UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI INGEGNERIA INDUSTRIALE

Corso di Laurea Magistrale in Ingegneria Chimica e dei Processi Industriali

Tesi di Laurea Magistrale in
Ingegneria Chimica e dei Processi Industriali

A microalgae based wastewater treatment suitable for biopolymer production: polyhydroxyalkanoates and cyanophycin

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ANNO ACCADEMICO 2016 – 2017

Alla mia famiglia

Abstract

The aim of this thesis is to evaluate the possibility to apply microalgae and cyanobacteria for the production of valuable compounds, based on wastewater exploitation. Different strategies are explored. A first process is investigated, where microalgae are cultured in the liquid obtained from the acidogenic fermentation of primary sludge, with the aim to remove nutrients, nitrogen and phosphorus, without reducing the COD content. This is mainly made of volatile fatty acids and is exploited for the production of polyhydroxyalkanoates by mixed cultures of PHA-storing biomass. A second process considers the cultivation of cyanobacteria in wastewater to produce directly polyhydroxyalkanoates. A screening of different microalgal and cyanobacterial species is performed. The influence of light, CO_2 insufflation and temperature on the growth of the selected microorganism is assessed. Nutrient consumption is measured as well. Particular attention is given to polyhydroxyalkanoates and cyanophycin production and the protocols for the extraction and quantification of these components from cyanobacteria are developed. Finally, a preliminary analysis on possible process schemes for the production of polyhydroxyalkanoates is carried out.

Contents

Introduction

Nowadays, fossil carbon (coal, oil, gas) is the major source of fuels as well as a building block for the production of chemicals and materials. Increased wealth, changed habits, and excessive consumption drastically increased the per capita use of energy and materials, so that fossil carbon is currently the dominant source of both energy and materials on which our society depends. However, fossil fuels are going to be depleted, making it necessary to explore other energy options. The use of biomass as source of materials and fuels has been reconsidered, although it is well known that in the old days (ninetieth century), overuse of biomass led to deforestation, soil degeneration, and desertification. For this reason, one of the most promising possibilities is the production of biomass by microorganisms, specifically microalgae and cyanobacteria, which have several advantages over traditional crops, including their lack of the requirement for arable land, their capacity to create biomass rich in protein, carbohydrate and lipids, their high growth rate and their capacity to create high value products servicing a number of markets (foods, fuels, nutraceuticals and plastics). Moreover, the cultivation of microalgae in wastewater offers the unique opportunity to achieve simultaneously nutrient removal and production of a high value algal biomass. For this reason phycoremediation, using waste streams as a source of secondary resource, is a key element within a circular economy concept.

In this work, the application of microalgae and cyanobacteria in the production process of polyhydroxyalkanoates integrated with wastewater treatment was evaluated.

In Chapter 1 the state of the art with information related to polyhydroxyalkanoates production are reported, while Chapter 2 details materials and methods used for the experimental investigations.

In Chapter 3 the experimental results are shown and discussed. A screening of different microalgal and cyanobacterial species was carried out. Subsequently the effect of light, CO_2, insufflation and temperature on the growth of selected species and on nutrient consumption was evaluated. Finally, the protocols for the extraction and the quantification of polyhydroxyalkanoates and cyanophycin from cyanobacteria has been improved.

In Chapter 4 with the support of mass and energy balances, a preliminary assessment on the process was performed.

I would like to thank dott. Nicola Frison, researcher at the University of Verona, for supplying wastewater and knowledge about PHA production process.

Chapter 1

State of the art

In this chapter the microorganism used are first presented, then a quick overview on the conventional wastewater treatment and on the application of microalgae in this process are reported. The most common cultivation systems of microalgae are shown as well. After a general introduction on renewable resources, particular attention to the production of biopolymers, especially cyanophycin and polyhydroxyalkanoates, is paid. Finally, a process for the production of polyhydroxyalkanoates is explained and the aim of the thesis is specified.

