I^* for both CO_2 conditions. From the analysis of the Table 3.3.1 it can be noticed that:

- the value of μ_{max} and I^* calculated when CO_2 was set at 0.035 %v, was half the value calculated when CO_2 was set at 2 %v.

These results confirmed that high concentration of CO_2 in the gas stream increased the microorganism growth performances as reported in literature by Olivieri *et al.* (2012).

Table 3.3.2: K_m, μ_{max}, and I^* for both CO_2 conditions.

	2 %v CO_2	**0.035 %v CO_2**
μ_{max} (h^{-1})	0.15	0.07
K_m (μE $m^{-1}s^{-2}$)	53	27
I^* (μE $m^{-1}s^{-2}$)	5	5

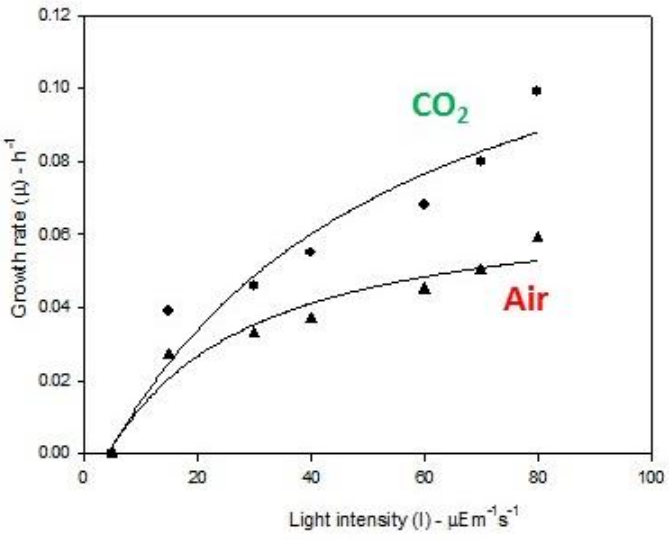

Figure 3.3.2: *Synechocystis* PCC6003 specific growth rate (μ) vs. light intensity (I) at CO_2 0.035 %v (CO_2 air concentration) (● plot) and at CO_2 at 2%v (▲plot).

Conclusions

The effect of different illuminations, ranging from 5 to 80 µEm^{-1}s^{-}1, on *Synechocystis* PCC6803 was studied in FPBRs. Two levels of CO_2 concentration were investigated at 0.035 %v and 2 %v.

A minimum value of light intensity under which no growth was observed can be determined. This value was 5 µE m^{-1}s^{-1} for both CO_2 condition.

The specific growth rate increased with light intensity for both CO_2 conditions. The species showed a maximum growth rate at 80 µE/m^2 s in both cases.

A modified Michaelis-Menten correlation was used to fit the experimental data. The results obtained from this correlation showed that the value of μ_{max} and I^* calculated when CO_2 was set at 0.035 %v, was half the value calculated when CO_2 was set at 2 %v.

These results confirmed that high concentration of CO_2 in the gas stream increased the cyanobacteria growth performances.

Nomenclature

PBR: phobioreactor;
FPBR: flat photobioreactor;
µ: microalgae specific growth rate;
μ_{max} : the maximum growth rate;
I^*: the minimum light intensity under which no growth was observed;
I: the light intensity;
$(K_m + I^*)$: the light intensity at which µ is half its maximum value (μ_{max})

4 KINETIC MODEL

This chapter reports about a kinetic model and adopted to characterize the PHB production from *Synechocystis* PCC6803. Batch tests were carried out in photoautotrophic conditions using different nitrate concentration in the media with, then the experimental data were used to implement the kinetic model.

A kinetic dynamic model of PHB production by *S.* PCC6803 has been developed by adopting the biochemical networks simulator COPASI.

4.1 Poly-β-hydroxybutyrate (PHB) production by *Synechocystis* PCC6803 from CO_2: Model development

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ABSTRACT

The biosynthesis of poly-β-hydroxybutyrate (PHB) by bioconversion of CO_2 is a sustainable alternative to the non-renewable, petroleum-based polymer production. Indeed, the PHB production by conversion of CO_2 contributes to the reduction of the greenhouse gas concentration in the atmosphere. A kinetic dynamic model of PHB production by autotrophic cultures of *Synechocystis* PCC6803 was proposed and developed by means of the biochemical networks simulator COPASI.

Two classes of cells were assumed to be present in the broth: growing cells and PHB producing cells. The model included the two classes of cells and their nitrogen and phosphate internal quota. The dynamics of the cell growth and PHB production were described taking into account: the cellular growth rate; the lysis rate; the nitrate and phosphate utilization rate; the PHB production rate. The assessment of the kinetic parameters and of the yields was carried out by the regression of experimental data. Tests were carried out in photobioreactors under dynamic light system (light/dark cycle) using media characterized by initial nitrate concentration ranging between 0 and 1.5 g/L.

The proposed model successfully reproduced the measured experimental data (cell concentration, nitrogen and phosphate concentration and PHB content): the square correlation coefficient of the investigated variable concentrations ranged between 0.81 and 0.99. Parameter sensitivity analysis was also carried out to assess the role of the chosen parameters on cell growth and PHB accumulation. The dynamics of cellular growth were not significantly affected by a ±20% variation of maximum specific growth rate, specific of velocity of conversion to PHB producing cells, and of maximum uptake rate of nitrate. The PHB accumulation dynamics were particularly sensitive to the variation of the value of the investigated parameters.

The proposed model may support the design and the optimization of a PHB production process by means of autotrophic cultures.

Keywords: polyhydroxybutyrate (PHB); cyanobacteria; nitrate concentration; kinetic model; COPASI

INTRODUCTION

The continuous increase of production and consumption of plastic in modern society, and the growing concern about solid waste management, are shifting attention to renewable and biodegradable polymers (Dias *et al.*, 2006). Therefore, the bioplastics market is destined to increase rapidly. According to the market data delivered by European Bioplastics association, the global production capacity of bioplastics is predicted to quadruple in the medium term. In particular, the European Bioplastics association reports that the global production of poly-β-hydroxybutyrate (PHB) – a biodegradable plastic - was approximately 34 ktonnes in 2014 and is forecasted to grow up to a 100 ktonnes in 2019. This interest in PHB is particularly due to its interesting features: thermoplastic processability, hydrophobicity, complete biodegradability and biocompatibility (Braunegg *et al.*, 1998). PHB is synthesized by and accumulates as intracellular granules in many bacteria, as a store of carbon and energy (Hahn *et al.*, 1995).

Biomaterials can also be produced by means of photoautotrophic organism. This approach is particularly attractive because it couples capturing of CO_2 from the atmosphere to its utilization for the production of chemicals (Campbell & Lobell, 2009). Photosynthetic life forms have attracted enormous interest as vehicles to capture light energy and subsequently convert that into the free energy of organic compounds, using water as the ultimate electron donor. Nowadays, two major technologies are employed with photoautotrophic organisms: i) plant-based organic compound production; ii) algae derived biomaterial production (Angermayr *et al.*, 2009). Plant-based systems are very attractive because no extra carbon source needs to be supplemented, a welcome economic requisite for large-scale production systems (Goddijn & Pen, 1995). However, plant-based systems compete directly with food production (Hempel *et al.*, 2011). A potential solution is could be to use algae-based biomaterial production: no extra carbon source is necessary and production systems may be developed in any area not used for the agro-food market.

Cyanobacteria (which belong to the Gram-negative bacteria) share all the advantages of photosynthetic microorganisms. Indeed they can convert CO_2 and sunlight directly into a product of interest, such as PHB (Gopi *et al.*, 2014; Gupta *et al.*, 2013; Wijffels *et al.*, 2013). The strain *Synechocystis* sp. PCC6803 is the best-characterized species and has been selected for various applications (Mikami *et al.*, 2002). *Synechocystis* sp. PCC6803, just as many other non-diazotrophic cyanobacteria, is characterized by its ability to induce drastic shifts of its central carbon metabolism, by tuning of the minerals concentration in the culture medium. Anfelt *et al*. (2015) have shown that under nitrogen depletion conditions the intracellular carbon flux is directed to lead to accumulation of compounds such as PHBs.

The optimization of PHB production is not a trivial issue because its production by microorganisms involves a combination of different metabolic pathways (Luengo *et al.*, 2003). For an effective design, scale-up, and optimization of PHB production process, it is necessary to have insight into the mechanisms of these metabolic routes. Indeed, an adequate model to describe the process is a key issue to address the conditions to maximize the fraction of PHB in the cell. Kasiri *et al*., (2015) proposed to uncouple the kinetics of nutrient uptake and cell growth. They proposed a nonlinear dynamic model to assess the optimal CO_2 concentration, phosphate concentration, and light intensity for CO_2 uptake and algal growth. The model proposed by Kasiri *at al.,* (2015) was focused on algal growth and it did not include the production of metabolites. Kim *et al*., (2015) proposed a multi-component kinetic model, applied to the cyanobacterium *Synechocystis* sp. PCC6803, and they applied the model to batch

tests to assess the kinetic parameters. Jeon *et al*., (2014) proposed to integrate a metabolic network model with a semi-empirical model (Droop model) to predict the simultaneous lipid accumulation and cell growth for the microalgal species *Chlamydomonas reinhardtii*. The model proposed by Jeon *et al*., (2014) did not include the effect of light intensity on microalgal growth. Mozumder *et al*., (2015) described the autotrophic PHB production in *Cupriavidus necator* by using a model based on a two-phase process: biomass growth and subsequent PHB production. The aim was to simulate the two distinct phases to achieve a high PHB production rate and PHB content. Faccin *et al*., (2012) presented a model to describe the kinetics of growth of the bacterium *Bacillus megaterium* for the production of PHB. Khanna and Srivastava (2005) proposed a structured unsegregated model for PHB accumulation in *Ralstonia eutropha*, based on detailed investigations on substrate limitations and inhibition by carbon-, nitrogen-, and phosphorus sources. Although the reported model considered PHB production in microorganisms, they did not include autotropic growth conditions of photosynthetic microorganisms. To the best of this author's knowledge, no model was reported in the literature regarding the PHB production by autotrophic cultures of cyanobacteria.

This paper proposes a kinetic dynamic model to describe photoautotrophic PHB production by cyanobacteria in photobioreactors under a light/dark cycle. Two types of cells were considered in the models: growing cells - able to grow - and PHB producing cells. The model is based on transient mass balances applied to the growing cells, the PHB producing cells, the PHB, the inorganic nitrogen-containing and phosphate-species, and it included the nitrate and phosphate utilization rate, the PHB production rate, the cell growth and the lysis rate of the cells. The effect of significant factors and nutrients on the cyanobacterial cells were taken into account. In particular, the proposed assessment of the kinetic parameters and of the yields was carried out by the regression of experimental data. Tests were carried out in photo-bioreactors subjected to a dynamic light regime (light/dark cycle) using media characterized by an initial nitrate concentration ranging between 0 and 1.5 g/L. Parameter sensitivity analysis was also carried out with the aim to assess the relevance of the proposed mechanisms on the performance of the PHB production process.

MATERIALS AND METHOD

MICROORGANISM AND MEDIUM

Synechocystis sp. PCC6803 was provided by the Laboratory of Microbiology of the Universiteit van Amsterdam. The strain was grown under photoautotrophic growth conditions in four growth media, each characterized by a different nitrogen concentration (Table 4.1.1). The features of the four media were: BG_{11} contained the optimal concentration of nitrate (1.5 g/L) for cyanobacterial growth according to Waterbury & Stainer (1981); BG_0 did not contain nitrate; $BG_{1/2}$ contained half the optimal nitrate concentration; $BG_{1/4}$ contained one fourth of the optimal nitrate concentration.

Table 4.1.1: The composition of BG_0, $BG_{1/4}$, $BG_{1/2}$ and BG_{11} in g/L, after Carpine et. al, (Carpine *et al.*, 2015).

	BG_0	$BG_{1/4}$	$BG_{1/2}$	BG_{11}
$NaNO_3$	0	0.375	0.75	1.5
K_2HPO_4	0.04			
$MgSO_4$	0.075			
$CaCl_2*2H_2O$	0.036			
Citric acid	0.006			
Ammonium ferric citrate	0.006			
$Na_2EDTA*2H_2O$	0.001			
$NaCO_3$	0.02			
HBO_3	2.86			
Mn Cl_2 * $4H_2O$	1.81			
$ZnSO_4$ * $7H_2O$	0.22			
Na_2MoO_4 *2 H_2O	0.39			
$Co(NO_3)_2$	0.05			

OPERATING CONDITIONS AND PROCEDURES

The cyanobacteria were transferred from a Petri plate into 250 mL Erlenmeyer flasks containing 50 mL medium. Pre-cultures were incubated in a climatic chamber (Gibertini) at 28 °C. The chamber was equipped with daylight fluorescent Philips lamps (TLD 30W/55), set at 150 µEm-2s-1 for 24/24 (Carpine *et al.*, 2015). After about two weeks of culturing, the pre-cultures were inoculated into photobioreactors: 10% (v/v) of actively growing pre-cultures were added.

Inclined bubble column photo-bioreactors (volume: 800 mL) were used for cyanobacterial cultures. The photobioreactors were housed in a climate chamber kept at constant temperature (28 °C). The chamber was equipped with fluorescent lamps (Philips TLD 30 W/55). Cultures were grown in inclined column photo-bioreactors under light/dark cycles: 16 h light/8h dark at 150 $\mu Em^{-2}s^{-1}$.

A gas stream was sparged at the bottom of the photobioreactors by means of multiple-orifice (1 mm ID) Teflon tube. A hydrophobic filter (0.2 µm) sterilized the gas stream fed to the photobioreactors. The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations (Olivieri *et al.*, 2013). A gas mixing device (M2M engineering) provided the selected concentration of CO_2 in the gas stream fed to the photobioreactors by mixing air and pure CO_2 from a pressurized vessel. The CO_2 concentration in the air stream was set at 2% (v/v). The gas flow rate was set at 4 volume/volume/min.

The cultures were sampled every 48 h. Samples were characterized in terms of pH and concentration of biomass, nitrate, phosphate and PHB concentration. Each test was carried out in biological triplicate and the mean values are reported as results. The error was typically within 5%.

ANALYTICAL METHODS

The pH and the total cell concentration were measured in cultures via sampling from the photobioreactors. Each sample was centrifuged at 5,000 g for 15 min. The liquid phase was characterized in terms of the concentration of nitrate and phosphate. The solid phase was processed to assess the PHB content of the cells.

The biomass - cyanobacteria - concentration of the culture broth was measured by means of a spectrophotometer (Specord 50 – Analytic Jena) at 730 nm and expressed as dry cell weight, according to the procedure reported by Carpine et al. (Carpine *et al*., 2015) with the conversion factor: 1 OD_{730} = 0.23 g_{DM}/L.

pH was measured with a Mettler Toledo pH meter.

The nitrate concentration was measured according to a modified method proposed by (Collos *et al.*, 1999). The method was based on ultraviolet absorption spectrometry. The absorbance of the liquid phase recovered after the centrifugation was measured at 220 nm, after supplementing the spent medium with 20 µL of 1 M HCl to 1 mL of sample.

The phosphate concentration was measured with the molybdate colorimetric test for ortho-phosphate. This colorimetric test is based on the monitoring of the redox state of the antimonyl-phosphomolybdate complex produced during the reaction between ammonium molybdate, potassium antimonyl tartrate and ortho-Phosphate. Upon reduction this complex forms an intense blue-coloured (molybdenum blue) complex with addition of ascorbic acid (Pierzynski & Simis, 2000). The preparation of the reagents required the mixing of: 250 mL of 4.5 M H_2SO_4; 45 mL of ammonium molybdate solution 95 g/L; 5 mL of potassium antimonyl tartrate solution 32.5 g/L; 50 mL of ascorbic acid solution 70 g/L. 30 µL of the reagent solution and 30 µL of ascorbic acid were added to 940 µL of diluted sample. The absorption of each sample was measured by means of the spectrophotometer at 704 nm.

Cells were ruptured to recover the PHB granules. The cells were broken according to a procedure based on that proposed by Schlebusch & Forchhammer, 2010. Cells (10 mL of culture at 1 OD) were harvested by centrifugation (20 min, 5,000 g, 4° C) and the pellet was dried overnight at 85 °C. The dry pellets were boiled in 1 mL 96 % (v/v) H_2SO_4 for 40 min, diluted with 9 mL of 0.007 M H_2SO_4, and filtered by means of a polyvinylidene difluoride membrane. Quantitative analysis of the PHB content in the solution produced during the H_2SO_4 treatment was carried out by means of HPLC (Agilent 1100 system) equipped with a multi-wave detector set at 210 nm. The separation column used was Aminex HPX-87H (300x7.8 mm). Crotonic acid was used as the external standard for HPLC analysis. PHB was converted into crotonic acid prior to measurements by treatment with sulphuric acid (Karr *et al.*, 1983).

THEORETICAL FRAMEWORK

MODEL

The kinetic model for the cyanobacterial system was aimed to describe the effects of significant factors and nutrients on the growth rate of the cyanobacteria, the nutrient uptake, and the PHB production. The proposed model includes the nitrate- and phosphate-utilization rate, the PHB production rate, the cellular growth rate and lysis rate (Kim *et al.*, 2015). A scheme of the proposed cyanobacterial system is shown in Figure 4.1.1.

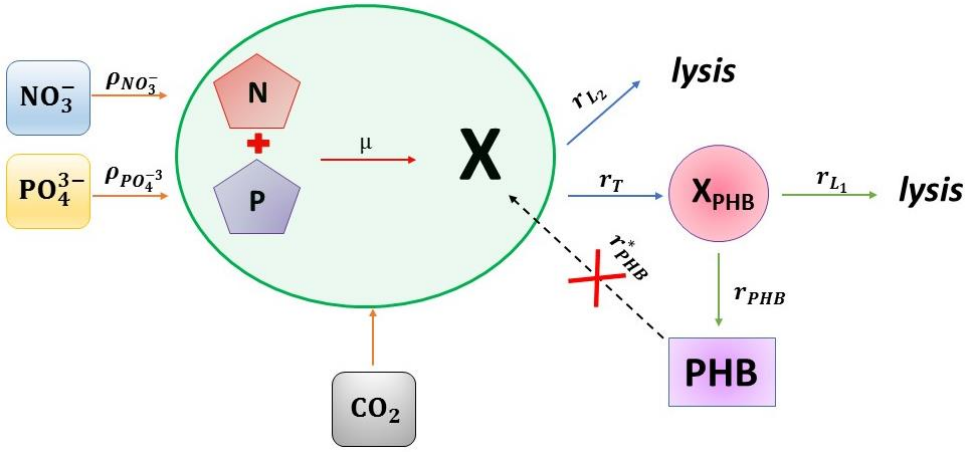

Figure 4.1.1. Scheme of the simplified mechanism assumed for the PHB production

The relevant species in this system were: the nitrogen source (concentration of NO_3^-); the phosphorous source (concentration of PO_4^{3-}); the internal quota of nitrogen (mass fraction with respect to the dry cell Q_n, concentration N); the internal quota of phosphorous (mass fraction with respect to the dry cell Q_p, concentration P); the inorganic carbon source as sum of the dissolved CO_2 (aq) and of the HCO_3^- (overall concentration C_i); the growing cellular biomass (concentration of X); the PHB producing cells (concentration of X_{PHB}); the product of interest (concentration of PHB). Seven steps were taken into account to describe the process sketched in Figure 4.4.1. The reactions associated to each step are listed in in Table 4.1.2 and are explained hereinafter:

a) the phosphate and nitrate uptake (Eq. T.4.1.2.1 and T.4.1.2.2). In the Table 4.1.2, $\rho_{PO_4^{-3}}$ is the phosphate specific uptake rate, $\rho_{NO_3^-}$ the nitrate specific uptake rate, $Y_{P/PO_4^{3-}}$ is the phosphate uptake yield, and Y_{N/NO_3^-} the nitrate uptake yield;

b) the cell growth (Eq. T.4.1.2.3). In the Table 4.1.2, μ is the cell-specific growth rate, $Y_{C_i/X}$ the C_i uptake yield, $Y_{N/X}$ the internal nitrogen quota (Q_n) uptake yield, $Y_{P/X}$ the internal phosphorous quota (Q_P) uptake yield;

c) the cellular transformation from growing cells (X) to PHB-producing cells (X_{PHB}) (Eq. T.4.1.2.4). In the Table 4.1.2, r_T is the rate of formation of PHB-producing cells;

d) the lysis of the growing cells and of the PHB-producing cells (Eq. T.4.1.2.5 and T.4.1.2.6). In the Table 4.1.2, r_{L_2} is the lysis rate of growing cells, r_{L_1} the lysis rate of PHB producing cells;

e) the PHB production (Eq. T.4.1.2.7). In the Table 4.1.2, r_{PHB} is the PHB production rate, Y_{PHB/CO_2} the CO_2 to PHB yield.

f) the PHB uptake r^*_{PHB}. A decrease in PHB concentration may be due to PHB utilization for cell functioning (Ansari & Fatma 2016). This path has not been included in the model because the rate is expected to be negligible under the operating conditions investigated.