1.1 Microorganisms

Microorganisms can be grouped according to the carbon and energy sources necessary for their metabolism. *Chemotrophs* are the microorganisms that oxidize chemical compounds to gain energy, instead *phototrophs* use light as energy sources. Microorganisms that use organic compounds as their major carbon source are called *heterotrophs*, while microorganisms that use inorganic carbon, such as CO_2 or bicarbonates, are called *autotrophs*. Combining the carbon source with energy source, it results a classification in four categories, which are shown in *Table 1.1*.

Table 1.1 *Nutritional types of microorganism (adapted from Mara and Horan., 2003)*

Nutritional types	Energy source	Carbon source	Examples of microorganisms
Chemoautotrophs	inorganic compounds	inorganic compounds	Nitrifying, hydrogen, iron and sulphur bacteria
Chemoheterotrophs	organic compounds	organic compounds	Most bacteria, fungi, protozoa and animals
Photoautotrophs	light	inorganic compounds	Purple and green sulphur bacteria; algae; plants; cyanobacteria
Photoheterotrophs	light	organic compounds	Purple and green non sulphur bacteria

The growth of microorganisms in batch cultures occurs in five main phases, as shown in *Figure 1.1*:

1. phase of latency (or lag phase): at this stage, there is a rapid consumption of nutrients due to the fast microorganism metabolism, followed by a slow increase in the number

of cells. It is a phase of acclimation of the culture to the new conditions in which it is set to grow;

2. first exponential phase: at this stage the culture begins to exhibit a certain acclimation to the experimental conditions, which allows the cells to exit the latency phase;
3. exponential phase (or log phase): this phase is characterized by an active cell proliferation and the growth rate is exponentially dependent on growth conditions (nutrients, light, temperature);
4. stationary phase: during this phase, there is no significant increase in the cells number due to the starvation of nutrients or light;
5. phase of cell death: at this stage, the vitality of the cells is compromised. Furthermore, the number of cell contributes to inhibit further growth.

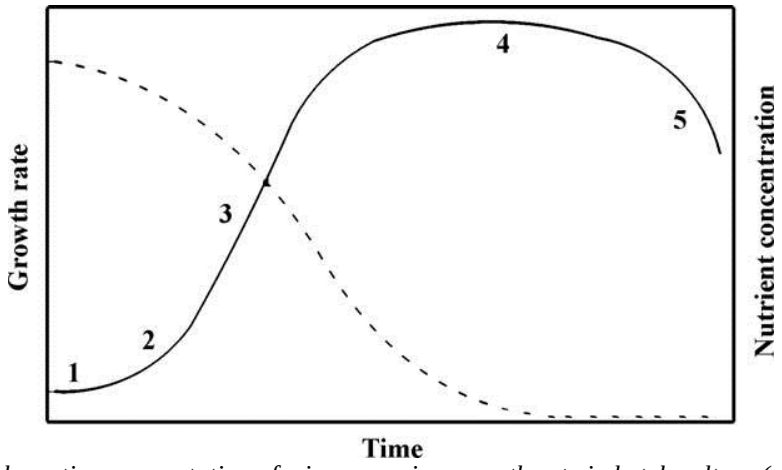


Figure 1.1 *Schematic representation of microorganism growth rate in batch culture (solid line) and nutrient concentration (dashed line) (Mata et al., 2010)*

1.1.1 Microalgae

Microalgae are eukaryotic unicellular microorganisms, with a size ranging from 1 μm to 2 mm. Species of diatoms, dinoflagellates and green flagellates are included in this category (Subashchandrabose et al., 2011). Microalgae may differ for morphology (e.g. motile and non-motile unicells and colonies, unbranched and branched filaments), cytology (e.g. eyespot presence or absence, cell wall structure, flagella number and length) and for their reproduction characteristics (e.g. type of sexual life cycle, morphology of asexual spores). They are generally classified according to their colour/pigments, storage products, cell covering and organelles, but taxonomy is under continuous revision based on evolutionary and genetic information. Classes of microalgae commonly employed for environmental purposes are *Chlorophyceae* (green algae), *Euglenophyceae* (euglenoids), *Bacillariophyceae* (diatoms) and *Cyanophyceae* (Valigore, 2011). This last class refers to cyanobacteria, a phylum of bacteria (prokaryotic) used to be called and classified in the past as “blue-green algae”. Depending on the environmental conditions, microalgae may

assume different types of metabolism: autotrophic, heterotrophic and mixotrophic (i.e. performing photosynthesis as the main energy source, though both organic compounds and CO_2 are essential). *Chlorella vulgaris*, *Haematococccus pluvialis*, *Arthtospira platensis* are examples of strains found to grow under all of these three conditions (Mata et al., 2010).
Several factors influence algal growth: abiotic ones such as light (spectrum and intensity), temperature, nutrient concentration, O_2, CO_2, pH, salinity, and toxic chemicals; biotic factors such as pathogens (bacteria, fungi, viruses) and competition from other algae; operational factors such as shear produced by mixing, dilution rate, depth, harvest frequency. After light availability, temperature is the most important limiting factor for culturing microalgae in both open and closed reactors. Salinity affects the growth and the cells composition. Indeed, each species of algae has a different optimum salinity range. In open reactors, salinity could increase during hot weather condition due to high evaporation. In any case mixing is essential to promote the fast circulation of microalgae cells from dark to light zone of the reactor, to homogenize the distributions of cells and to facilitate the transfer of gases. It is however necessary to find an optimum to prevent shear stress from damaging the cells (Mata et al., 2010).

1.1.2 Cyanobacteria

Cyanobacteria ("blue-green algae") are Gram-negative prokaryotes classified on the base of their morphology. They are an ancient (occurring as long ago as 3500 million years) and diverse group of microorganisms that can exist in a wide range of habitats, including those that are considered extreme, such as frozen lakes, hot springs and salt works. Under phosphorus and nitrogen rich conditions, cyanobacteria are able to uptake and then intracellularly store these components. This property has evolved over several billion years to allow them to exploit extreme environmental conditions. Generally, cyanobacteria cells are larger than most of other bacteria, ranging from 1 µm for unicellular types to over 30 µm for multicellular species. Three basic morphological forms are described: unicellular, filamentous with heterocysts and filamentous without heterocysts. Heterocysts are differentiated and specialized cells that can fix nitrogen, thus allowing survival in a low nitrogen concentration environment. Cyanobacteria are mainly photoautotrophic microorganisms, thus producing oxygen and sequestering carbon dioxide. Some of them show also the capability to perform nitrogen fixation. The process of nitrogen fixation is inhibited by oxygen, and cyanobacteria deal with this problem by effectively separating the processes, both spatially (role of heterocysts) and temporally (fixing carbon during day, nitrogen during night).
Cyanobacterial growth is promoted by the presence of specific trace metals, such as iron, which is considered to enhance both photosynthesis and nitrogen fixation, and molybdenum, which seems to increase the rate of carbon fixation. On the other hand, some

cyanobacteria produce a range of cyanotoxins as neurotoxins, hepatotoxins, tumourogenic toxins and toxins that are irritants affecting the skin and gastrointestinal tract. These toxins are produced intracellularly and are then released into the environment as a consequence of cells lyse. Finally, cyanobacteria can grow prolifically under suitable conditions, thus generating so called "algal blooms" in eutrophic freshwater lakes and reservoirs (Percival and Williams, 2014) (*Figure 1.2*).