Table 4.1.2: The simple reactions of the steps reported in the Figure 4.1.1.

Rate	REACTION	Eq.
$\rho_{PO_4^{-3}}$	$PO_4^{3-} \rightarrow Y_{P/PO_4^{3-}} \cdot P$	T.4.1.2.1
$\rho_{NO_3^-}$	$NO_3^- \rightarrow Y_{N/NO_3^-} \cdot N$	T.4.1.2.2
μ	$Y_{N/X} \cdot N + Y_{P/X} \cdot P + Y_{C_i/X} \cdot C_i \rightarrow X$	T.4.1.2.3
r_T	$X \rightarrow X_{PHB}$	T.4.1.2.4
r_{L_1}	$X_{PHB} \rightarrow$ lysis	T.4.1.2.5
r_{L_2}	$X \rightarrow$ lysis	T.4.1.2.6
r_{PHB}	$CO_2 \rightarrow Y_{PHB/CO_2} \cdot PHB$	T.4.1.2.7

Mass balances that hold for the cells (X and XPHB), the mineral substrates (nitrate and phosphate), and PHB under transient conditions, according to the scheme reported in the Figure 4.1.1 are, respectively:

$$\frac{dX(t)}{dt} = \mu X - r_T - r_{L_2} \quad (1)$$

$$\frac{dX_{PHB}(t)}{dt} = r_T - r_{L_1} \quad (2)$$

$$\frac{dNO_3^-(t)}{dt} = -\rho_{NO_3^-} \cdot X \quad (3)$$

$$\frac{dPO_4^{3-}(t)}{dt} = -\rho_{PO_4^{-3}} \cdot X \quad (4)$$

$$\frac{dN(t)}{dt} = Y_{N/NO_3^-} \cdot \rho_{NO_3^-} \cdot X - Y_{N/X} \cdot \mu \cdot X \quad (5)$$

$$\frac{dP(t)}{dt} = Y_{P/PO_4^{3-}} \cdot \rho_{PO_4^{-3}} \cdot X - Y_{P/X} \cdot \mu \cdot X \quad (6)$$

$$\frac{dPHB(t)}{dt} = r_{PHB} \quad (7)$$

The main assumptions made to assess the kinetics and the stoichiometric parameters of the model are:

- the substrates uptake and cyanobacterial growth are not coupled;
- the Droop model was used to describe the contribution of nitrogen and phosphorous to the cellular growth rate (Minkyu *et al*., 2014);
- the cell specific growth rate depended on: the light intensity, the internal quota of nitrogen and phosphorous, and the availability of inorganic carbon Ci. Regarding the latter, Ci is the sum of the concentration of $CO_2(aq)$ and HCO_3^- in the liquid phase and is described according to the Eq. 8 proposed by Kim *et al*., (2015):

$$C_i = CO_2(aq) + HCO_3^- \quad (8)$$

- the $CO_2(aq)$ concentration in the liquid phase was constant: the photo-bioreactor was assumed differential with respect to the CO_2. The CO_2 concentration was the liquid phase concentration in equilibrium with the gas phase as expressed by the Henry's law. According to these hypothesis, Eq. 9 holds:

$$CO_2(aq) = \frac{P_{CO_2}}{k_H} \quad (9)$$

The Henry's constant k_H of CO_2 was calculated from the CO_2 solubility at standard conditions (pure gas at 30° C and 1 atm);

- the rate of the formation of PHB producing cells (r_T) was proportional to the concentration of growing cells (X) and the specific velocity (k_T) of PHB formation depended on the initial concentration of nitrate. As reported in literature (Monshupanee & Incharoensakdi, 2013), the cyanobacterial cells significantly start to produce PHB after nitrogen starvation. The instant at which the nitrogen starvation occurred depends on the initial nitrate concentration.
- the nitrogen and phosphate released during the cell lysis are not included in the nitrogen and phosphate medium source.

Cell growth rate

The specific growth rate of cyanobacteria depends on several variables, e.g. nutrient concentration and light intensity (Cheng *et al.*, 2013; Ho *et al.*, 2012). A potential un-structured, un-segregated model is expressed by the multiplication of rate expressions for each of the influencing factors (Bastin *et al*., 1990):

$$\mu = \mu(C_i) \cdot \mu(I) \cdot \mu(N) \cdot \mu(P) \quad (10)$$

where $\mu(C_i)$, $\mu(I)$, $\mu(N)$ and $\mu(P)$ are the contribution to the growth rate depending on the inorganic carbon concentration, light intensity, nitrogen concentration, and phosphorous concentration, respectively.

The inorganic carbon contribution $\mu(C_i)$. The Monod-like model described the influence of C_i on the growth rate of cyanobacteria (Bastin *et al*., 1990):

$$\mu_{CO_2} = \left(\frac{C_i}{k_{X_{C_i}} + C_i}\right) \quad (11)$$

where μ_m and k_{X_i} are the constants of the model.

The light intensity contribution, $\mu(I)$. The Monod model captured the effect of light intensity on the growth of *Synechocystis* PCC6803 (Béchet *et al.*, 2013; Bordel *et al.*, 2009):

$$\mu_I = \left(\frac{I_{av}}{k_{S_I} + I_{av}}\right) \quad (12)$$

where I_0 is the incident light intensity (μE m^{-2} s^{-1}), σ the extinction coefficient, and "l" the depth of the culture. In this study, σl was assumed to be 0.0147 L/mg as reported by He et al. [34]: the culture depth (l) was about 0.04 m and the σ about 370 m^2/kg.

where I_{av} is the average light intensity (μE m^{-2} s^{-1}) and k_{S_I} is the half saturation constant of light intensity. I_{av} can be estimated by the Beer-Lambert law for light distribution (He *et al.*, 2012; Martínez *et al.*, 1997):

$$I_{av} = \frac{I_0}{(\sigma l)X}\left(1 - e^{-(\sigma l)X}\right) \quad (13)$$

where I_0 is the incident light intensity (μE m^{-2} s^{-1}), σ the extinction coefficient, and "l" the depth of the culture. In this study, σl was assumed to be 0.0147 L/mg as reported by He et al. (He *et al.*, 2012): the cyanobacteria culture depth (l) was about 0.04 m and the σ about 370 m^2/kg.

The N and P contribution, $\mu(N)$ *and* $\mu(P)$. The effects of the nitrate and of the phosphate concentration on the growth of the cells are typically described in the literature according to the Monod model or the Droop model (Zhang *et al.*, 2015a). The Monod model is mainly applied to the growth of bacteria - such as *E. coli* - and yeasts (Sommer, 1991): the cellular growth rate is directly related to the presence of nutrients. In contrast, microalgae can still grow after depletion of nutrients and the Monod model

is not able to describe this behaviour (Minkyu *et al*., 2014). The Droop model is based on the concept of "intracellular quota" (Q_i): a term representing substrate storage for mass unit of cyanobacteria dry matter. The substrates - such as nitrate and phosphate – accumulated in the cells (as a storage supply) and may be available for further cell growth under external substrate depletion. According to Zhang et al. (Zhang *et al.*, 2015b) the cyanobacteria growth rate as a function of the nitrogen and phosphorous content is described by Eq. 14:

$$f(Q_i) = \left(1 - \frac{q_i}{Q_i}\right) \quad (14)$$

where q_i is the minimum intracellular quota required for the subsistence of the cell (Lemesle & Mailleret, 2008). The codomain $f(Q_i)$ ranges between 0 and 1: for Q_i = high - abundance of nutrient in the medium - $f(Q_i)$ approaches l; for Q_i = low – nutrient depletion in the medium - $f(Q_i)$ approaches 0 (Minkyu *et al.*, 2014). In the investigated system, the use of the substrates nitrate and phosphate may be interpreted according to a storage phenomenon: an extension of the cellular growth phase may be observed even under nutrient depletion in the medium until complete depletion of the intracellular quota.

The combination of the contributions Eq.s 12, 13 and 14 (expressed for both N and P) yields the relationship Eq. (T4.1.3.1).

Substrate uptake

The substrate uptake rate by cyanobacteria was introduced in the model to uncouple the uptake of nutrients from growth. The uptake rate of phosphate and nitrate were described by Michaelis–Menten (Kasiri *et al.*, 2015) and Haldane (Islam Mozumder *et al.*, 2015) type kinetics, respectively. The uptake kinetics are reported in Table 4.1.3 (Eq. 1 and T4.1.3.2). The equations T4.1.3.1 and T4.1.3.2 include the constants: $\rho_{m_{PO_4^{-3}}}$, $k_{\rho_{PO_4^{-3}}}$, $\rho_{m_{NO_3^-}}$, $k_{\rho_{NO_3^-}}$ and the inhibition constant k_{In}. In particular, $\rho_{m_{PO_4^{-3}}}$ is the maximum uptake rate of phosphate, $\rho_{m_{NO_3^-}}$ the maximum uptake rate of nitrate. NO_3^- and PO_4^{-3} are the nitrate and the phosphate concentration, respectively, in the liquid phase.

Cells Transformation Pathway

Growing cells (X) and PHB producing cells (X_{PHB}) contributed to the total-measured cell concentration (X_{Tot}):

$$X_{Tot} = X + X_{PHB} \quad (15)$$

The rates of the transformation cell pathways were described by kinetics reported in Table 3: Eq.s T4.1.3.4, T4.1.3.5 and T4.1.3.6, where k_T, k_1 and k_2 are constants.

PHB Production

The PHB production rate was described by a linear relationship in CO_2 concentration and growth-cell concentration: Eq.T4.1.3.7 (Mozumder *et al.*, 2015), where k_{PHB} is a constant.

Table 4.1.3: kinetics

Name	KINETICS	Ref.	Eq.
$\rho_{PO_4^{-3}}$	$\rho_{PO_4^{-3}} = \rho_{m_{PO_4^{-3}}} \cdot \frac{PO_4^{-3}}{K_{\rho_{PO_4^{-3}}} + PO_4^{-3}}$	a, b	T4.1.3.1
$\rho_{NO_3^-}$	$\rho_{NO_3^-} = \rho_{m_{NO_3^-}} \cdot \frac{NO_3^-}{k_{\rho_{NO_3^-}} + NO_3^- + \frac{(NO_3^-)^2}{K_{In}}}$	a, b	T4.1.3.2
μ	$\mu = \mu_m \cdot \left(\frac{C_i}{kx_{Ci} + C_i}\right) \cdot \frac{I_{av}}{k_{S_I} + I_{av}} \cdot \left(1 - \frac{q_n}{Q_n}\right) \cdot \left(1 - \frac{q_p}{Q_p}\right)$	a, c	T4.1.3.3
r_T	$r_T = k_T \cdot X$	d	T4.1.3.4
r_{L_1}	$r_{L_1} = k_1 \cdot X_{PHB}$	e	T4.1.3.5
r_{L_2}	$r_{L_2} = k_2 \cdot X$	e	T4.1.3.6
r_{PHB}	$r_{PHB} = k_{PHB} \cdot CO_2 \cdot X_{PHB}$	d	T4.1.3.7

[a](Kasiri *et al*., 2015); [b](Zhang *et al*., 2015a); [c](Minkyu *et al*., 2014); [d](Mozumder *et al*., 2015); [e](Yoshida *et al*., 2015)

COMPUTATION PROCESS AND SENSITIVITY ANALYSIS

The simulation model was made of 7 differential equations (Eq.1, Eq.2, Eq.3, Eq.4, Eq.5, Eq.6 and Eq.7) and 23 parameters were required. The biochemical network simulator software COPASI was used for the process simulation (Hoops *et al.*, 2006; Raganati *et al.*, 2015). The annealing method – an optimization algorithm of COPASI – was used to process experimental data and to assess the kinetic parameters (named assessed values).

Sensitivity analysis was carried out to assess the soundness of the proposed model (Fouchard *et al.*, 2009). The assessment regarded the impact on the asymptotic concentration of endpoint cells and PHB as the kinetic parameters (in Table 4.1.3) increased/decreased of 20%. The assessment was carried out for test performed at initial nitrate concentration of 0.75 g/L ($BG_{1/2}$).

The sensitivity function defined for cell and PHB concentration are (Shinto *et al.*, 2007):

$$\beta_X = \frac{X_{end\pm 20\%} - X_{end\ control}}{X_{end\ control}} \quad (16)$$

where $X_{end\pm 20\%}$ is the total cells concentration at 35 h given a ±20% increase/decrease in each kinetic parameter and $X_{end\ control}$ is total cells concentration at 35 h calculated setting the kinetic parameters at the assessed values.

$$\beta_{PHB} = \frac{PHB_{end\pm 20\%} - PHB_{end\ control}}{PHB_{end\ control}} \quad (17)$$

where $PHB_{end\pm 20\%}$ is PHB concentration at 35 h given a ±20% increase/decrease in each kinetic parameter and $PHB_{end\ control}$ is PHB concentration at 35 h calculated setting the kinetic parameters at the assessed values.

RESULTS AND DISCUSSION

BATCH CULTURE

Figure 4.1.2 reports the experimental data regarding autotrophic cultures of *Synechocystis* PCC6803 carried out in the four investigated culture media (BG_0, $BG_{1/4}$, $BG_{1/2}$ and BG_{11}). In particular, Figure 4.1.2 reports biomass, PHB, nitrate and phosphate concentrations measured during the tests using the four investigated media. From the experimental data reported in Figure 4.1.2 it can be noted that the cyanobacteria continued to grow even though nitrogen starvation occurred. This observation may be explained by taking into account the spectrum of resources

available for growth of the microorganism: extracellular substrates are consumed under nutrient replete conditions; stored intracellular nutrients are consumed under nutrient depletion conditions (Palabhanvi *et al.*, 2014). The analysis of the data points out that the best medium to produce PHB was $BG_{1/2}$ (Carpine *et al.*, 2015).

The high accumulation of PHB under nitrogen-starvation conditions may be interpreted by taking into account the absence of nitrogen in the medium while the photosynthesis was still active and cells continued to fix CO_2. Indeed, the cells cannot synthesize proteins and they accumulate the carbon/energy as PHB (Smith, 1982).

MODEL

The experimental data reported in Figure 4.1.2 were processed according to the proposed model to assess the kinetic parameters and yields of the metabolic pathways of *Synechocystis* PCC6803. The procedure is reported in previous section. The kinetic and stoichiometric parameters assessed by processing the experimental data measured during the cyanobacteria growth are reported in Table 4.1.4 and Table 4.1.5, respectively.

Figure 4.1.2 also reports the plots of the results of the proposed model for total cell concentration (Figure 4.1.2 a), PHB concentration (Figure 4.1.2 b), extracellular nitrate- (Figure 4.1.2 c) and phosphate-concentration (Figure 4.1.2 d). The agreement of the proposed model with the experimental data is very satisfactory. The analysis of Figure 4.1.2 and Table 4.1.6 points out that the proposed model is characterized by a high likelihood regarding the PHB concentration (average R^2 0.97) and a satisfactory likelihood regarding the nitrate concentration (average R^2 0.89). The correlation coefficient (R^2) assessed for the other variables was 0.92 for biomass and phosphate concentration, when the average value assessed from data of the tests carried out with the four growth media is used. In any case, the R^2 of the overall model was about 0.92 (Table 4.1.6). It is relevant to note the saw-tooth like behavior of the total cell concentration (Figure 4.1.2 a): the model is able to catch the growth of cyanobacterial growth during the daytime and their rest during the night. The time evolution of the concentration of nitrate, phosphate and PHB is not characterized by the saw-tooth like behavior because the uptake/production rate is not affected by the irradiance. The decrease of the biomass concentration under dark condition was too small to be detected as a reduction of the uptake/production rate under dark condition. The simulations are characterized by the PHB concentration approaching constant values (Figure 4.1.2 b). Although the experimental data are quite scattered under this condition (±10%), the hypothesis step f) holds.

The time-evolution of estimated value of Q_n and Q_P - the nitrogen and phosphorous internal quota fraction with respect to cells mass, respectively – are reported in Figure 4.1.2 e and f, respectively. The simulation were carried out setting the ratio q_n/Q_n and q_P/Q_P at 0.9: Q_i is 10% larger than q_i. As expected both Q_P and Q_N are characterized by a maximum at the instant at which external source depleted. According to this observation and to the initial values of the external source, the time at which Q_P is maximum does not change with the operating conditions investigated. The instant at which Q_N is maximum increases with the initial concentration of nitrate. The endpoint of both Q_n and Q_P were about equal to the minimal internal quota. PHB synthesis appears to be initiated when the q_n/Q_n ratio drops below about 8 %.

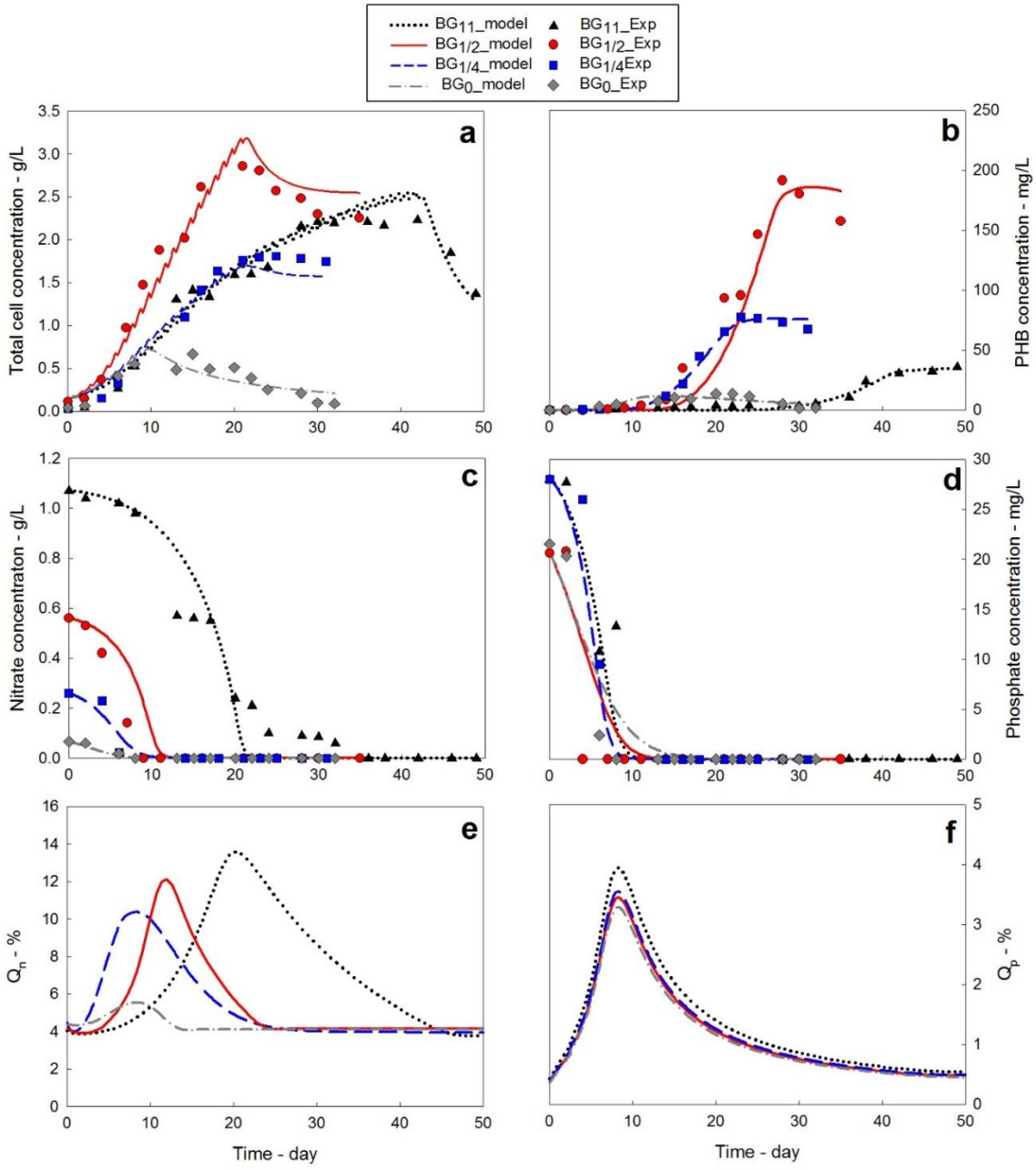

Figure 4.1.2: Time-resolved experimental data (after Carpine et al., 2015]) and simulation results of concentration of biomass, substrate and target metabolites. Tests and simulation were carried out using the media BG_0, $BG_{1/4}$, $BG_{1/2}$ and BG_{11}.

Table 4.1.4: Estimated kinetic parameters

KINETIC	PARAMETER	
μ	k_{S_I} ($\mu E/m^2 \cdot s$)	8.9
	$k_{X_{C_i}}$ (g_{C_i}/L)	0.002
	μ_m (d^{-1})	2.2
	q_n (g_N/g_X)	0.04
	q_p (g_P/g_X)	0.0038
r_{PHB}	k_{PHB} ($g_{PHB} \cdot L/h \cdot g_{X_{PHB}} \cdot g_{CO_2}$)	0.026
r_T	k_TBG_0 (d^{-1})	0.31
	$k_TBG_{1/4}$ (d^{-1})	0.26
	$k_TBG_{1/2}$ (d^{-1})	0.15
	k_TBG_{11} (d^{-1})	0.017
r_{I_1}	k_1 (d^{-1})	0.02
r_{I_2}	k_2 (d^{-1})	0.21
$\rho_{PO_4^{-3}}$	$k_{\rho_{PO_4^{-3}}}$ ($g_{PO_4^{3-}}/L$)	0.0042
	$\rho_{m_{PO_4^{-3}}}$ ($g_{PO_4^{3-}}/g_X \cdot d$)	0.0042
$\rho_{NO_3^-}$	$k_{\rho_{NO_3^-}}$ ($g_{NO_3^-}/L$)	5.89
	$\rho_{m_{NO_3^-}}$ ($g_{NO_3^-}/g_X \cdot d$)	0.028
	k_{In} ($g_{NO_3^-}/L$)	0.16

Table 4.1.5: stoichiometric parameters

REACTION	STOICHIOMETRIC PARAMETERS		
	Assessed values		Maximum theoretical values
$Y_{N/X} \cdot N + Y_{P/X} \cdot P + Y_{C_i/X} \cdot C_i \rightarrow X$	$Y_{N/X}$ (g_N/g_X)	0.07	0.13
	$Y_{P/X}$ (g_P/g_X)	0.0085	0.013
	$Y_{C_i/X}$ (g_{Ci}/g_X)	0.33	0.51
$CO_2 \rightarrow Y_{PHB/CO_2} \cdot PHB$	Y_{PHB/CO_2} ($g_{PHB}/g_{X_{PHB}}$)	0.42	0.56
$PO_4^{3-} \rightarrow Y_{P/PO_4^{3-}} \cdot P$	$Y_{P/PO_4^{3-}}$ (g_P/g_P)	0.28	0.31
$NO_3^- \rightarrow Y_{N/NO_3^-} \cdot N$	Y_{N/NO_3^-} (g_N/g_N)	0.13	0.22

Table 4.1.6: average squared correlation coefficients (R^2) between simulation results and experimental data.

R^2	BG_0	$BG_{1/4}$	$BG_{1/2}$	BG_{11}	AVERAGE
X	0.94	0.92	0.91	0.90	0.92
PHB	0.98	0.99	0.94	0.96	0.97
$\mathbf{PO_4^{3-}}$	0.98	0.95	0.81	0.93	0.92
$\mathbf{NO_3^-}$	0.96	0.84	0.82	0.94	0.89
AVERAGE	0.97	0.92	0.87	0.91	**0.92**

The analysis of data reported in Table 4.1.5 and Table 4.1.6 and of the data reported in the literature is very interesting and the most notable observations are reported hereinafter.

- The estimated value of μ_m (2.2 d^{-1}) is about the experimental value (2.8 d^{-1}) reported by Kim *et al.*, 2015.
- The estimated value of $k_{X_{C_i}}$ was 0.002 g_{C_i}/L. The value of $k_{X_{C_i}}$ suggests that as CO_2 concentration in the air stream is higher than about 0.7 %, the cellular growth rate is of the order "0" with respect to the CO_2

concentration, as pointed out by Kim *et al.*, 2015. Indeed, Eq. (11) yields μ_{CO_2}> 0.9 as P_{CO2} in the air stream is larger than 0.7 %. Under these conditions, the assumption "the $CO_2(aq)$ concentration in the liquid phase was constant" is sound.

- The estimated value of k_{S_I} was 9 $\mu E/m^2 \cdot s$. Therefore, as the light intensity is higher than about 84 $\mu E/m^2 \cdot s$ the cellular growth rate is of order "0" with respect to the light intensity I_{av}, as pointed out by He *et al.*, 2012. Indeed Eq. (12) yields μ_{I_2}>0.9 as I_{av} is larger than 84 $\mu E/m^2 \cdot s$.
- The estimated value of q_n and q_P - the nitrogen and phosphorous internal quota fraction with respect to cells mass, respectively - were 4% and 0.38%, respectively. These value are close to that assessed by Minkyu Jeon *et al*. (2014) by using a Flux Balance Model.
- The estimated specific rate of X_{PHB} formation (k_T) was inversely proportional to the initial nitrate concentration. This observed behaviour would suggest that the size of the nitrogen reservoir per cell decreased with the initial nitrate concentration, to be very low when the growth is carried out in a balanced (*i.e.* BG_{11}) medium,
- The k_T estimated for cultures carried out in BG_0, $BG_{1/4}$ and $BG_{1/2}$ medium was about one order of magnitude higher than the specific X_{PHB} lysis rate (k_1) and that estimated for cultures in BG_{11} medium was about the k_1. The observed behaviour suggests that there is the same chance for the growing-cells in optimal growth medium (BG_{11}) to convert in PHB-producing cells and for PHB-producing cells to have lysis. This observation should address the low level of PHB production for cultures in BG_{11} medium even though the cell concentration was high.
- The estimated value of $k_{\rho_{PO_4^{-3}}}$ was 0.0042 $g_{PO_4^{3-}}/L$. Therefore, as the initial phosphate concentration is about 22 mg/L the phosphate uptake rate does not depend remarkably on PO_4^{-3} concentration. Indeed, the Eq. (T.4.1.3.1) yields $\rho_{PO_4^{-3}}$ larger than the (90%) of the maximum value as PO_4^{-3} is larger than 38 mg/L. As a result of the simulation, it is to be expected that the PO_4^{-3} concentration should be increased by about 50 % to operate the cultures such that there will be no effect of the PO_4^{-3} concentration.
- Assessed parameters regarding the nitrate uptake kinetics allow one to estimate the nitrate concentration at which $\rho_{NO_3^-}$ is maximum (Eq. 18):

$$\sqrt{k_{\rho_{NO_3^-}} \cdot k_{In}} = 0.97\ g_{NO_3^-}/L \qquad (18)$$

  The initial nitrate concentration of BG_{11} medium is higher than this value. Therefore, the inhibition effect of nitrate can be observed only when using BG_{11} medium as growth medium.
- The assessed stoichiometric parameters (Table 4.1.5) were lower than their maximum theoretical value (Kim *et al.*, 2011). These departures from the theoretical values could be expected because the tests were carried out under operating conditions there were not optimal for the natural life of the microorganism.

SENSITIVITY ANALYSIS

Sensitivity analysis was carried out to assess the firmness of the proposed model. Results of the sensitivity analysis are reported in terms of the sensitivity function β_X and β_{PHB} defined by Eq.s (16) and (17). The assessments were carried out with reference to experimental test carried out at initial nitrate concentration of 0.75 g/L (BG_{11}). The investigation regarded the assessment of the impact on endpoint cells and PHB concentration given a ±20 % increase/decrease in each kinetic parameter. The results of the sensitivity analysis on the endpoint of the nitrate and phosphate concentration as well as of the initial internal quota of nitrogen and phosphorous were not reported because the impact of ±20 % increase/decrease in each kinetic parameter was not significant (within |1| %).

Figure 4.1.3 reports the results of β_X and β_{PHB} and the main observations are:

a) The sensitivity of the cell concentration at the endpoint of the experiment on μ_m, k_T and $\rho_{m_{NO_3^-}}$ is low. The β_X is typically within 1/3 of the imposed variations of the parameters.

b) The sensitivity of the endpoint PHB concentration on μ_m, k_T and $\rho_{m_{NO_3^-}}$ is significant. The β_{PHB} is typically larger than the imposed variation of the parameters. It is surprising that the endpoint PHB concentration typically decreases for any variation of the parameter: a sort of maximum of the endpoint PHB concentration was found for the kinetic parameters reported in table 4.1.4.

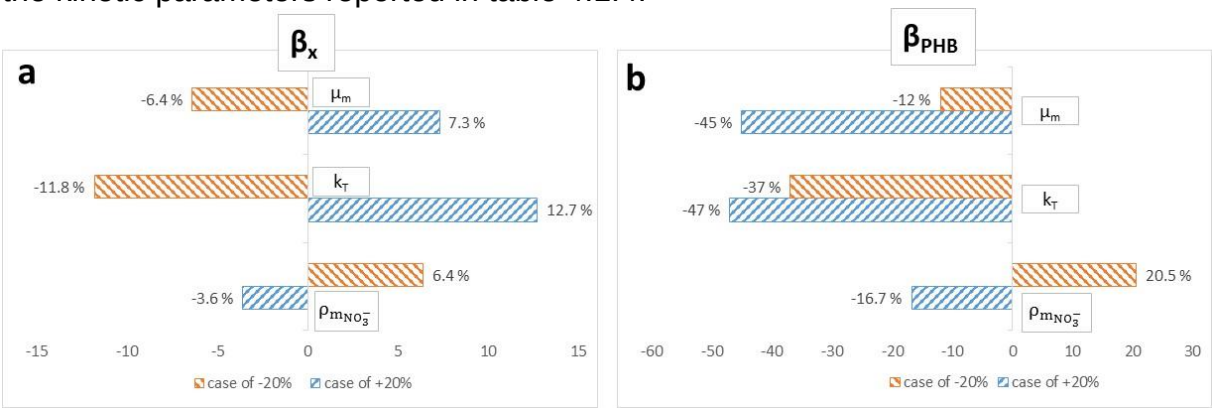

Figure 4.1.3: Sensitivity functions for amount of cells at the endpoint and PHB concentration for the variation of the model parameters: μ_m, k_T and $\rho_{m_{NO_3^-}}$

The dynamics of cell growth and of PHB production were simulated by changing the kinetic parameters (μ_m, k_T and $\rho_{m_{NO_3^-}}$) according to the values set for the sensitivity analysis: ±20% variation with respect to the estimated values (Table 4.1.5). The simulations were carried out with reference to the culture carried out in the $BG_{1/2}$ medium.

Figure 4.1.4 a and b report the simulation keeping fixed k_T and $\rho_{m_{NO_3^-}}$ and changing μ_m; Figure 4.1.4 c and d report the simulation keeping fixed μ_m and $\rho_{m_{NO_3^-}}$ and changing k_T; figures 4c and 4d report the simulation keeping fixe k_T and μ_m and changing $\rho_{m_{NO_3^-}}$. The analysis of Figure 4.1.4 points out that:

- As expected, the increase of μ_m positively affected the cyanobacteria cell growth (Figure 4.1.4 a). However, a negative effect on the final PHB concentration was observed (Figure 4.1.4 b). The adverse effect observed regarding the PHB production may be due to a bottleneck of the PHB synthesis. Although the parallel path T.4.1.2.4 and T.4.1.2.6 are both favorited by a high biomass content (X) and an increase of X_{PHB} is expected

at high μ_m, the path T.4.1.2.5 limits the PHB production while cells continue to lyse (route T.4.1.2.6);

- As expected, the increase of k_T did not affect remarkably the total cell concentration (Figure 4.1.4 c) and positively affected the final PHB concentration (Figure 4.1.4 d);
- An increase of $\rho_{m_{NO_3^-}}$ did not affect remarkably the cell growth (Figure 4.1.4 e). However, an increase of $\rho_{m_{NO_3^-}}$ is desirable because this contributes to the accumulation of nitrogen quota, then the final PHB concentration (Figure 4.1.4 f).

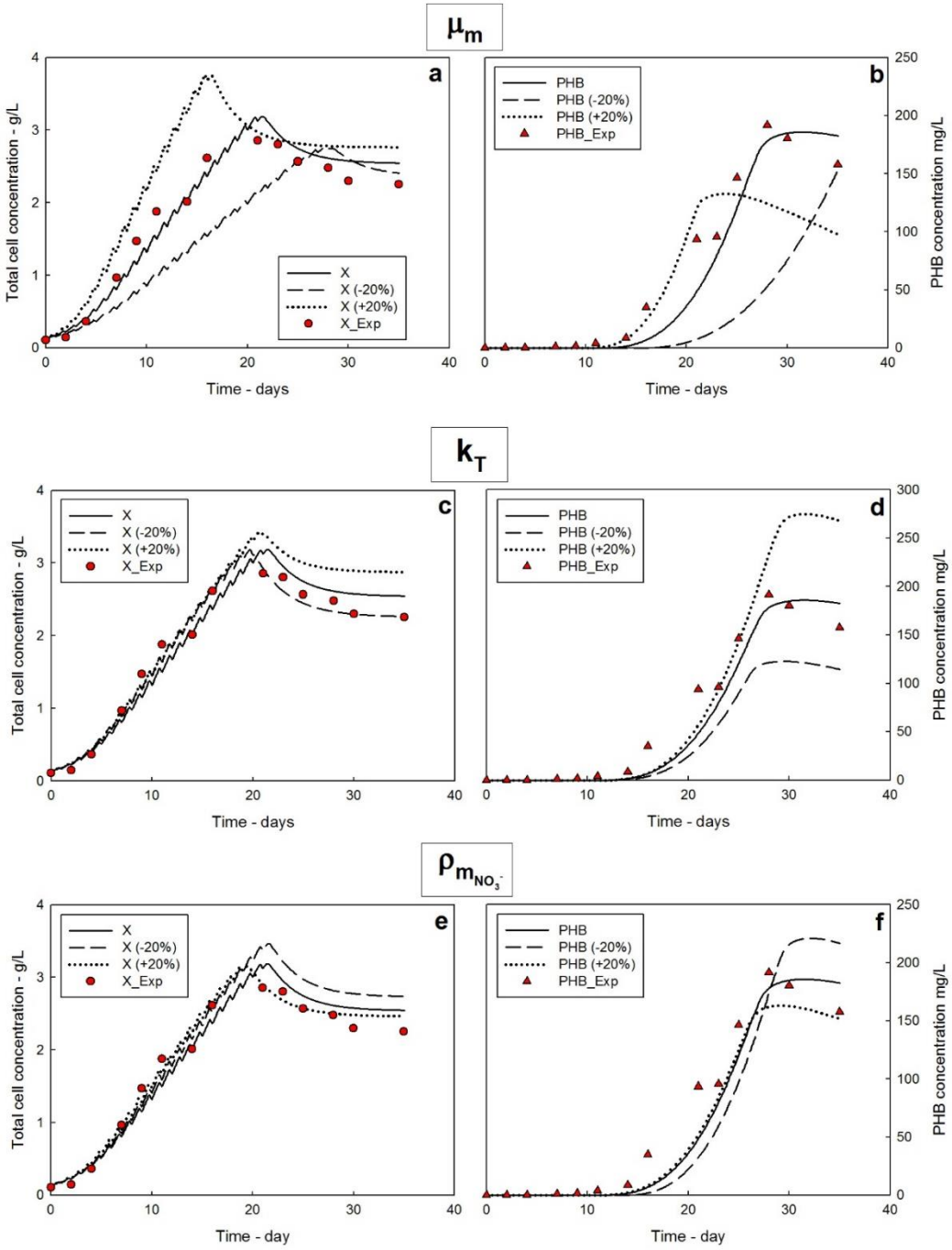

Figure 4.1.4: Plots of the concentration of total amount of cells and PHB vs. time. Experimental test (data points) carried out in the $BG_{1/2}$ medium. Simulation results: solid line) kinetic parameter in table 5; dashed line) -20%; dotted line) +20%. a) and b) sensitivity analysis on μ_m; c) and d) sensitivity analysis on k_T; e) and f) sensitivity analysis on $\rho_{m_{NO_3^-}}$

CONCLUSIONS

A kinetic/dynamic model of poly-β-hydroxybutyrate (PHB) production by *Synechocystis* PCC6803 from CO_2 has been proposed and developed with the biochemical networks simulator COPASI. The proposed model describes successfully the cyanobacterial growth, phosphate uptake, nitrate uptake and PHB production of *Synechocystis* PCC6803 cultivated in growth media characterized by different nitrate concentrations and using a light/dark cycle. Two types of cells were considered in the cultures: growing cells - able to grow - and PHB producing cells. The PHB production was related to the internal quota of the nitrogen- and the phosphate source. The effect of significant factors and nutrients on the cyanobacterial system were taken into account. In particular, the proposed model includes the nitrate- and phosphate-utilization rate, the PHB production rate, the growth rate of the cells and the lysis rate.

Sensitivity analysis was performed to investigate the simulation accuracy and the effect of parameter variation on growth of the cells and PHB accumulation.

To the authors' knowledge, the model elaborated in this work, is the first reported in literature in which PHB production by cyanobacteria has been evaluated. The proposed model is useful for analysis of the process to effectively design, scale-up, and optimize the PHB production process.

ACKNOWLEDGEMENTS

The grant PRIN "Innovative downstream processing of conversion of algal biomass for the production of jet fuel and green diesel" from Ministero dell'Università, della Ricerca is acknowledged.