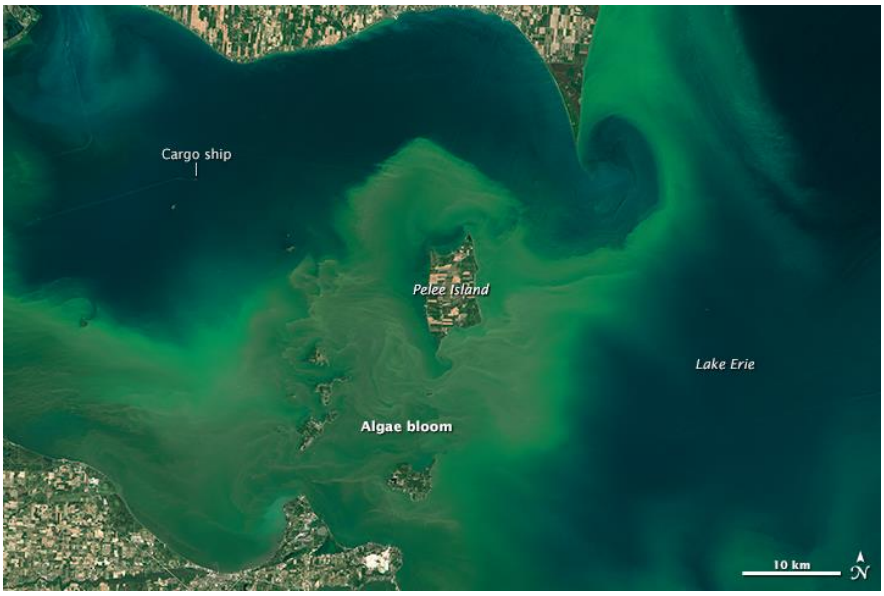


Figure 1.2 *Satellite view of western Lake Erie's algal bloom (USA), July 28, 2015 Credit: NASA/Landsat-8*

1.1.3 Bacteria

Bacteria are prokaryotic microorganism surrounded by a rigid cell wall. Bacteria exists as unicellular, pairs, chains and cluster and they can be motile or non-motile. Except for filamentous forms, cells have a diameter of 1-2 µm (Mara and Horan, 2003). Mostly three shapes are observed, i.e. spheres (*cocci*), spirals (*spirilla*) and rods (*bacilli*). Under optimal growth conditions, some bacteria can double their population through asexual reproduction in less than 30 min, which makes them the fastest growing organisms known. Bacteria present in wastewater are usually chemoheterotrophs and the phyla mostly present are *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* (Unnithan et al., 2014), including *Zooglea*, *Pseudomonas*, *Chromobacter*, *Achromobacter*, *Alcaligenes* and *Flavobacterium* (Mara and Horan, 2003).

1.2 Conventional wastewater treatment

Wastewater from domestic, industrial and agricultural processes has been considered a serious problem for a long time, but in the past few years, a new way of looks at wastewaters arose, as they could be no more seen as a problem but as a source of energy and other precious resources, including water itself.

Usually, wastewaters are classified based on their sources, and the most common types are:

- domestic wastewaters, which are discharged from residences, services, commercial facilities and similar activities;
- industrial wastewaters;
- infiltration/inflow waters, which enter directly or indirectly the collection system through leaking joints, cracks and breaks;
- stormwater, which involves runoff resulting from rainfall and snowmelt.

Industrial wastewaters have to be specifically treated before entering in conventional wastewater treatment plants. Runoff can be collected into a separate collection system with respect to the first three types of wastewater but also in a combined collection one. In both cases, meteorological conditions and the period of the year influence the composition of wastewater (Metcalf and Eddy, 2003). Water should undergo different treatment steps before being discharged into the environment:

- preliminary treatments remove all materials that can be easily collected from the raw sewage before they damage or clog the pumps and other downstream process units. Objects commonly removed during pre-treatment include trash, tree limbs, leaves, branches, and other large objects. Pre-treatment may include a sand or grit channel or chamber, where the velocity of the incoming sewage is adjusted to allow the settlement of sand, grit, stones, and broken glass. Fat and grease are removed by passing the sewage through a small tank where skimmers collect the fat floating on the surface;
- primary treatments remove part of the suspended solids and organic matter from the sewage. This step could be enhanced by chemical addition or filtration;
- secondary treatments remove biodegradable organic matter and suspended solids. Disinfection and nutrient removal could be performed in this step;
- tertiary treatments process provide a final treatment stage to further improve the effluent quality before it is discharged to the receiving environment;
- advanced treatments are generally based on technologically complex techniques, such as chemical precipitation, ozonation, reverse osmosis or carbon adsorption.