NOMENCLATURE

$\mathbf{BG_0}$	medium without nitrate
$\mathbf{BG_{1/4}}$	medium in which nitrate concentration was one fourth of the optimal concentration
$\mathbf{BG_{1/2}}$	medium in which nitrate concentration was the half of optimal concentration
$\mathbf{BG_{11}}$	medium with the optimal nitrate concentration
$\boldsymbol{\beta_{PHB}}$	sensitivity function for PHB concentration
$\boldsymbol{\beta_X}$	sensitivity function for active cells concentration
$\mathbf{CO_2}$	carbon dioxide
$\mathbf{C_i}$	inorganic carbon concentration
$\mathbf{f(Q)}$	function of quota
$\mathbf{HCO_3^-}$	bicarbonate concentration
$\mathbf{I_{av}}$	average light intensity (μE m^{-2} s^{-1})
$\mathbf{I_0}$	incident light intensity (μE m^{-2} s^{-1})
$\mathbf{k_1}$	specific velocity of PHB producing cells inactivation (d^{-1})
$\mathbf{k_2}$	specific velocity of PHB growing cells inactivation (d^{-1})
$\mathbf{k_{In}}$	inhibition parameter (g_N/L)
$\mathbf{k_{PHB}}$	PHB production constant ($g_{PHB} \cdot L/h \cdot g_{cell} \cdot g_{CO_2}$)
$\mathbf{k_T BG_0}$	specific velocity of PHB producing cells formation in BG_0 (d^{-1})
$\mathbf{k_T BG_{1/4}}$	specific velocity of PHB producing cells formation in $BG_{1/4}$ (d^{-1})
$\mathbf{k_T BG_{1/2}}$	specific velocity of PHB producing cells formation in $BG_{1/2}$ (d^{-1})
$\mathbf{k_T BG_{11}}$	specific velocity of PHB producing cells formation in BG_{11} (d^{-1})
$\mathbf{k_{S_I}}$	half saturation constant of light intensity ($\mu E/m^2 \cdot s$)
$\mathbf{k_{\rho_{NO_3^-}}}$	uptake half-saturation constant of nitrate (g_N/L)
$\mathbf{k_{\rho_{PO_4^{-3}}}}$	uptake half-saturation constant of phosphate (g_P/L)
$\mathbf{k_{X_{CO_2}}}$	half saturation constant of CO_2 (g_{CO_2}/L)
$\mathbf{l}$	depth of the algal culture (m)
μ	specific growth
$\mu(\mathbf{CO_2})$,	growth rates influenced by the CO_2 concentration

$\mu(\mathbf{I})$,	growth rates influenced by light intensity
$\mu(\mathbf{N})$	growth rates influenced by the nitrogen concentration
$\mu(\mathbf{P})$	growth rates influenced by phosphorous concentration
$\mu_{\mathbf{m}}$	maximum growth rate (1/day)
$\mathbf{N}$	nitrogen internal quota concentration (g/L)
$\mathbf{NO_3^-}$	nitrate concentration (g/L)
$\mathbf{P}$	phosphorous internal quota concentration (g/L)
$\mathbf{PO_4^{-3}}$	phosphate concentration (mg/L)
PHB	poly-β-hydroxybutyrate
$\mathbf{PHB_{end\pm 20\%}}$	PHB concentration at 35 h given a ±20% increase/decrease
$\mathbf{PHB_{end\ control}}$	PHB concentration at 35 h by proposed model
$\mathbf{Q_n}$	intracellular quota of nitrogen (g_N/L)
$\mathbf{Q_P}$	intracellular quota of phosphorous (g_P/L)
$\mathbf{q_n}$	minimum intracellular concentration of nitrogen quota (g_N/L)
$\mathbf{q_p}$	minimum intracellular concentration of phosphorous quota (g_P/L)
$\mathbf{r_{I_1}}$	inactivation rate of PHB producing
$\mathbf{r_{I_2}}$	inactivation rate of growing cells
$\mathbf{r_{PHB}}$	PHB production rate
$\mathbf{r_T}$	rate of PHB producing cells formation
$\mathbf{r_X}$	growth rate of biomass
$\boldsymbol{\rho}_{\mathbf{NO_3^-}}$	uptake rate of nitrate
$\boldsymbol{\rho}_{\mathbf{PO_4^{-3}}}$	uptake rate of phosphate
$\boldsymbol{\sigma}$	extinction coefficient (m^2/kg)
$\mathbf{X}$	growing cells
$\mathbf{X_{end\pm 20\%}}$	active cells concentration at 35 h given a ±20% increase/decrease
$\mathbf{X_{end\ control}}$	active cells concentration at 35 h by proposed model
$\mathbf{X_{PHB}}$	PHB producing cells
$\mathbf{X_{Tot}}$	measured cell concentration
$\mathbf{Y_{CO_2/X}}$	CO_2 uptake yield (g_{CO2}/g_X)
$\mathbf{Y_{PHB/CO_2}}$	CO_2 to PHB yield (g_{PHB}/g_X)
$\mathbf{Y_{N/NO_3^-}}$	nitrate uptake yield (g_N/g_N)
$\mathbf{Y_{P/PO_4^{3-}}}$	phosphate uptake yield (g_P/g_P)
$\mathbf{Y_{N/X}}$	internal nitrogen quota (Q_n) uptake yield (g_N/g_X)
$\mathbf{Y_{P/X}}$	internal phosphorous quota (Q_P) uptake yield (g_P/g_X)

5 GENETIC ENGINEERING

Results of the investigation regarding the genetic engineering approach to improve PHB production from CO_2 by *Synechocystis* PCC6803 is reported. Seven mutant strains were produced (section 5.1) by single and combined mutations: the deletion of phosphotransacetylase (*pta*); the deletion of and acetyl-CoA hydrolase (*ach*); the overexpression of phosphoketolase (*xfpk*). The tests were carried out on BG_{11} media.

In the section 5.2 the mutant strain that gave best performance on BG_{11} medium was tested on optimized medium ($BG_{1/2}$ medium).

5.1 Genetic engineering of *Synechocystis* sp. PCC 6803 for poly-β-hydroxybutyrate overproduction

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ABSTRACT

The biosynthesis of poly-β-hydroxybutyrate (PHB) directly from carbon dioxide is a sustainable alternative for non-renewable, petroleum-based polymer production. *Synechocystis* sp. PCC6803 can naturally accumulate PHB using CO_2 as the sole carbon source, particularly when major nutrients such as nitrogen become limiting. Many previous studies have tried to genetically engineer PHB overproduction mostly by increasing the expression of enzymes directly involved in its biosynthesis pathway. Here, we have instead concentrated on engineering the central carbon metabolism of *Synechocystis* such that *(i)* the PHB synthesis pathway becomes deregulated, and/or *(ii)* more of its substrate, acetyl-CoA, becomes accessible to it. Seven different mutants were constructed harboring, separately or in combination, three different genetic modifications to the metabolic network. These were the deletions of phosphotransacetylase (Pta) and acetyl-CoA hydrolase (Ach), and the overexpression of a heterologous phosphoketolase (XfpK) from *Bifidobacterium breve*. The wild type *Synechocystis* and derivative strains were compared in terms of biomass and PHB production capability during photoautotrophic growth. This was performed in a photobioreactor exposed to a circadian rhythm and using standard BG11 as the growth medium. We found that the strain that combined all three genetic modifications, *i.e. xfpk* overexpression in a double *pta* and *ach* deletion background, showed the highest levels of PHB production from all the strains tested here. Encouragingly, the production levels obtained: 232 mg/L, ~12% (w/w) of the dry biomass weight, and a productivity of 7.3 g m^{-3} d^{-1}; are to the best of our knowledge, the highest ever reported for PHB production directly from CO_2.

Keywords: poly-β-hydroxybutyrate; cyanobacteria; genetic engineering; phosphotransacetylase; acetyl-CoA hydrolase; phosphoketolase

INTRODUCTION

The growing world population and the accompanying increase demand of plastic materials drive our need for more sustainable production of biodegradable polymers (Dias *et al.*, 2006). In particular, poly-β-hydroxybutyrate (PHB) has received special attention for its interesting features, such as thermoplastic processability, hydrophobicity, complete biodegradability and biocompatibility (Braunegg *et al.*, 1998). The global production of PHB was approximately 34 ktonnes in 2014 and is forecasted to grow by 100 ktonnes in 2019 (www.en.european-bioplastics.org).

Under optimal conditions, bacteria such as *Cupriavidus necator* (previously known as *Ralstonia eutropha*) H16 can produce PHB up to 80% of their cellular dry weight while relying on fructose as the carbon source (Budde *et al.*, 2011b). However, costs of raw materials for PHB production by microbial fermentation are still very high, making it of paramount importance to find other sustainable production routes. This has brought plants into focus as an alternative low cost photosynthetic production system (Mittendorf *et al.*, 1998; Poirier *et al.*, 1992). Yet, plant-based expression systems compete directly with subsistence crops for agricultural acreage, and raise further ethical concerns, as the dissemination of transgenic plants is difficult to control. The latter has led to strict regulatory restrictions of transgenic plants in many countries (Hempel *et al.*, 2011). Cyanobacteria, share all the advantages of photosynthetic microorganisms when compared to chemoheterotrophs, indeed having the potential to use (sun)light energy to directly convert CO_2 into product of interest such as PHB, but circumvent the drawbacks of competing with the agro-food market for resources (Gopi *et al.*, 2014; Gupta *et al.*, 2013; Wijffels *et al.*, 2013).

In many cyanobacterial cells, PHB is a native carbon-storing polymer generated via the polyhydroxyalkanoate (PHA) biosynthetic pathway. The latter relies on the activities of three key enzymes: (i) β-ketothiolase (encoded by *phaA*), catalysing the conversion of acetyl-CoA to acetoacetyl-CoA; (ii) acetoacetyl-CoA reductase (encoded by *phaB*), which produces the intermediate 3-hydroxybutyryl-CoA (3HB-CoA); and (iii) PHB synthase (encoded by *phaC/phaE*), finally resulting in the storage polymer PHB (Hondo *et al.*, 2015b). The main flux of carbon in light-driven CO_2-based synthesis of PHB by cyanobacterial cells goes from the Calvin–Benson–Bassham (CBB) cycle, through the lower part of glycolysis, to pyruvate and then via acetyl-CoA to PHB (Figure 1). One of the key intermediates in this route is acetyl-CoA, which acts as the last branching point between PHB synthesis and other competing metabolic pathways. For instance, acetyl-CoA can be metabolized in multiple ways to acetate such as via phosphotransacetylase (encoded by *pta*) and acetate kinase (encoded by *ackA*) generating ATP in the latter step, or directly catalyzed by an acetyl-CoA hydrolase (encoded by *ach*) (Tielens *et al.*, 2010). Acetyl-CoA synthase (encoded by *acs*), in contrast, can recover acetate to reconvert it into the much more versatile intermediate acetyl-CoA (Thiel *et al.*, 2017).

The model cyanobacteria, *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*), has been metabolically engineered to synthesize a variety of chemical commodities, such as ethanol, hydrogen, glycerol, ethylene, 2,3-butanediol, iso-butanol, lactic acid, and also PHB (Atsumi *et al.*, 2009; Deng & Coleman, 1999; Du *et al.*, 2016; Savakis *et al.*, 2015, Wang *et al.*, 2013). The introduction of heterologous pathways and/or deletion or overexpression of specific native enzymes can divert the metabolic flux originating from CO_2, from biomass to a product of interest (Branco dos Santos *et al.*, 2014). The central carbon metabolism of cyanobacteria comprises the CBB cycle, glycolysis, the pentose-phosphate (PP) pathway and the TCA cycle, which collectively form a very complex and interconnected network (Figure 5.1.1). In this

study, we concentrated on engineering the native metabolic network of *Synechocystis* to further improve PHB production.

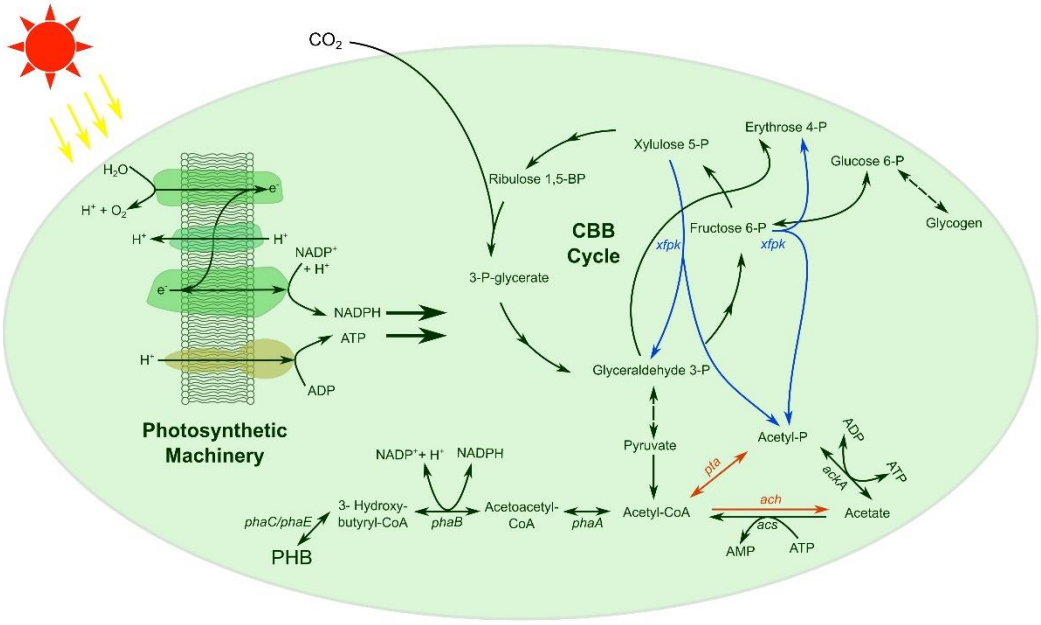

Figure 5.1.1: Overview of the metabolism of *Synechocystis* sp. PCC6803 for PHB production. In this study, genes coloured in red (*pta* and *ach*) were knocked out, while the gene coloured in blue (*xfpk*) was overexpressed.

The genetic engineering strategies deployed here aimed at increasing acetyl-CoA availably in the expectation that this would pressure cells to redirect their carbon flux towards PHB synthesis. The deletion of *pta* and *ach* in *Synechocystis* was used to test the effect of reducing the drainage of acetyl-CoA to acetate. In *E. coli* the effect of the former has been tested, demonstrating that a reduced phosphotransacetylase activity leads to an increase in acetyl-CoA levels, which ultimately results in an increased PHB accumulation (Miyake *et al*., 2000). Moreover, the overexpression of phosphoketolase (encoded by *xfpK*) was also used as a strategy to increase acetyl-CoA levels, and subsequently, PHB production. In yeast, such a strategy has been shown to lead to improved free fatty acid production (de Jong *et al.*, 2014) and PHB accumulation (Kocharin *et al.*, 2013). Phosphoketolases catalyze the cleavage of xylulose 5-phosphate (Xu5P) or fructose 6-phosphate (F6P) to acetyl-P and either glyceraldehyde-3-P or erythrose-4-P, respectively (Sánchez *et al.*, 2010), and have only recently been characterized in cyanobacteria (Xiong *et al.*, 2015). This activity confers flexibility in carbon and energy metabolism, and can be exploited to increase the efficiency of cyanobacterial central metabolism and photosynthetic productivity (Xiong *et al.*, 2015). The utility of phosphoketolase in redirecting autotrophic metabolism, which can be used in addition to other metabolic engineering strategies, has been used to improve butanol production in *Synechocystis* (Anfelt *et al.*, 2015), and acetone in another cyanobacterium, *Synechococcus elongatus* PCC 7942 (Chwa *et al.*, 2016).

Here, we report the first study in which *pta* and *ach* deletions and *xfpk* overexpression were evaluated, separately and in combination, as strategies to improve PHB accumulation in cyanobacteria. Ultimately, while relying solely on

engineering the metabolic network of the production host, and not the production pathway itself, this has resulted in a 6-fold increase in PHB production.

MATERIALS AND METHOD

GENETIC ENGINEERING

Strains and general cultivation conditions

All the strains used in this study are listed in Table 1. Molecular cloning procedures were carried out in *E. coli* DH5α, grown either on solidified LB plates containing 1.5% (w/v) agar or in liquid LB medium at 37°C agitated at 200 rpm. When appropriate, antibiotics were added for propagation of specific plasmids. Concentrations of antibiotics used, alone or in combination, were 100 µg ml^{-1} for ampicillin and 50 µg ml^{-1} for kanamycin.

A glucose-tolerant *Synechocystis* was obtained from D. Bhaya, University of Stanford, Stanford, CA. Unless indicated otherwise, it was cultivated in BG11 medium (J.B. Waterbury, 1981) at 30°C in a shaking incubator at 120 rpm (Innova 43, New Brunswick Scientific) under constant white-light illumination (around 30 µE m^{-2} s^{-1}, measured with a LI-250 light meter). For *Synechocystis* mutant construction, kanamycin or nickel sulphate was added to the medium up to a final concentration of 50 µg ml^{-1} or 20 µM, respectively. Growth was monitored by recording the optical density at 730 nm (OD_{730}; lightwave II, Biochrom).

Plasmid construction

All plasmids used are listed in Table 5.1.1. For each markerless gene knockout mutant construction, two plasmids were needed. One contains only the 1kb homologous regions up- and downstream of the deletion target. While for another plasmid, extra selection cassette including both kanamycin resistance fragment and nickel-induced *mazF* expression fragment were used. For individual *ach* and *pta* gene knock out plasmid construction, each homologous region was amplified from the genomic DNA of *Synechocystis* by PCR and fused together using Pfu DNA Polymerase (Thermo Scientific). After gel extraction and purification (Zymo Research), extra adenosine (“A-”) was added to the 3’ overhang of each fused fragment using Taq DNA Polymerase (Thermo Scientific). The fragment with the A-overhang was then ligated to the BioBrick “T-” vector pFL-SA (Zhou *et al.*, 2012), resulting in pWD026 and pWD028, respectively. Since the designed fusion primers possess an XbaI restriction site between the homologous regions, and the selection cassette contains a XbaI restriction site on both sides, it can be easily inserted into pWD026 and pWD028, resulting pWD027 and pWD029, respectively. Since *ach* and *pta* are neighbouring genes in the genome with no obvious other overlapping transcripts (Mitschke *et al.*, 2011), same strategy was applied to delete these two genes simultaneously. The plasmids constructed for this purpose are pWD030 and pWD031. All the fragments amplified in this study were confirmed by Sanger sequencing (Macrogen Europe, The Netherlands), and all the primers used are listed in Table 5.1.1 as well.

Table 5.1.1. Plasmids and strains used in this study

Plasmids, strains and primers[1]	**Relevant characteristics**	**Reference**
pFL-SA	BioBrick "T" vector with SpeI and AvrII restriction sites	(Zhou *et al.*, 2012)
pWD026	pFL-SA derivate, Amp^r, containing *ach* gene upstream and downstream homologous regions	This study
pWD027	pFL-SA derivate, Amp^rKm^r, containing *ach* gene upstream homologous region, selection cassette, and downstream homologous region	This study
pWD028	pFL-SA derivate, Amp^r, containing *pta* gene upstream and downstream homologous regions	This study
pWD029	pFL-SA derivate, Amp^rKm^r, containing *pta* gene upstream homologous region, selection cassette, and downstream homologous region	This study
pWD030	pFL-SA derivate, Amp^r, containing *ach-pta* gene upstream and downstream homologous regions	This study
pWD031	pFL-SA derivate, Amp^rKm^r, containing *ach-pta* gene upstream homologous region, selection cassette, and downstream homologous region	This study
pCYJ2	*trc* promoter driving overexpression of a phosphoketolase from *Bifidobacterium breve*; Km^r	(Anfelt *et al.*, 2015b)
Synechocystis	*Synechocystis* sp. PCC6803 wild type	D. Bhaya
WD113 (ACH)	*Synechocystis ach* gene knock out	This study
WD114 (PTA)	*Synechocystis pta* gene knock out	This study
WD115 (DKO)	*Synechocystis ach-pta* double gene knock out	This study
WD116 (XFPK)	*Synechocystis* overexpression of *xfpk*; Km^r	This study
WD117 (ACHX)	*Synechocystis ach* gene knock out and overexpression of *xfpk*; Km^r	This study
WD118 (PTAX)	*Synechocystis pta* gene knock out and overexpression of *xfpk;* Km^r	This study
WD119 (DKOX)	*Synechocystis ach-pta* gene knock out and overexpression of *xfpk*; Km^r	This study
ach-upst-Fwd	GGACTATTCACCCAAGAACT	This study
ach-upst-Rev	AGAAAGAAGACGGCTCAACtctagaTGTTCCTGGCGATCAGAG	This study
ach-downst-Fwd	CTCTGATCGCCAGGAACAtctagaGTTGAGCCGTCTTCTTTCT	This study
ach-downst-Rev	CATCGCCACCACTAAATG	This study
pta-upst-Fwd	CTCAATCGAGGCGATATTC	This study
pta-upst-Rev	ACAACCCCGACTTAGATCAtctagaATAACCTCAACGTATCCAG	This study
pta-downst-Fwd	CTGGATACGTTGAGGTTATtctagaTGATCTAAGTCGGGGTTGT	This study
pta-downst-Rev	GATGCTTTACTTCATCAATCGG	This study
ach-pta-upst-Fwd	GGACTATTCACCCAAGAACT	This study
ach-pta-upst-Rev	AAACAACCCCGACTTAGATtctagaTTCCTGGCGATCAGAGT	This study
ach-pta-downst-Fwd	ACTCTGATCGCCAGGAAtctagaATCTAAGTCGGGGTTGTTT	This study
ach-pta-downst-Rev	GATGCTTTACTTCATCAATCGG	This study
xfpk-upst-Fwd	GCCTACACTTCGGCTATTAGC	(Anfelt *et al.*, 2015)
xfpk-downst-Rev	GCTAAACCCACCTCTTGCC	(Anfelt *et al.*, 2015)

[1] primer sequences are given from 5′→3′

Synechocystis markerless mutant construction

Using a previously reported method (Cheah *et al.*, 2013), it takes two rounds of transformation to make a clean deletion (*i.e.* without leaving behind selection cassettes) of one locus in the *Synechocystis* chromosome. First round transformation is to isolate a fully segregated mutant with the selection cassette inserted into the chromosome, while second round of transformation is to completely remove the latter. Briefly, for the first round transformation, *Synechocystis* wild type cells were collected either directly from the plate or from liquid culture. After washing twice with fresh BG11 medium by centrifuging at 5000 rpm for 5 min, cells were further concentrated to a total liquid volume of 200 µl. Plasmid containing selection cassette was added and mixed with cells to a final concentration of 10 µg mL^{-1}. After illumination under moderate light for 4 to 6 hours, the mixture was spread on commercial membranes (Pall Corporation, USA) and left resting on BG11 plates (without antibiotic). After being further illuminated for about 16-24 hours, the membrane containing the mixture was transferred to another BG_{11} plate with kanamycin. One week later, the colonies that appeared on the plate were picked and seeded sequentially on BG11 plates supplemented with either kanamycin or nickel. Colonies that grew on the BG11 plate with kanamycin but not on the BG11 plate with nickel were candidates for further PCR confirmation. Further segregation in the liquid culture with higher concentration (> 50 µg ml^{-1}) of kanamycin was used when necessary until the desired clone was obtained. For the second round transformation, which removes the selection cassette, plasmid with only upstream and downstream homologous regions was used for subsequent transformation. Following the protocol just described for the first round transformation, colonies appeared after about one week on the BG_{11} plate with nickel sulphate. Again, colonies were picked and seeded sequentially on new BG_{11} plates supplemented with either kanamycin or nickel, but now the colonies that grow on the BG11 plate with nickel and not on BG11 plate with kanamycin were chosen for the final PCR confirmation.

For the construction of *xfpk* overexpression mutants, transformation basically followed as described above, but now using kanamycin as the only selection pressure. The cells were transformed using pCyJ02 plasmid, kindly provided by the Hudson group (KTH, Royal Institute of Technology), which harbours a cassette with a strong promoter (Ptrc) upstream of *xfpk* from *Bifidobacterium breve* flanked by regions homologous to the neutral site *slr0168* of the chromosome of *Synechocystis*. The wild type, the strain with *pta* deletion, the strain with *ach* deletion and the strain with the double knock-out (*pta* and *ach*) were transformed with pCyJ02 plasmid. The integration of the cassette at the *slr0168* site in all the mutants constructed was confirmed by PCR (Figure 5.1.2).

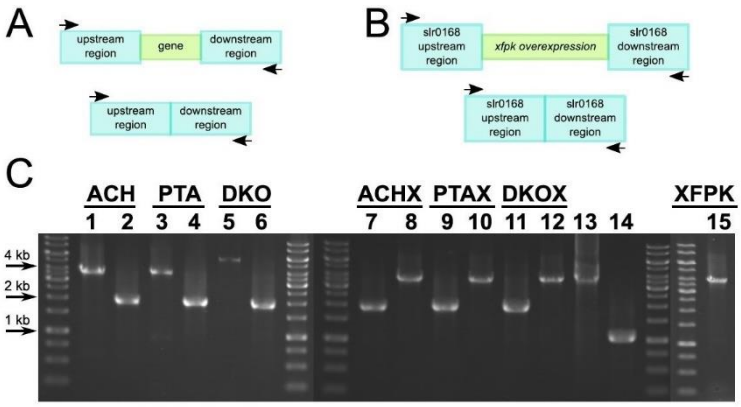

Figure 5.1.2: PCR confirmation of all the mutants constructed in this study. (A) For gene knock out mutants, each homologous region is about 1 kb. Therefore, with the primers indicated as black arrows, a fully segregated markerless knock out mutant gave a single fragment of approximate 2 kb. While for *Synechocystis* wild type, the size of the fragment is larger than its corresponding mutant due to the presented gene. (B) For *xfpk* overexpression mutants, the primers designed would result a fragment of approximate 1 kb for wild type and 4 kb for the mutant. (C) Lane 1, 3, 5 are the results using wild type genomic DNA as controls with each primers specific to each gene, while lane 2, 4, 6, 7, 9,11 are for their corresponding gene knock out mutants, respectively. Lane 8, 10, 12, 15 are the fragments amplified for *xfpk* overexpression mutants, with lane 13 and 14 as their respective control through using the plasmid and wild type genomic DNA as template, respectively.

PHB PRODUCTION IN PHOTOBIOREACTOR

Cultivation conditions and operating procedures

Pre-cultures of 50 mL inoculated from a single isolate picked from a solid plate were grown in 250 mL Erlenmeyer flasks housed in a climatic chamber (Gibertini, Italy) at 28 °C. The chamber was equipped with daylight fluorescent lamps (Philips TLD 30W/55) set continuously at 150 µE m^{-2} s^{-1} for 24/24 h (Carpine *et al.*, 2015). After about two weeks the pre-cultures were used to inoculate the photobioreactors (10% v/v). Inclined bubble column photobioreactors (working volume of 800 mL) were adopted for cyanobacterial growth. The photobioreactors were housed in a climate chamber (Solar Neon) at 28 °C. The chamber was also equipped with fluorescent lamps (Philips TLD 30 W/55). A sterilized gas stream using a hydrophobic filter (0.2 µm) was sparged at the bottom of the photobioreactors by means of multiple-orifices (1 mm ID) in a Teflon tube. The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations as previously described (Olivieri *et al.*, 2013). A gas mixing device (M2M engineering, Italy) provided the selected concentration of 2 % (v/v) of CO_2 in the gas stream fed to the photobioreactors by mixing air and pure carbon dioxide from a pressurized vessel at a final gas flow rate of 4 vessel volumes per minute (vvm). Cultures in inclined column photobioreactors were grown under light/dark cycles using a circadian rhythm: 18 h light varying between 10 and 260 µE m^{-2} s^{-1}, followed by 6 h darkness. The cultures were sampled every 48 h. Samples were characterized in terms of pH and concentration of biomass, nitrate, phosphate and PHB concentration. Each test condition was carried out in biological triplicate and the mean values are reported, typically with a variation of < 5 %.

Measured data were processed to assess the PHB fraction (ω_{PHB}) and PHB productivity (P_{PHB}). ω_{PHB} was calculated according to eq. 1:

$$\omega_{PHB} = \frac{[PHB]}{[X]} \cdot 100 \qquad \text{(eq. 1)}$$

where $[\mathrm{X}]$ is the cyanobacterial cell concentration, and $[\mathrm{PHB}]$ the poly-β-hydroxybutyrate concentration. The PHB productivity was calculated according to eq. 2:

$$\mathrm{P_{PHB}} = \frac{[\mathrm{PHB}]|_t}{\mathrm{t}} \qquad \text{(eq. 2)}$$

where $[\mathrm{PHB}]|_t$ is the poly-β-hydroxybutyrate concentration measured at the instant t.

Analytical methods

The pH and the total cell concentration were measured in the culture sampled from the photobioreactors. The sample was then centrifuged at 5000 g for 15 min. The liquid phase was characterized in terms of concentration of nitrate and phosphate. The solid phase was processed to assess the PHB content.

The biomass concentration was measured with a spectrophotometer (Specord 50, Analytic Jena) at 730 nm. Analysis of the dry cell weight (dcw) was carried out using a gravimetric method as previously described (Du *et al*., 2015) by filtering, drying and weighing 10 mL aliquots of culture. The corresponding OD_{730} values were paired with the measured dry cell weight, and a correlation factor of 1 OD_{730} = 0.23 g_{dcw}/L was found. The pH was measured with a Mettler Toledo pH meter.

The nitrate concentration was determined using a modified method proposed by Collos *et al*. (1999). The method is based on ultraviolet absorption spectrometry. The absorbance of the liquid phase recovered after the centrifugation was measured at a single wavelength (220 nm) after supplementing 20 µL of HCl 1 M to 1 mL of sample.

The phosphate concentration was measured according to the molybdate colorimetric test for ortho-phosphate. The colorimetric test was based on monitoring the redox state the antimonyl-phosphomolybdate complex produced during the reaction among ammonium molybdate, potassium antimonyl tartrate and ortho-phosphate. This complex is reduced to an intense blue-coloured (molybdenum blue) complex by ascorbic acid (Pierzynski & Simis, 2000). The preparation of the reagents required the mixing of: 250 mL of 4.5 M H_2SO_4; 45 mL of ammonium molybdate solution 95 g/L; 5 mL of potassium antimonyl tartrate solution 32.5 g/L; 50 mL of ascorbic acid solution 70 g/L. 30 µL of reagent and 30 µL of ascorbic acid were added to 940 µL of diluted sample. The absorption time-evolution of each sample was measured unsing a spectrophotometer at 704 nm.

PHB granules were released by a slightly modified cell rupture protocol (Schlebusch & Forchhammer, 2010). Biomass (10 mL of culture at OD_{730} 1) was harvested by centrifugation (20 min, 5000 g, 4° C). The cell pellets were dried overnight at 85°C. The dry pellets were boiled in 1 mL of 18 M H_2SO_4 for 40 min, cooled down to room temperature and diluted with 9 mL of 7 mM H_2SO_4, before being filtered through a polyvinylidene difluoride membrane. Quantitative analysis of PHB content in the solution produced from the chemical processing of the cells was carried out by means of HPLC (Agilent 1100 system) equipped with a multi-wave detector set at 210 nm. The separation column used was an Aminex HPX-87H (300x7.8 mm). PHB was converted in crotonic acid after treatment with sulphuric acid (Karr *et al.*, 1983b) and hence the latter was used as the external standard for HPLC analysis.

RESULTS AND DISCUSSION

Genetically engineered Synechocystis

In the present study, the native genes *pta* and *ach* of *Synechocystis* were knocked out, separately and in combination, using a two-step transformation protocol

resulting in three markerless derivative mutant strains. All these constructs, along with the *Synechocystis* wild type, were further transformed by integrating a cassette that harbors a heterologous *xfpk* under control of Ptrc in the neutral locus *slr0168*. In total, seven different constructs were obtained, which were subsequently characterized specifically in terms of biomass and PHB production capability. These constructs may however prove to be useful as chassis for the production of different compounds whose biosynthetic pathways also depend on the availability of acetyl-CoA [e.g. acetone (Zhou *et al.*, 2012), or 1-butanol (Lan & Liao, 2012), amongst others].

The specific growth rate of all mutant strains was reduced in comparison to the wild type *Synechocystis* from which they derive (Table 5.1.2). The deletion of *pta* had the biggest deleterious effect followed by the *ach* mutant, leading to a 63% and 51% drop in comparison to the wild type, respectively. Somewhat unexpectedly, the double deletion of *pta* and *ach* combined did not lead to a reduction in growth rate as strong as observed for the single deletions, although it was still quite substantial (44 % drop in comparison to wild type). Not surprisingly, the overexpression of *xfpk* from *B. brevis* had a negative effect on growth rate relative to the wild type (35% drop). However, it is interesting to note that this effect was reversed when the growth rate of the different mutant backgrounds were compared with the derivative overexpressing strain. Invariably, the single *pta*-, the single *ach-,* and the double *pta* and *ach* deletions recover growth rate when combined with *xfpk* overexpression, increasing 65%, 17% and 10%, respectively.

Table 5.1.2: Specific growth rate, maximum biomass concentration, and PHB concentration, fraction and productivity of *Synechocystis* wild type and derivative strains used in this study.

STRAIN	μ (h^{-1})	Maximum Biomass conc g(/L)	Maximum PHB conc (mg/L)	Maximum PHB fraction (%)	Maximum PHB productivity (g/m^3d)
SWT	0.036 ± 0.001	3.45 ± 0.32	50 ± 0.75	1.8 ± 0.2	3.05 ± 0.05
PTA	0.013 ± 0.002	1.97 ± 0.18	44 ± 0.5	3.5 ± 0.14	1.3 ± 0.01
ACH	0.017 ± 0.001	3.01 ± 0.05	17 ± 1.25	0.86 ± 0.04	1.4 ± 0.08
DKO	0.020 ± 0.002	3.66 ± 0.03	20 ± 0.25	0.58 ± 0.016	0.8 ± 0.01
XFPK	0.023 ± 0.002	3.07 ± 0.05	150 ± 10	5.3 ± 0.6	6.3 ± 0.57
PTAX	0.022 ± 0.002	1.47 ± 0.09	91 ± 6.25	6.2 ± 0.03	2.7 ± 0.16
ACHX	0.020 ± 0.001	2.73 ± 0	130 ± 10	5 ± 0.29	3.7 ± 0.29
DKOX	0.022 ± 0.001	2.42 ± 0.03	232 ± 7.5	12.4 ± 0.25	7.3 ± 0.18

Biomass and PHB production in photobioreactors

Synechocystis wild type and mutant strains were grown in BG11 medium under photoautotrophic conditions exposed to a circadian rhythm (18 h light/6 h dark). The biomass concentration and PHB accumulation therein, were periodically monitored in time during the 40 days of cultivation (Figure 5.1.3). Similar to what was observed for the growth rate, the single *pta* deletion has a great negative effect (43 % drop) when it comes to maximum biomass formation, leading to the lowest drop in final biomass concentration. The single ach deletion has relatively only a very slight effect on the maximum biomass concentration (13 % drop), while the double knockout completely reverts the trend observed for the single *pta* deletion, not showing any measurable effect on the biomass concentration (Table 5.1.2). The *xfpk* overexpression, now in stark contrast to what was observed for the growth rate, was found to consistently have a negative impact on the maximum biomass concentration when compared to the respective background not harboring the heterologous cassette. This apparent trade-off between rate and yield has been noted before in organisms displaying different metabolic strategies (Bachmann *et al.*, 2013).

The deletions of *pta* and *ach*, both separately, or in combination, did not lead to a major increase in the maximum PHB concentration reached throughout the photocultivations. However, unlike what is observed for the wild type and single ach mutant, whenever *pta* is knocked out, it seems cells are not able to utilize during the late stage of cultivation the PHB storages that they have accumulated earlier (Figure 5.1.3). Regarding the overexpression of *xfpk*, irrespective of the genetic background tested, it had a much more drastic effect on final PHB concentration. This effect can be observed clearly for wildtype and single deletions of *pta* and *ach*, but more importantly, it has a cumulative effect when both of these competing pathways are deleted simultaneously, leading to a PHB accumulation of 232 mg/L in the DKOX strain cultured under photoautotrophic conditions (Table 5.1.2).

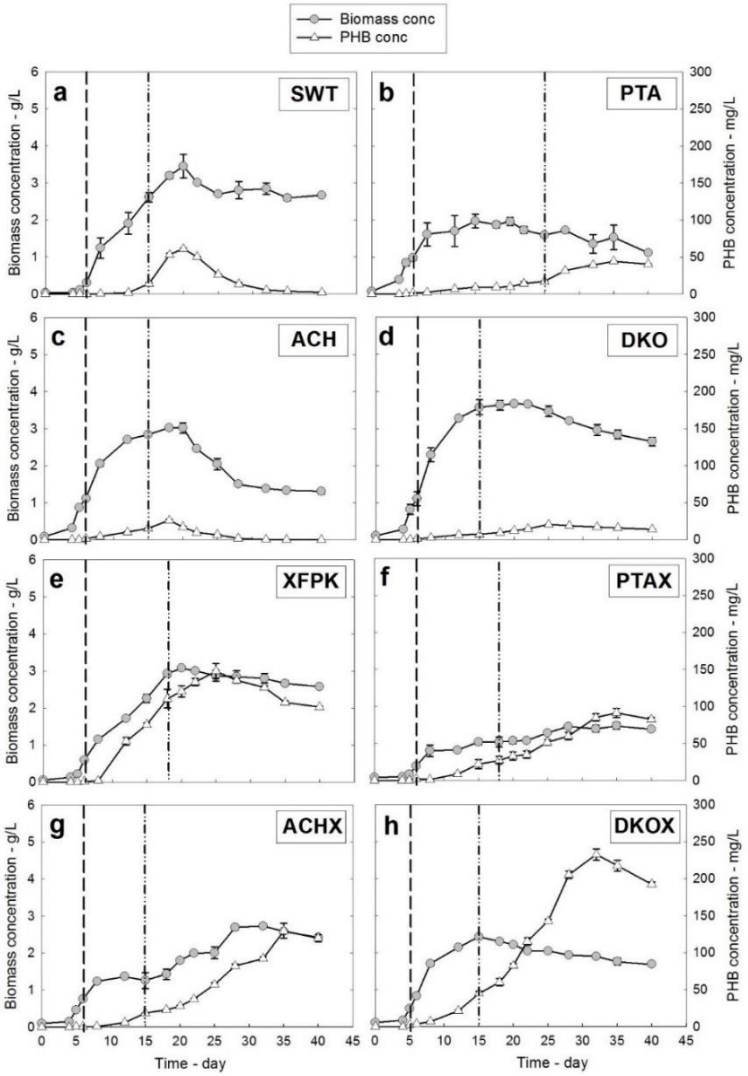

Figure 5.1.3 Biomass and PHB concentration in the photocultivations of Synechocystis and derivative strains. a) depicts *Synechocystis* wildtype; b) PTA strain carrying a single pta deletion; c) ACH strain carrying a single ach deletion; d) DKO strain carrying a double pta and ach deletion; e) XFPK strain harbouring the xfpk overexpression under a wildtype background; f) PTAX strain harbouring the xfpk overexpression under a PTA background; g) ACHX strain harbouring the xfpk overexpression under an ACH background; h) DKOX strain harbouring the xfpk overexpression under a DKO background. Grey circle indicates the biomass concentration, while white triangle indicates PHB concentration. Vertical lines mark the depletion of phosphate (long dash line) and nitrogen (dash dot dot line).

Effect of phosphate and nitrogen depletion on biomass formation and PHB production

During the multiple photocultivations under a circadian rhythm, residual phosphate and nitrogen concentration was determined periodically (Figure 5.1.4). Phosphate is rapidly depicted without any measurable phenotypic consequence for biomass formation or PHB accumulation. The rapid depletion of extracellular phosphate pools by *Synechocystis* has been previously described and linked to the rapid build up of intracellular phosphate storages (*e.g.* by increasing the number of chromosome copies) that are used in later stages of growth (Zerulla *et al*. 2016). This phenomenon is thought to be part of the survival strategies deployed by microorganisms that have evolved in aquatic environments, which are at times limited by this nutrient (Oliver, 2000).