In the biological wastewater treatment the biodegradable constituent are transformed through oxidation into simple end products and biomass, and nutrients such as nitrogen and phosphorus are removed. Many microorganisms, especially bacteria, perform these reactions, assimilating nutrients that are necessary for their metabolism according to the following equation (Metcalf and Eddy, 2014)

$$\nu_1 organic\ matter + \nu_2 O_2 + \nu_3 NH_3 + \nu_4 PO_4^{--} \xrightarrow{microorganism} \nu_5 new\ cells + \nu_6 CO_2 + \nu_7 H_2O \qquad (1.1)$$

where ν_i are the stoichiometric coefficient. As a result of the oxidation process, microorganisms convert organic matters into new biomass (new cells). Removal of

inorganic nitrogen is accomplished in a two step process. First, ammonia is oxidized to nitrites and nitrates by two different autotrophic bacteria, *ammonia-oxidizing bacteria* (AOB) and *nitrite-oxidizing bacteria* (NOB). Afterwards, nitrites and nitrates are reduced biologically into molecular nitrogen by heterotrophic bacteria under anoxic conditions. Moreover, some bacteria have the capability to uptake and store large amount of phosphorus, thus contributing to the reduction of this element in the wastewater.

There are two main categories of biological processes used for wastewater treatment, attached growth systems and suspended growth processes. In the first one, the microorganism are attached to an inert packing material, named biofilm. In this layer, the degradation of organic matter and the removal of nutrients take place. The packing material could be completely submerged or partially submerged. In the suspended growth processes, the microorganisms are maintained in suspension in the medium through continuous mixing. Activated sludge process is the most common of the suspended systems for municipal wastewater treatment (*Figure 1.3*).