Nitrogen depletion, under the conditions tested, occurred invariably much later than that of phosphate (Figure 5.1.4). This instant tended to match when the culture was reaching its maximum biomass concentration, and for the wild type, *pta* mutant, *ach* mutant and double *pta* and *ach* mutant strains, it also marked when PHB began to be accumulated by the cells. This corroborates previous studies in which it was found that upon N-depletion, cyanobacterial cells initially respond by driving the fixed carbon towards storage compounds such as PHB (Anfelt *et al.*, 2015). The genes coding for precursor biosynthesis of PHB in *Synechocystis*, *phaA* (*slr1993*) and *phaB* (*slr1994*), are organized in one operon. PHB synthase, the enzyme catalyzing the polymerization reaction to polyhydroxybutyrate, is encoded in a second operon and forms a heterodimer of PhaE (*slr1829*) and PhaC (*slr1830*). Expression of both operons has been reported to be up-regulated upon nitrogen starvation (Hauf *et al.*, 2013). A remarkable feature of overexpressing *xfpk* observed for all the genetic backgrounds tested was that this re-routing of carbon flux towards PHB storage upon N-depletion appears to be deregulated. We observed an accumulation of PHB long before N-depletion was reached. Mechanistically, this might be a consequence of the increased acetyl-CoA pools, which accompanies an increased phosphoketolase activity (Anfelt *et al.*, 2015), and which would increase the thermodynamic drive to route carbon through the PHB pathway (Kocharin *et al.*, 2013). Alternatively, or in combination with the latter, this could also be a consequence of increased acetyl-P levels, which are known to activate PhaA, the first step of the PHB synthesis pathway (Miyake *et al.*, 1997; Sonderegger *et al.*, 2004). Irrespective of the molecular basis for this phenotype, the finding that *xfpk* overexpression misleads the cells towards behaving as if they were N-limited, may be used to route carbon towards other products as well, while the cells are still under non nutrient-limited conditions.

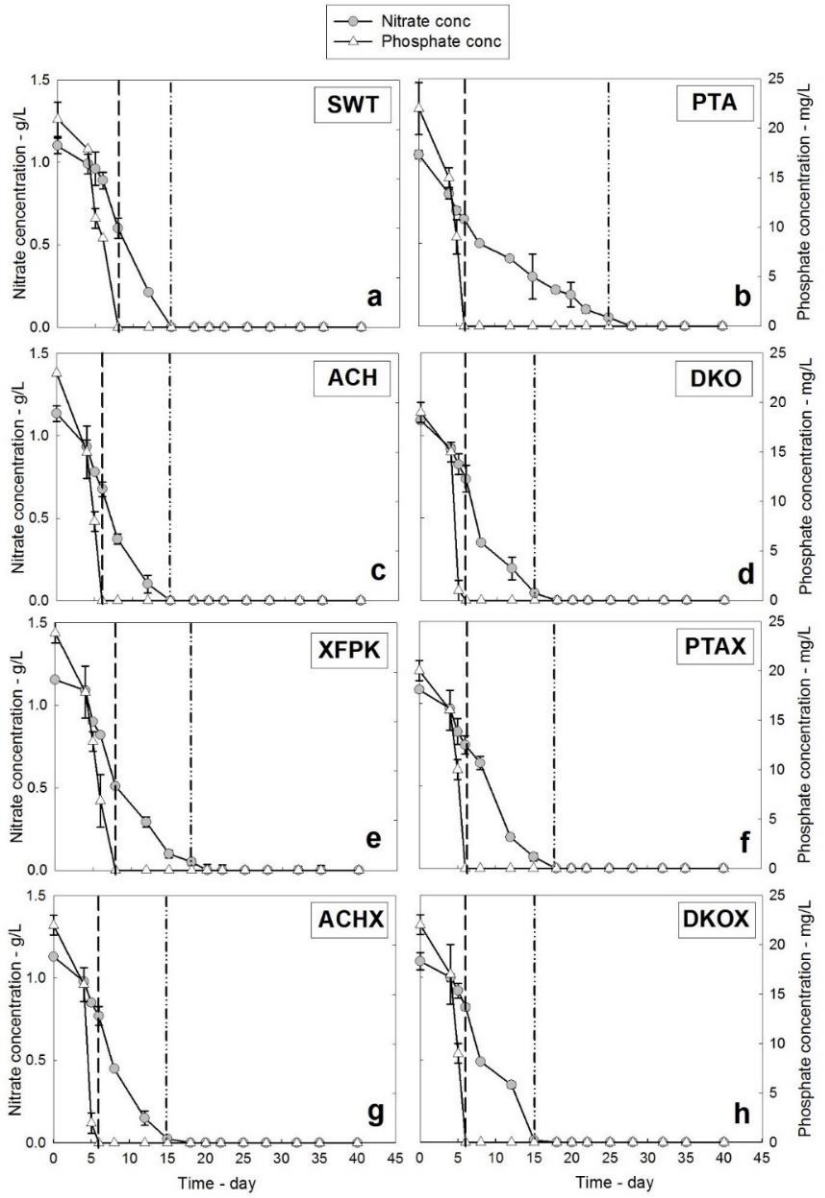

Figure 5.1.4 Residual nitrate and phosphate concentration in the photocultivations of Synechocystis and derivative strains. A) depicts *Synechocystis* wildtype; B) PTA strain carrying a single pta deletion; c) ACH strain carrying a single ach deletion; d) DKO strain carrying a double pta and ach deletion; e) XFPK strain harbouring the xfpk overexpression under a wildtype background; f) PTAX strain harbouring the xfpk overexpression under a PTA background; g) ACHX strain harbouring the xfpk overexpression under an ACH background; h) DKOX strain harbouring the xfpk overexpression under a DKO background. Nitrate (grey circles) was found in all cases to be depleted after phosphate (white triangle). Vertical lines mark the sampling point in which depletion of phosphate (long dash line) and nitrogen (dash dot dot line) was considered to occur.

Characterization of PHB fraction in biomass and overall PHB productivity in the photobioreactors

The PHB fraction in biomass (ω_{PHB}) and overall productivity (P_{PHB}) throughout the photoautotrophic cultivations under circadian rhythm were calculated based on the biomass and PHB concentration levels (Figure 5.1.5).

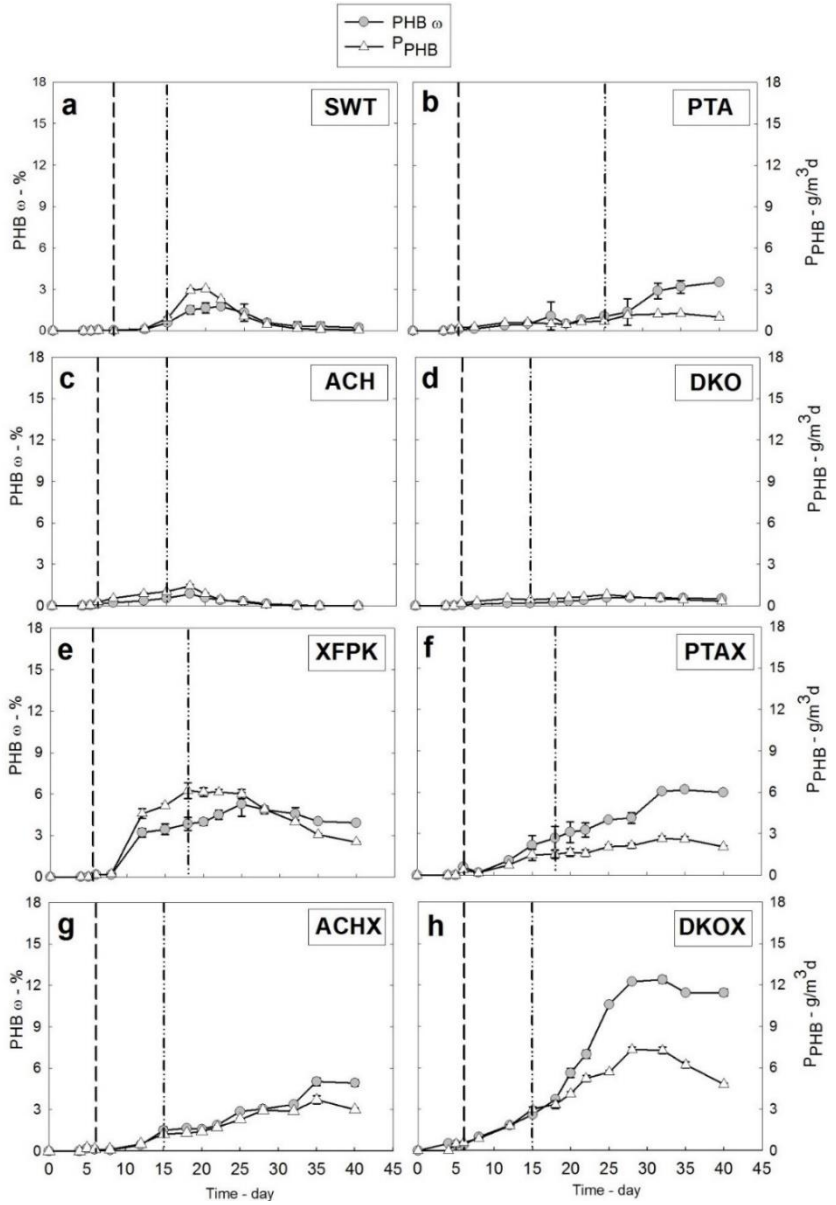

Figure 5.1.5. PHB fraction and productivity in the photocultivations of *Synechocystis* and derivative strains: a) depicts *Synechocystis* wildtype; b) PTA strain carrying a single *pta* deletion; c) ACH strain carrying a single *ach* deletion; d) DKO strain carrying a double *pta* and *ach* deletion; e) XFPK strain harboring the *xfpk* overexpression in a wildtype background; f) PTAX strain harboring the *xfpk* overexpression in a PTA background; g) ACHX strain harboring the *xfpk* overexpression in an ACH background; h) DKOX strain harboring the *xfpk* overexpression in a DKO background. Grey circle indicates the PHB fraction, while white triangle indicates PHB productivity. Vertical lines mark the depletion of phosphate (long dash line) and nitrogen (dash dot dot line).

The ω_{PHB} and P_{PHB} are important indicators because the different genetic modifications performed led to changes in both biomass formation and PHB accumulation, which may be misleading if analyzed separately regarding the potential of the different constructs as photoautotrophic cell factories of PHB. The *ach* deletion had a negligible impact on both ω_{PHB} and P_{PHB}, regardless of whether it was combined or not with the *pta* deletion as well. Strain PTA, carrying the single *pta* deletion, did display a sustained increase of ω_{PHB}. However, since this was accompanied as well by a sharp drop in biomass formation, ultimately the overall productivity was not greatly increased. The effect of the *xfpk* overexpression on both ω_{PHB} and P_{PHB} was much more pronounced than that of any of the other genetic modifications tested. The mimicking of N-depletion before it actually sets in, which triggers cells to start

accumulating PHB immediately at earlier stages of growth plays a huge role here. Particularly, when the overexpression of *xfpk* was combined with the elimination of both *pta* and *ach* (strain DKOX). This mutant, ultimately reached a PHB fraction of about 12 % (w/w) of the dry cell weight, a value 6-fold higher than that of the wild type strain, and a maximal PHB productivity of ~7 g m^{-3} d^{-1} (Table 5.1.2).

Many different genetic engineering approaches to increase PHB content in cyanobacteria have been described in literature with varying degrees of success (Balaji *et al.*, 2013). The ω_{PHB} and P_{PHB} reported are not always directly comparable, specially because in some instances PHB is not being produced strictly photoautotrophically, *i.e.* directly from CO_2 alone. We have strived to contextualize the achieved ω_{PHB} and P_{PHB} obtained here by surveying the aforementioned reports in respect to: (i) host used; (ii) genetic modifications performed; (iii) carbon source used; (iv) culture condition; and (v) reactor type. Unfortunately, we still found that the overall productivity cannot be compared, because there is not enough data collected in some studies, or the production conditions are simply too distinct. Nonetheless, we found it useful to still compare the different ω_{PHB} obtained (Table 5.1.3).

The several studies that have evaluated PHB accumulation using mixotrophic growth conditions (Khetkorn *et al.*, 2016; Sudesh *et al.*, 2002; Takahashi *et al.*, 1998; Wu *et al.*, 2002) in mutants of either *Synechocystis* or *Synechococcus* PCC7942, added acetate in the growth medium. Rationale has been that acetate can be converted into acetyl-CoA via mostly the activity of acetyl-CoA synthetase, and thereby support many metabolic pathways such as the TCA cycle for cell growth, glycogen synthesis and fatty acid metabolism relevant for biofuel production, and also PHB biosynthesis (Khetkorn *et al.*, 2016). The heterologous expression of PHA-synthesizing gene operon from the bacterium *C. necator* in *Synechocystis* sp. PCC 6803 increased the PHB content from 7% to 11% of dcw when grown in N-deprived medium with 10 mM acetate (Sudesh *et al.*, 2002). Similar results had already been reported for the cyanobacterium *Synechococcus* sp. PCC 7942 heterologously expressing the entire *A. eutrophus* PHB-synthesizing machinery. These recombinant *Synechococcus* cells accumulated 25% PHB of dcw under N- deprivation with 10 mM acetate (Takahashi *et al.*, 1998). The PHB content of the phaAB strain obtained by Khetkorn et al. (Khetkorn *et al.*, 2016), increased to 35% of dcw in the presence of 4 mM acetate under nitrogen deprived condition when compared to about 13% PHB of dcw in the wild type under the same condition.

The numbers reported when ω_{PHB} is evaluated under photoautotrophic growth conditions using only CO_2 as carbon source tend to be somewhat more modest (Hondo *et al.*, 2015; Osanai *et al.*, 2013). The overexpression of the native *sigE* gene integrated in the *Synechocystis* chromosome, increased the PHB content from 0.6% to 1.4% when grown in N-deprived medium (Osanai *et al.*, 2013). Hondo et al. (Hondo *et al.*, 2015) transformed *Synechocystis* cells with the vector pAM461c harboring a PHA biosynthetic operon from *Microcystis aeruginosa* NIES-843 and reached a PHB content about 7% in N-deprived medium. We have obtained a similar ω_{PHB} by simply overexpressing from a genomic integration *xfpk* under control of a strong promoter (Ptrc). Moreover, when the overexpression of *xfpk* was combined with the double deletion of both *pta* and *ach*, we reached a PHB content in biomass above 12%. To the best of our knowledge, this value is almost 2-fold higher than ever reported using CO_2 alone as the direct carbon source.

Table 5.1.3: Comparison of achieved PHB fraction in engineered cyanobacteria cultured under different conditions

Cyano-bacterial strain	Engineered genes	Carbon source	Culture cond.	Reactor	Polymer fraction (% dcw)	Ref
Syne-chocystis derivative	The *agp* gene was inserted	CO_2 0.035%, Acetate 5mM	Nitrogen deprived, phototrophic growth for 7 days	flask	18.6%	(Wu *et al.*, 2002)
Syne-chococcus derivative	A PHB synthesizing enzyme from *A. eutrophus* was expressed from a plasmid	CO_2 5%, Acetate 10 mM	Nitrogen deprived, phototrophic growth for 14 days	flask	25%	(Takahashi *et al.*, 1998)
Syne-chocystis derivative	PHA synthase from *C. necator* was expressed from a plasmid	CO_2 1%, Acetate 10 mM	Nitrogen deprived, phototrophic growth	flask	11%	(Sudesh *et al.*, 2002)
Syne-chocystis derivative	The native *sigE* was expressed from the chromosome	CO_2 1%	Nitrogen deprived, phototrophic growth for 9 days	flask	1.4%	(Osanai *et al.*, 2013b)
Syne-chocystis derivative	A PHA biosynthetic operon from *M. aeruginosa* was expressed from a plasmid	CO_2 2 %	Nitrogen deprived, phototrophic growth for 8 days	flask	7%	(Hondo *et al.*, 2015b)
Syne-chocystis derivative	The native *phaAB* was overexpressed from the chromosome	CO_2 0.035%, Acetate 4 mM	Nitrogen deprived, phototrophic growth for 9 days	flask	35%	(Khetkorn *et al.*, 2016)
Syne-chocystis derivative	*xfpk* from *B. breve* was expressed in a double *pta* and *ach* knock out.	CO_2 2 %	BG11, phototrophic growth for 40 days	photo reactor	**12%**	**This work**

CONCLUSIONS

Genetic engineering of cyanobacteria for the overproduction of PHB directly from CO_2 has been targeted by many previous studies. Most have focused on increasing the levels of the enzymes in the PHB synthesis pathway in order to improve PHB accumulation (Hondo *et al.*, 2015b; Khetkorn *et al.*, 2016; Takahashi *et al.*, 1998). We have instead concentrated in trying to engineer the central carbon metabolism of the host such that *(i)* the PHB synthesis pathway becomes deregulated, and/or *(ii)* that more acetyl-CoA becomes available to it. For this purpose, we constructed seven different mutants, which harboured either separately or in combination, three different genetic modifications to the metabolic network – phosphotransacetylase deletion, acetyl-CoA hydrolase deletion and heterologous phosphoketolase overexpression. These, along with the wildtype *Synechocystis*, were tested for PHB production in photobioreactors under photoautotrophic conditions while exposed to a circadian rhythm and using standard BG_{11} as the growth medium. Ultimately, the strain that combined all three genetic alterations, led to highest levels reported of PHB production directly from CO_2: 232 mg/L, ~12% (w/w) of the dry biomass weight, and a productivity of 7.3 g m^{-3} d^{-1}.

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5.2 PHB production improvement from mutant strain of *Synechocystis* PCC6803 on optimized growth media

In this section the strain of *Synechocystis* with the three mutations provided by (DKOX) the investigation reported in section 5.1 was grown under optimized growth conditions to further improve the PHB production. Indeed, DKOX was grown on the $BG_{1/2}$ medium (section 3.2).

Media

Mutant strain of *S. sp. PCC6803* (DKOX) was grown under photoautotrophic conditions in $BG_{1/2}$ media to improve PHB production.

Procedures

The cyanobacteria were transferred from a Petri plate into 100 mL Erlenmeyer flasks containing 30 mL medium. After about two weeks the pre-cultures were inoculated into photobioreactors: 10% (v/v) of actively growing pre-cultures.

Cultures in PBR photobioreactors were grown in light/dark cycles: 18 h light at light intensity between 260 $\mu E/m^2s$ and 6 h of dark. The cultures were sampled for 22 day every day for two times per day (one at the end of light phase and one at the end of dark phase). Samples were characterized with respect to biomass concentration, PHB concentration, fraction and productivity and nitrate and phosphate concentration. Each test condition was carried out in biological triplicate and the mean values are reported, typically with a variation of < 5 %.

Analysis

Biomass concentration:

Biomass concentration was measured using the protocol reported in section 5.1.

Nitrate concentration:

Nitrate concentration was measured using the protocol reported in section 3.2.

Phosphate concentration:

Phosphate concentration was measured using the protocol reported in section

PHB concentration:

PHB was quantified as reported in section 3.2.

Results

Synechocystis wild type and mutant strain (DKOX) were grown in $BG_{1/2}$ medium under photoautotrophic conditions exposed to a circadian rhythm (18h light/6h dark). The biomass concentration and PHB accumulation were periodically monitored in time during the 22 days of cultivation (Figure 5.2.1). Indeed, the cultures were sampled for 22 days every day for two times per day (one at the end of light phase and one at the end of dark phase). As shown in Figure 1 A, that reports the cell concentration for SWT strain and DKOX strain, the shift from light phase to dark phase had a consequence on biomass behaviour. Indeed, the total cell concentration had a wavelike trend: growth (crests) and inactivation (trough) cycles (as reported in section 4.1). Both WT and DKOX maximum cell concentration reached a value of about 2.7 g/L.

The Figure 5.2.1 B reports the PHB concentration for SWT and DKOX strains. It can be noticed that PHB concentration reached a value about 410 mg/L which is twice the value reached by SWT strain in $BG_{1/2}$ and by DKOX strain in BG_{11} medium (section 5.1).

The Figures 5.2.1 C and D report the results about PHB fraction and productivity respectively. It is evident that PHB content in DKOX strain reached a value about 21% of dry cell weight, that is 3-fold higher with respect to SWT strain in $BG_{1/2}$ and 2-fold higher with respect to DKOX strain in BG_{11} (results reported in section 5.1). The productivity obtained with DKOX strain (Figure 1D) is about 31 g/m^3d, which is twice the value of WT strain in $BG_{1/2}$ and four times the value of DKOX strain in BG_{11}.

During the multiple photocultivations under a circadian rhythm, residual phosphate and nitrogen concentration was determined periodically (Figure 1 E and F). Phosphate was rapidly depicted (after about 100 h) without any measurable phenotypic consequence for biomass formation or PHB accumulation.

Nitrogen depletion, under the conditions tested, occurred later than that of phosphate (after 210 h of cultivation). This instant tended to match when the culture was reaching its maximum biomass concentration, and it also marked when PHB began to be accumulated by the cells for both SWT and DKOX strains.

It is also interesting to note that PHB started to decrease after 12 days of culture (Figure 5.2.1 B). Indeed, as reported in literature (Ansari & Fatma, 2016) PHB is used by the cells for their functioning during stress conditions (nitrogen-depletion).

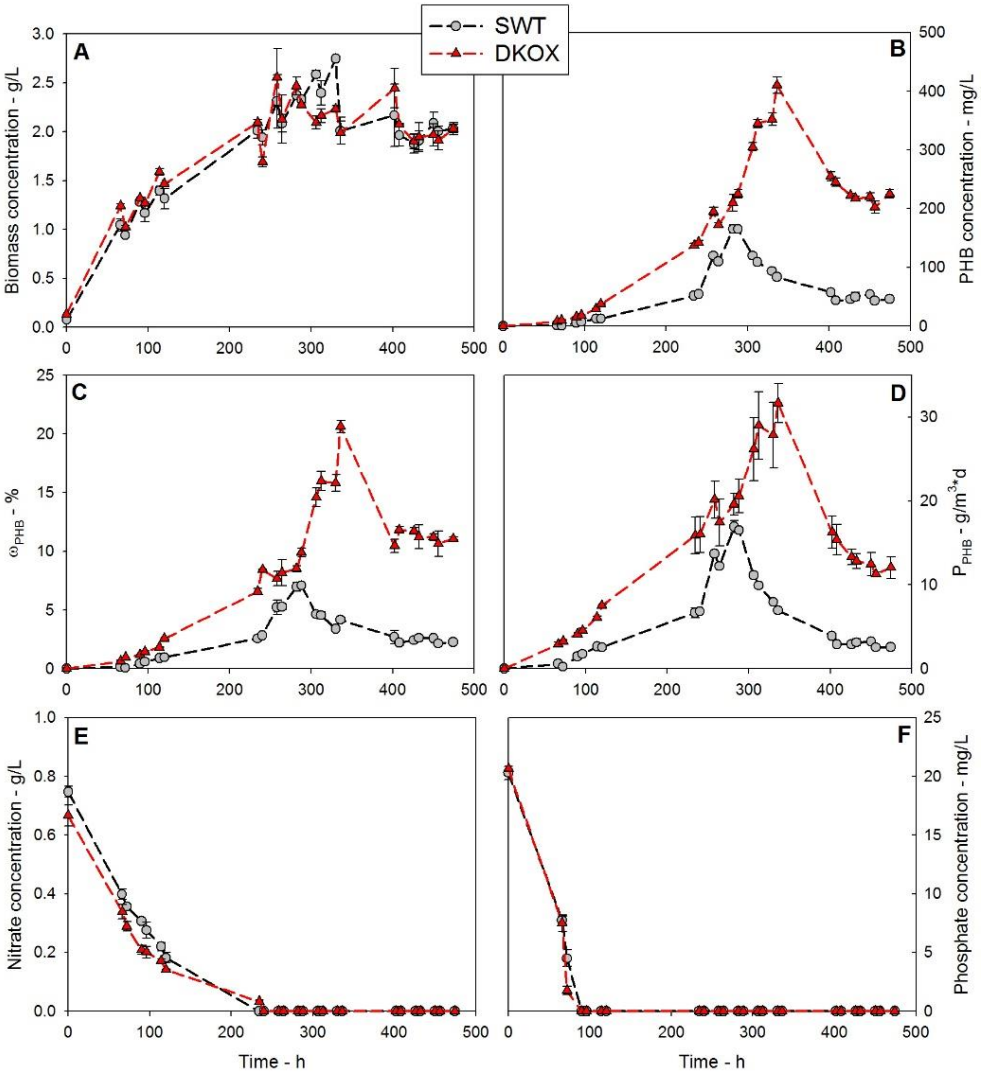

Figure 5.2.1: A) Biomass, B) PHB concentration, C) PHB fraction, D) PHB productivity, E) nitrate concentration and F) phosphate concentration in the photocultivations of Synechocystis WT and DKOX strains. Grey circle indicates the SWT strain, while red triangle indicates DKOX strain.

Table 5.2.1 reports a comparison between SWT strain growth in BG_{11} and in $BG_{1/2}$ media at 150 $\mu Em^{-2}s^{-1}$ (section 3.2) and SWT strain growth in BG_{11} and in $BG_{1/2}$ media at 260 $\mu Em^{-2}s^{-1}$ (section 5.1 and 5.2 respectively). It should be noted that even though the maximum irradiation was different for the two series of run, the light energy irradiated each day was almost the same: 0.70 μEm^{-2} for tests in section 3.2 and 0.80 μEm^{-2} for tests in section 5.1 and 5.2. Moreover, the photobioreactor design used in the two series of run was different: inclined bubble coloumn photobiorectors for tests in section 3.2 and and coloumn bubble photobioreactors for test in section 5.1 and 5.2. It can be noted that a similar value of PHB concentration and fraction were reached whatever the light irradiation strategy for both media. On the contrary, the PHB productivity was 2.5 fold higher if the light intensity used for growth was 260 $\mu Em^{-2}s^{-1}$ for both media. Hence, the light irradiation strategy and reactor design affected just the cultivation time and not the performance of growth and PHB production.

In Table 5.2.1, a comparison between DKOX strain grown on BG_{11} and $BG_{1/2}$ at 260 $\mu Em^{-2}s^{-1}$ (section 5.1 and 5.2) is also reported. The best performance in terms of PHB concentration, content and productivity, was obtained on $BG_{1/2}$ at 260 $\mu Em^{-2}s^{-1}$ (this section). The high accumulation of PHB under nitrogen-starvation conditions may be interpreted taking into account the absence of nitrogen into the medium while the photosynthesis was still active and fixed CO_2. Indeed, the cells cannot synthesize proteins and they accumulate the carbon/energy as PHB (Smith, 1982).

Table 5.2.1: Comparison between SWT strain and DKOX strain in different conditions.

STRAIN	MEDIUM	Light intensity ($\mu Em^{-2}s^{-1}$)	Maximum Biomass conc g(/L)	Maximum PHB conc (mg/L)	Maximum PHB fraction (%)	Maximum PHB productivity (g/m^3d)	Additional Information
SWT	BG_{11}	150	2.3	48	2	0.8	Section 3.2
SWT	$BG_{1/2}$	150	2.7	210	8	7	Section 3.2
SWT	BG_{11}	260	3.45	50	1.8	3.05	Section 5.1
SWT	$BG_{1/2}$	260	2.7	240	7	17	This section
DKOX	BG_{11}	260	2.42	232	12.4	7.3	Section 5.1
DKOX	$BG_{1/2}$	260	2.55	410	21	32	This section

Conclusions

Genetic engineering approach on *Synechocystis* PCC6003 was used as techniques to improve PHB production. The central carbon metabolism of the host was engineered: (i) the PHB synthesis pathway becomes deregulated, and/or (ii) that more acetyl-CoA becomes available to it. The mutant strain with three different genetic modifications to the metabolic network – phosphotransacetylase deletion, acetyl-CoA hydrolase deletion and heterologous phosphoketolase overexpression – was tested. These, along with the wildtype *Synechocystis*, were tested for PHB production in photobioreactors under photoautotrophic conditions while exposed to a circadian rhythm and using standard $BG_{1/2}$ as the growth medium. The strain that combined all three genetic alterations, led to highest levels reported of PHB production directly from CO_2: 410 mg/L, ~21% (w/w) of the dry biomass weight, and a productivity of 32 g/m^3d.

6 DISCUSSION

About 140 million tons of plastic are consumed every year worldwide, which necessitates the processing of approximately 150 million tons of fossil fuels and directly causes immense amounts of waste that can take thousands of years to naturally deteriorate (Hempel *et al.*, 2011). Consequently, bioplastics are a feasible alternative. They are not based on fossil resources and can easily be biodegraded. In the current scenario of increasing environmental problems, polyhydroxyalkanoates are becoming the focus of attention as a potential substitute for non biodegradable polymers (Sharma *et al.*, 2007). Poly-β-hydroxybutyrate is the most widespread and completely characterized PHAs found in bacteria.

Poly-(3-hydroxybutyrate) (PHB) is a metabolite product that may be accumulated in microorganisms as carbon and energy reserve source. It can be produced by various bacteria - e.g. such as *Cupriavidus necator*, strains of *Pseudomonas*, *Bacillus*, *Azotobacter* - and cyanobacteria such as *Synechocystis* PCC6893, *Nostoc muscorum* and *Spirulina platensis* (Peña *et al.*, 2014). The use of PHB produced by bacterial fermentation as a commodity polymer is limited by its high production cost compared to some widely used petroleum derived plastics. The high cost of PHB production process depends on different issues among which the use of conventional carbon sources (e.g. glucose, sucrose, lactose) in growth media.

A solution to the cost of the feedstock could be the use of autotrophic based systems because their photosynthetic apparatus allows to use CO_2 as feedstocks. Two potential routes may exploit the photosynthetic apparatus: plants and microorganisms. The bacterial pathway for PHB production can be expressed in the cytosol or targeted to different compartments of the plant cell leading to high amounts of PHB accumulation. Nevertheless the high potential productivity provided by plants, plant-based expression systems compete directly with subsistence crops for agricultural acreage, and raise further ethical concerns as the dissemination of transgenic plants is difficult to control, which has led to strict regulatory restrictions of transgenic plants in many countries (Hempel *et al.*, 2011). Cyanobacteria can be considered as an alternative system for PHB production. Cyanobacteria share all the advantages of photosynthetic microorganisms when compared to chemoheterotrophs. Cyanobacteria use (sun)light energy to directly convert CO_2 into product of interest such as PHB without the drawbacks of competing with the agro-food market for resources (Gopi *et al.*, 2014; Gupta *et al.*, 2013; Wijffels *et al.*, 2013). Indeed, cyanobacteria can be cultivated in saline/brackish water/coastal seawater on non-arable land (Singh *et al.*, 2016). A conceptual model about the role of cyanobacteria in sustainable agriculture and environmental management has been proposed in Figure 6.1.

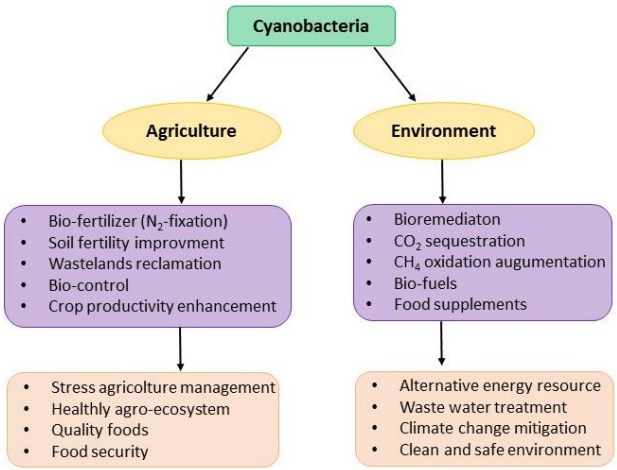

Figure 6.1: A model exhibiting the potential roles of cyanobacteria in sustainable agriculture and environmental management.

6.1 Cyanobacteria as attractive alternatives for the PHB production

Among short-chain-length-PHAs (SCL-PHAs), polyhydroxybutyrate (PHB) is the most common and wide- spread PHA produced in various bacterial and cyanobacterial species. The PHB features make this PHA particularly attractive for the bioplastic market.

Cyanobacteria, also known as blue-green algae, are photoautotrophic Gram-negative prokaryotes with the ability to carry out oxygenic photosynthesis. Cyanobacteria need some simple inorganic nutrients such as phosphate, nitrate, magnesium and calcium as macro and ferrous, manganese, zinc, cobalt, and copper as micronutrients for their growth and multiplication (Balaji *et al.*, 2013; Campbell *et al.*, 1982). There are also some nitrogen-fixing cyanobacteria, usually grown without supplementation of nitrogen sources (Capone *et al.*, 2005; Sharma *et al.*, 2007). Cyanobacteria exhibit three modes of cell growth, i.e., photoautotrophic, mixotrophic, and heterotrophic. The first cyanobacterial species, which reported the presence of PHB, is *Chlorogloea fritschii* in the year 1966.

To date, the occurrence of PHB has been demonstrated for several cyanobacteria such as *Spirulina* sp., *Aphanothece* sp., *Gloeothece* sp., *Synechococcus* sp. and *Synechocystis* sp. (Miyake *et al*., 2000; De Philippis *et al.*, 1992; Wu *et al.*, 2001).

The metabolic pathways for PHB production representing the conversion of acetyl-CoA to PHB. The acetyl coenzyme A (acetyl-CoA), referred to as a central point of metabolism. It is usually obtained from various routes including glycolysis pathway by oxidative conversion of pyruvate catalyzed by pyruvate dehydrogenase, and glycogenosis pathway by degrading glycogen into glucose-6-phosphate (G6P) which then enters glycolysis (Khetkorn *et al.*, 2016). The biosynthetic pathway for PHB production (Figure 6.2) consists in three enzymatic reactions. The synthesis starts with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. The enzyme that catalyse this reaction is 3-ketothiolase, encoded by the phbA gene. Acetoacetyl-CoA reductase (NADPH dependent enzyme), encoded by phbB, converts the acetoacetyl-CoA to 3-hydroxybutyryl-CoA. During the last step, the enzyme PHA synthase (encoded by phbC) polymerizes the 3-hydroxybutyryl-CoA monomers to PHB, liberating CoA (Balaji *et al.*, 2013; Peña *et al.*, 2014; Singh *et al.*, 2016).

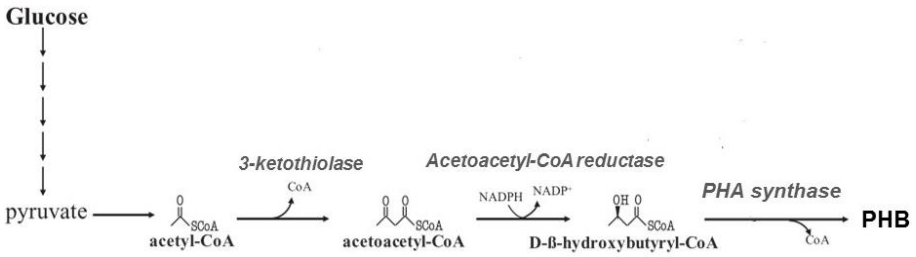

Figure 6.2: Biosynthetic pathway for PHB production (modified from Peña *et al.*, 2014).

6.2 Improvement of PHB production in cyanobacteria

6.2.1 Growth conditions optimization

Nutrient deficiency or limitation (mainly deficiency or limitation of nitrogen or phosphate) stimulate the accumulation of the PHB in cyanobacteria under photoautotrophic growth conditions (Bhati & Mallick, 2012; Samantaray & Mallick, 2014; Takahashi *et al.*, 1998). PHB production of 71% (dcw) has been measured in *Nostoc Muscorum* Agardh when medium was provided with glucose coupled with nitrogen and phosphate limitations (Bhati & Mallick, 2015). The PHB pool in *Spirulina* sp. LEB 18 reached 31% (dcw) under photoheterotrophic condition together with a deficiency of nitrogen and phosphorus (medium provided with sodium bicarbonate) (Coelho *et al.*, 2015). *Aulosira fertilissima* CCC 444 showed an accumulation of 77% (dcw) when medium was provided with fructose and valerate coupled with phosphorus deficiency (Samantaray & Mallick, 2014). For *Synechocystis* sp. PCC 6803, PHB accumulation of 38% (dcw) under nitrogen depletion with fructose and acetate supplementation was recorded (Panda & Mallick, 2007). The same cyanobacterium showed an accumulation of PHB to 4% (dcw) under photoautrophic conditions together with nitrogen depletion (Wu *et al.*, 2001). As reported by Haase *et al*., (2012), *Nostoc muscorum* reached a PHB content of 22% under photoautotrophic condition together with a deficiency of nitrogen. *Calothrix scytonemicola* TISTR 8095 showed an accumulation of PHB of 25% (dcw) in photoautotrophic growth conditions together with a deficiency of nitrogen. The high accumulation of PHB under nitrogen-starvation conditions may be interpreted taking into account the absence of nitrogen into the medium while the photosynthesis was still active and fixed CO_2. Indeed, the cells cannot synthesize proteins and they accumulate the carbon/energy as PHB (Smith, 1982).

The results reported in this work (Carpine *et al*., 2017a) showed that *Synechocystis* sp. PCC 6803 reached a PHB accumulation of 8% in photoautrophic conditions (CO_2 2%) when the nitrate concentration is the half of the optimal concentration ($BG_{1/2}$ medium).

A critical review of the data reported above highlights that cells growth under unbalanced feeding of nitrogen and phosphorus is necessary to activate PHB production (Singh *et al.*, 2016).

As reported by Bhaty & Mallick (2015), Panda & Mallick (2007), Samantary & Mallick (2014), the maximum PHB content was obtained under **heterotrophic** growth conditions: 78, 77 and 38 % dwc respectively. However, the PHB production requires the supplying of the carbon source. Coelho *et al.* (2015), Haase *et al*. (2012) and Kaewbai-ngam *et al.*, (2016) reported interesting results for **autotrophic** growth cultures carried out in flasks: 31, 21 and 25 % dcw respectively. They cultivated *Nostoc muscorum*, *Calothrix scytonemicola* and *Spirulina* sp. LEB 18, respectively. However, these strains are biofilm forming strains (Di Pippo *et al.*, 2013) and their performance

may strongly reduce when cultivated in photobioreactors. *Synechocystis* PCC6803 was characterized by high PHB productivity under heterotrophic conditions (Panda *et al.*, 2006). However, the performance drastically reduced when growth under autotrophic conditions (Wu *et al.*, 2001). Indeed, the PHB fraction reported by Wu et al. (4% of PHB) was half the value reported in this work.

6.2.2 Genetic engineering approach

Rational design strategies typically developed within the microbial biotechnology allow the construction of microbial cell factories. Cyanobacteria can be considered the cell factories of choice. The introduction of an exogenous biosynthetic pathway into a cyanobacterial cell will lead to product formation from CO_2 (Angermayr *et al.*, 2012). Cyanobacteria are convenient for genetic manipulation both via plasmid introduction or by chromosome modification compared to other algae and plants (Berla *et al.*, 2013). Genetic engineering and recombinant DNA technology, together with metabolic engineering, have emerged as one of the strategies towards the production of high PHB accumulating strains with the ability to produce a large quantity of biomass (Singh *et al.*, 2016).

Different literature works regarding the genetic engineering approach to improve PHB from cyanobacteria were reported in section 1.6 (Introduction). In this work (see Carpine *et al*. 2017c) a higher PHB content was obtained by simply overexpressing from a genomic integration phosphoketolase (*xfpk)* under control of a strong promoter (Ptrc). Moreover, when the overexpression of *xfpk* was combined with the double deletion of phosphotransacetylase *(pta)* and acetyl-CoA hydrolase *(ach)*, a PHB content in biomass above 12% was reached, (in balanced conditions using BG_{11} as growth medium), almost 2-fold higher than ever reported using CO_2 alone as the direct carbon source.

The strains in which *xfpk* was overexpressed were tested in growth medium characterized by a different nitrate concentration to further increase PHB production (**Section 5.2**). Indeed, a high amount of PHB - approximately 21% w/w of the dry cell weight - was obtained in the strain in which *xfpk* gene was overexpressed and *pta* and *ach* genes were knocked out. In particular, the PHB fraction of this mutant strain reached a value 3-fold higher than the one obtained with the wild type strain using $BG_{1/2}$ medium (medium in witch nitrate concentration is the half of optimal) as growth medium.

6.3 Kinetic model assessment

The optimization of the production process of PHB is not a trivial issue because the PHB production by microorganisms involves different metabolic pathways. However, an adequate model to describe the process is a key issue to address the conditions to maximize the PHB fraction in the cell. For an effective design, scale-up, and optimization of PHB production process, it is necessary to have insight into the mechanisms of the process. There are many contributions in the literature about modeling photosynthetic microorganism growth and about PHB production from non-photosynthetic bacteria. They are summarized hereinafter and compared with the activity carried out in the present PhD thesis.

Kasiri *et al*., (2015) considered to uncouple the kinetics of nutrients uptake and of cell growth. They proposed a nonlinear dynamic model to assess the optimal CO_2 concentration, phosphate concentration, and light intensity for CO_2 uptake and algal growth. The model proposed by Kasiri at al., (2015) was focused on the algal growth and it did not include the production of metabolites. Kim *et al*., (2015) proposed a multi-

component kinetic model applied to the cyanobacterium *Synechocystis sp*. PCC6803 and tested it in batch experiments that allowed to assess kinetic parameters for each factor (light irradiance and the concentration of an inorganic nutrient) independently each other. Jeon *et al*., (2014) proposed to integrate a metabolic network model with a semi-empirical model (Droop model) to predict the simultaneous lipid accumulation and cell growth for the microalgae specie *Chlamydomonas reinhardtii*. The model proposed by Jeon *et al*., (2014) did not include the effect of light intensity on microalgal growth. Mozumder *et al*., (2015) described the autotrophic PHB production in *Cupriavidus necator* by using a model based on a two-phase processes: biomass growth and subsequent PHB production. The aim was to simulate the two distinct phases to achieve a high PHB production rate and PHB content. Faccin *et al*., (2012) presented a model to describe the culture kinetics of the bacterium *Bacillus megaterium* for the production of P(3HB). Khanna & Srivastava (2005) proposed a structured unsegregated model for PHB accumulation in *Ralstonia eutropha* based on detailed investigations on substrate limitations and inhibitions by carbon, nitrogen, and phosphorus sources. Although the model proposed by Mozumder *et al*., (2015), Faccin *et al*., (2012) and Khanna & Srivastava (2005) regarded the PHB production in microorganisms, they did not include autotropic growth conditions of photosynthetic microorganisms. To the author knowledge, no model has been reported in the literature regarding the PHB production by autotrophic cultures of cyanobacteria.

In Carpine *et al*. (2017b) was proposed a mathematical model to describe photoautotrophic PHB production, using a light/dark cycle (18h light/6h dark) for cyanobacteria growth. The model was based on mass balances. The effect of significant factors and nutrients on the cyanobacteria system were taken into account. In particular, the proposed model includes the nitrate and phosphate utilization rate, the PHB production rate, the cell growth and lysis rate. Two types of cells were considered in the cultures: growing cells (X), which are able to grow, and PHB producing cells (X_{PHB}). The specific velocity (k_T) of X_{PHB} formation was considered dependent from the initial concentration of nitrate. Data measured during growth tests carried out at several initial nitrate concentration were processed to assess the kinetic and stoichiometric parameters of the proposed model. Parameter sensitivity analysis was carried out with respect to the parameters affecting the cell growth and the PHB accumulation.

6.4 Future perspectives about PHB production from cyanobacteria

The results obtained during this study have given a further understanding on the production of PHBs by cyanobacteria. Based on these results, the following experiments are suggested which could be carried out in the future (Figure 6.3):

a) Further genetic modification to improve PHB production from cyanobacteria (section 6.4.1);
b) Production of PHB from cyanobacteria using a continuous mode instead batch mode (6.4.2);
c) Design an environmental and economic recovery techniques of PHB granules from cyanobacteria (6.4.3).

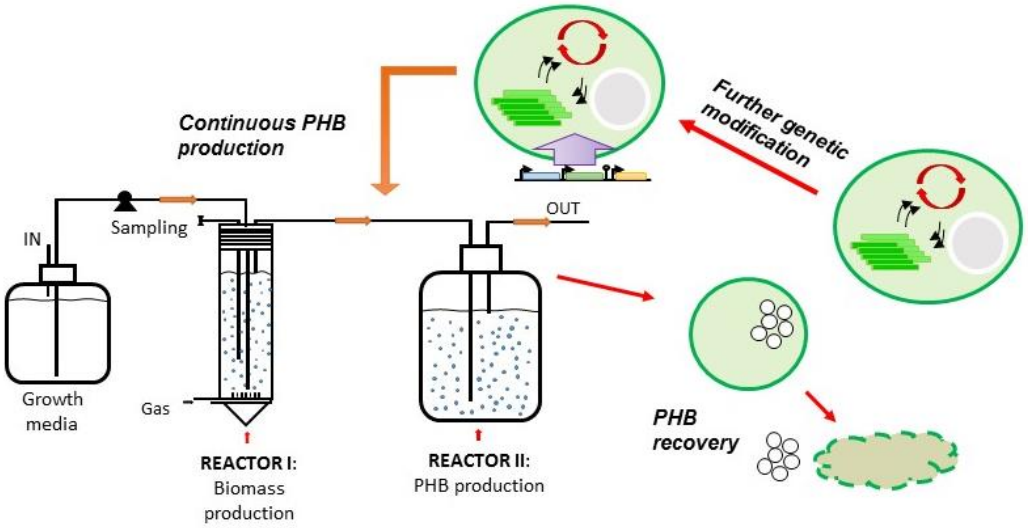

Figure 6.3: future perspectives

6.4.1 Genetic modification

Enhancement strategy of PHB production from biosources encompasses not only nutrient modification but also genetically engineered changes that enable cells to produce PHB at higher concentration/rate than its natural capacity. Only a few pathways have been investigated to improve PHB production from *Synechocystis* PCC6803. It could be useful to understand if with different genetic engineering approaches, higher amount of PHB can be obtained. The possible genetic modification are reported hereinafter:

a. *Synechocystis* sp. PCC 6803 contains two genes, phaC and phaE, of PHA synthase, which are located in the same operon (Khetkorn *et al.*, 2016). The other two genes in PHB biosynthesis pathway, phaA and phaB, were identified and the sequence analysis showed that both genes are also located in the same orientation in the chromosome (Takahashi *et al.*, 1998). Overexpression of native genes of PHA synthase pathway in *Synechocystis* PCC6803 (Khetkorn *et al.*, 2016), associated with the overexpression of phosphoketolase (*xfpk*) and the cyanobacteria growth on the optimized media ($BG_{1/2}$), could be a possible solution to improve PHB production;
b. Introduction of the PHA biosynthetic genes from other bacteria (e.g. *Ralstonia eutropha; Alcaligenes eutrophus*) (Sudesh *et al.*, 2002). Indeed, the bacterium *Ralstonia eutropha* accumulates high levels of PHB (about 80%) (Budde *et al.*, 2011a). The Gram-negative facultative chemolithoautotrophic hydrogen-oxidizing bacterium *Ralstonia eutropha* is the best-studied micro-organism with regard to the metabolism of polyhydroxyalkanoic acids (Pötter *et al.*, 2004). The transformation of *Synechocystis* PCC6803 with an exogenous PHA pathway, under optimized growth conditions, may be a good alternative to improve PHB production;
c. As shown in **Section 5.2**, PHB was used for cell functioning (Ansari & Fatma, 2016) (decrease of PHB concentration after 12 days). A genetic modification to remove the pathway involved in the utilization of PHB from cell could be designed.

6.4.2 Continuous PHB production

All tests reported in this work were carried out in batch conditions. A possible way

to implement the process of PHB production from a genetic modified strain of *Synechocystis* PCC6803 is to realize the process in continuous mode. As exhaustively described in literature, PHB production includes two easily distinguishable phases for most production strains. The first phase, a sufficient concentration of active biomass with only minor quantity of accumulated PHB is produced in a nutritionally balanced growth medium. The second phase, nutritional stress is provoked by restricting the supply of a growth-essential nutrient (e.g., nitrogen, phosphate, etc.) resulting in the deviation of the carbon flux from biomass production towards PHB accumulation. In the second phase, biomass formation is negligible if compared to PHB accumulation (Koller & Braunegg, 2015).

A potential reactor configuration could be the series of two reactors:

i. Reactor I for biomass production (optimal growth conditions);
ii. Reactor II for PHB accumulation (nitrate-starved conditions).

The advantages of this configuration are:

i. The possibility to separate the biomass growth phase from PHB production phase which are characterized by different optimal operating conditions;
ii. Minimization of equipment downtime and time loss due to the lag phase of the microbial culture (Braunegg *et al.*, 1995)
iii. The continuous cultivation can guarantee growth of microorganisms under defined nutrient limitations for extended time periods, and a long-term genetic stability of the organism provided can result in both high productivities and constant product quality (Koller & Braunegg, 2015);
iv. Harvest of biomass at a desired PHB-mass fraction;
v. The applied dilution rate D significantly impacts the molar mass of PHB (molar mass direct proportional to D) (Koyama & Doi, 1995).

6.4.3 PHB recovery

The main drawback to the commercial production and application of PHB in industry is its comparatively high cost compared with conventional plastics. The recovery of PHB granules from bacterial cytoplasm significantly increases total processing costs (Aramvash *et al.*, 2015). The lowest recovery costs were obtained with the sodium hydroxide and sulphuric acid treatments (1.02 and 1.11 € kg^{-1}, respectively) (López-Abelairas *et al.*, 2015). Efficient, economical, and environmentally friendly extraction of PHB from cells is required for its cost-effective industrial production. Several studies have proposed various recovery techniques that improve the yield and purity of extraction and reduce manufacturing costs.

Currently, most PHB solvent extraction strategies are based on halogenated organic solvents (e.g., chloroform), which are expensive, environmentally unfriendly, and may cause degradation of the biopolymer (Wang *et al.*, 2015). Thus, a simple, practical, efficient, and cost-effective system of PHB recovery using non-halogenated solvents should be developed. Different strategies for PHB recovery are reported hereinafter:

i. Recovery of PHB accumulated in cyanobacteria using ionic liquids which dissolve cyanobacteria but not PHB (Kobayashi *et al.*, 2014). Recently, ionic liquids (ILs) have attracted attention as a potential solvent for a wide area. ILs have melting point below 100 °C. They are characterized by interesting properties – e.g. high ionic conductivity and high thermal stability - which are difficult to achieve in general organic solvents (Kobayashi *et al.*, 2014). Furthermore, ILs are characterized by negligible vapour pressure. They are gathering attention as novel green solvents (Figure 6.4);

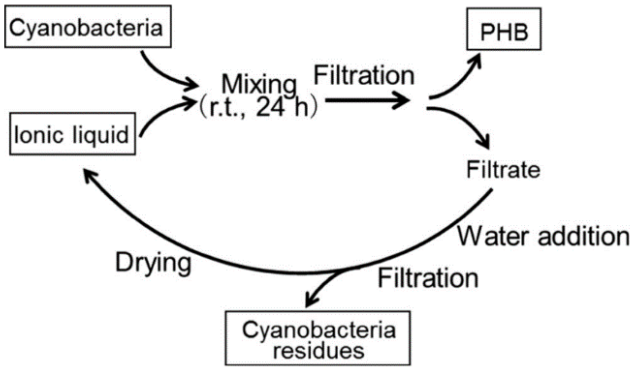

Figure 6.4: Process for the recovery of PHB accumulated in cyanobacteria using ILs (from Kobayashi *et al*. 2014)

ii. Spontaneous liberation of intracellular PHB granules using a genetic engineering approach. Indeed Jung et al. (2005) manipulating the initial inoculum size and the composition of the medium obtained an *Escherichia coli* strain that was able to produce PHB at a very high. After spontaneous cell's lysis, PHB granules was liberated into the medium (Jung *et al.*, 2005);

iii. Extraction of PHB using non-chlorinated solvents (e.g. anisole) (Rosengart *et al.*, 2015) or non-halogenated solvent (e.g. butyl acetate) (Wang *et al.*, 2015);

iv. Extraction of PHB with a solvent-free approach using enzyme digestion in an aqueous medium (Martino *et al.*, 2014). Martino et al. (2014) used biomass of *Cupriavidus necator* DSM 428 grown on used cooking oil (UCO), for extraction of the PHB granules using sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), and the enzyme Alcalase in an aqueous medium. The recovered PHB granules showed a degree of purity higher than 90% and no crystallization.

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1. **R. Carpine**, G. Olivieri, K. Hellingwerf, A. Pollio, The Cyanobacterial Route to Produce Poly- β -hydroxybutyrate, Chem. Eng. Trans. 43 (2015) 289–294.

2. **R. Carpine**, G. Olivieri, K. Hellingwerf, A. Pollio, G. Pinto, A. Marzocchella, Photoautotrophic production of poly-β-hydroxybutyrate (PHB) from cyanobacteria: nitrate effects and screening of strains, Submitted to *Environmental Engineering and Management Journal*.

3. **R. Carpine**, W. Du, G. Olivieri, A. Pollio, K. J. Hellingwerf, A. Marzocchella, F. Branco dos Santos, Genetic engineering of *Synechocystis* sp. PCC 6803 for poly-β-hydroxybutyrate overproduction, Submitted to *International Scientific Journal*.

4. **R. Carpine**, F. Raganati, G. Olivieri, K. J. Hellingwerf, A. Pollio, A. Marzocchella, Poly-β-hydroxybutyrate (PHB) production by Synechocystis PCC6803 from CO_2: Model development, Submitted to *International Scientific Journal*.

CONFERENCE PRESENTATIONS

1. **R. Carpine**, G. Olivieri, K. J. Hellingwer, A. Pollio, A. Marzocchella, The Cyanobacterial Route to Produce Poly- β –hydroxybutyrate**,** Italian Forum on Industrial Biotechnology and Bioeconomy, IFIB, 25-16 September 2014, Genoa (Italy), Poster contribution.

2. **R. Carpine**, G. Olivieri, K. J. Hellingwer, A. Pollio, A. Marzocchella, The Cyanobacterial Route to Produce Poly- β –hydroxybutyrate, International Conference on Chemical and Process Engineering, ICheaP, 19-22 May 2015, Milan (Italy), Oral Presentation.

3. **R. Carpine**, G. Olivieri, K. J. Hellingwer, A. Pollio, A. Marzocchella, Poly-β-hydroxybutyrate (PHB) Production by Cyanobacteria, European Congress on Biotechnology, ECB17, 3-6 July 2016, Cracovia (Polonia).

4. **R. Carpine**, W. Du, G. Olivieri, A. Pollio, K. J. Hellingwerf, A. Marzocchella, F. Branco dos Santos, Genetic engineering to improve poly-β-hydroxybutyrate (PHB) production by Synechocystis PCC6803 in photoautotrophic conditions, FITEMI, 6-7 April 2017, Palermo (Italy).

COLLABORATION WITH FOREIGN RESEARCH INSTITUTION

October 2015-February 2016: Research activity at University of Amsterdam-Molecular Microbial Physiology Group (The Netherlands) on the issue: **Genetic engineering of Synechocystis sp. PCC 6803 for poly-β-hydroxybutyrate overproduction**. Supervisor: Prof. Klaas J. Hellingwerf.

IMPROVERS COURSE

October 2014: Advanced School on the Present and Future of Bioenergy, Campinas, Brasil, 2014.

Attended course/seminaries

1. **Arben Markoci**: “Nanomaterials-based biosensing system for diagnostics and environment monitoring applications”. 28-04-2014
2. **Hakan Temur**: “Introduction: The challenge, universal energy carrier, potential market, boundary conditions. Biomass: energy from the sun: photosyntesys, carbon dioxide’s key role in climate change, carbon cycle, type of biomass, utilization of biomass”. 05-05-2014.
3. **Mehmet Emin Arztung**: “Molecular diffusion in liquids. Diffusion controlled mass transfer in solid-liquid systems and governing equations. Convective mass transfer on flat plate”. 05-05-2014.
4. **Mehmet Emin Arztung**: “Methods to dermine mass transfer coefficients. Mass transfer enhancement techniques: impinging jets”. 06-05-2014.
5. **Hakan Temur**: “Quality characteristics of bioenergy sources, solid bioenergy products, liquid bioenergy products, gaseous bioenergy products, possible technical uses, anaerobic digestion project retaliation, commissioning and start-up, operation and maintenance, economics”. 06-05-2014.

6. **Telma Teixeira Franco**: “The differences of bioreactor performance fed with carbon sources and the PBRs”. 12-06-2014.
7. **Roberto Lauri**: “Aspetti di sicurezza relative a processi industriali finalizzati alla produzione di biocombustibili e bioplastiche”. 20-01-2015.
8. **Biancamaria Pietrangeli**: “Biotecnologie per lo sviluppo sostenibile: applicazioni e sicurezza”. 20-01-2015.
9. **Piero Salatino**: “Fluidizzazione di solidi granulari e mobilità di flussi piroclastici densi”. 29-01-2015.
10. **Angelo Fierro**: “Il Grandfallon del bioetanolo di seconda generazione: il caso studio per la Regione Campania”. 11-02-2015.
11. **Alfredo Ronca**: “Bioactive composite scaffolds for bone regeneration: from the process to the biological validation”. 25-03-2014.
12. **Peter Gotz**: “Microbial production of polysaccharides as feed additives”. 16-04-2015.
13. Bennet Parker: “Structuring and writing manuscripts for publication in scholary journals”. 15/16/17-06-2015.
14. **Raffaele Porta**: “Le transglutaminase: dalle poliammine alle bioplastiche”. 30-06-2015.
15. **Blanca Garcìa**: “Microbial study of Listeria monocytogeness biofilms formation, biofouling and their control”. 09-07-2015.
16. **Carlos Regaldo**: “Edible films and coatings to increase shelf life of fresh foods”. 09-07-2015.
17. **Lars Rehmann**: “Fermentativebutanol production from unconventional resources”. 14-06-2016.
18. Angelo Fontana: “Research and exploitation of marine genetic resources from eco-physiology to biotech”. 13-07-2016.
19. **Tomaso Zambelli**: “Development of the FluidFM and its applications for 2D patterning as well as 3D microprinting”. 17-10-2016.
20. **Tomaso Zambelli**: “FluidFM for single cell manipulation”. 18-10-2016.
21. **Edgardo Filippone**: “Dalle piante alle microalghe, il mondo biotec si tinge di verde (e non solo)”. 26-10-2016.
22. **Dyonysios Dionysiou**: “Treatment of cyanotoxins and contaminates of emerging concern in water using advanced oxidation process”. 27-10-16.
23. **Tomas Morosinotto**: “Algae: metabolic engineering for the sustainable production of bio-commodities”. 06-12-16.

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