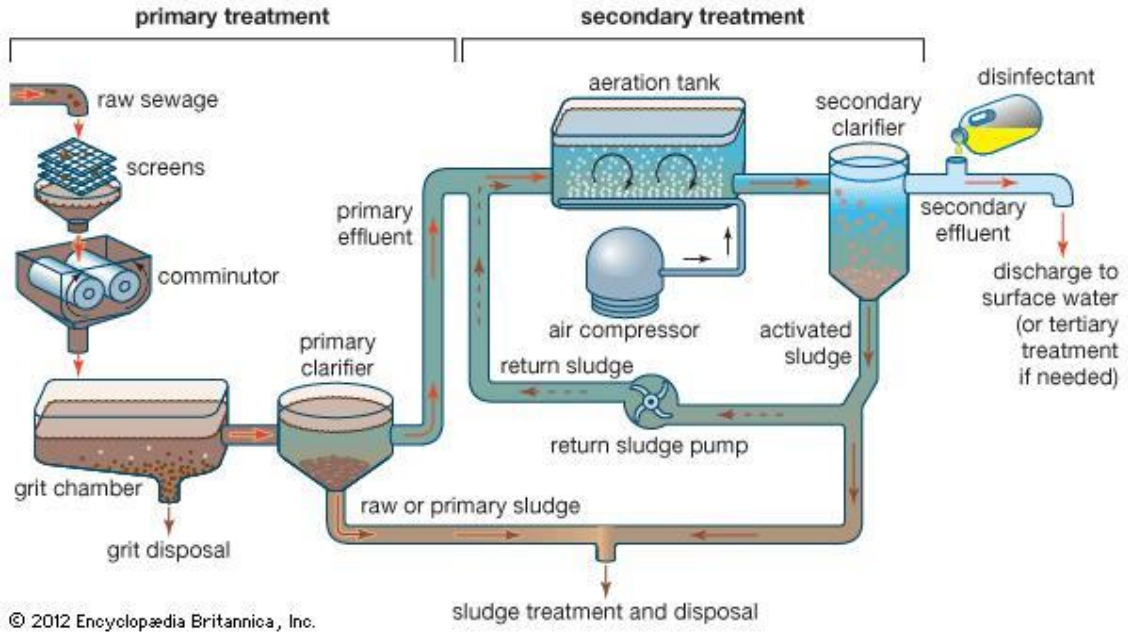


Figure 1.3 *Schematic of a typical activated sludge wastewater treatment plant*

It consists in the production of an activated mass of microorganisms which biodegrade organic material under aerobic conditions. The tank where the process takes place is continuously aerated and mixed, in order to ensure the homogeneous distribution of oxygen ant to avoid the sedimentation of the biomass. Then, the mixed liquor flows to a clarifier where the microbial suspension is settled and eventually partially recirculated back to the aeration tank, in order to increase the concentration of biomass. The settled biomass is named activated sludge. The main drawback of conventional biological processes is the

huge amount of oxygen that must be provided to microorganisms to allow the biological oxidation of organic matter. Moreover, the conventional treatment process generates large amounts of sludge, whose handling and disposal is one of the largest bottlenecks of the technology (Rawat et al., 2016). However, the nutrient and organic loads in the sludge treatment line are typically high, and side-stream treatment is an attractive option to avoid recycling these nutrients to the main treatment line (Frison et al., 2015).

1.3 Wastewater treatment with microalgae

Wastewater bio-treatment with microalgae is particularly attractive because of their photosynthetic capabilities, converting solar energy into useful biomass and incorporating nutrients such as nitrogen and phosphorus, which are the main causes of eutrophication in water bodies. Nitrogen is an essential macronutrient for the growth of microalgae, as it is required for the synthesis of peptides, proteins, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). In wastewater, inorganic nitrogen is present in the form of nitrite, nitrate and ammonium. Microalgae prefer ammonium because it is easily converted to the amino acid glutamine without any redox reaction, and thus requiring less cell energy, but they are able to exploit also nitrate. Phosphorus is present in wastewater due to human activities, particularly because of the use of fertilizers in agriculture. It is in the form of phosphates such as orthophosphate, polyphosphate and organic phosphates. Microalgae are able to take up these essential macronutrients and use them in the synthesis of various compounds such as nucleic acids, phospholipids and proteins via phosphorylation. Indeed, microalgae have the advantage of removing nutrients by storing them in the biomass. Thus, in comparison to conventional wastewater treatment, where nitrogen is finally converted to N_2 and released to atmosphere, this nutrient can be accumulated in microalgal biomass and then possibly recycled. A similar situation occurs for phosphorus. Moreover, many species of microalgae and cyanobacteria show the capability to remove heavy metals from aqueous solutions. Consequently, the ability of wastewater treatment systems to remove these compounds is of great interest (Abdel-Raouf et al., 2012). Other several advantages of microalgae wastewater treatment include a smaller sludge formation, lower energy requirements, the reduced production of greenhouse gases, lower costs and the concurrent production of energy-rich algal biomass which can be processes to obtain biofuels, bio-fertilizers, biopolymers, lubricants, paints, nutraceuticals, dyes and colourants. Specifically, microalgal biomass is a commercial source of bioenergy (bioethanol, biodiesel, biomethane, etc.), of feed for aquaculture and poultry, and of high-value products such as β-carotene, astaxanthin, Omega 6 (arachidonic acid) and Omega 3 (docosahexaenoic acid, eicosapentaenoic acid) fatty acids and phycobilin. As well, microalgae can accumulate large amount of carbohydrates in the form of starch, glucose, cellulose, hemicellulose and various kind of polysaccharides. These are suitable for biofuel,

especially bioethanol, production. On the other hand, high lipids accumulation is essential for the production of biodiesel. Hence, the cultivation of algae on nutrient-rich wastewater presents a unique opportunity to achieve simultaneously nutrient removal and production of high value algal biomass (Rawat et al., 2016).

1.4 Cultivation systems of microalgae

There are two types of systems to cultivate microalgae, open and closed photobioreactors. The main challenges in any bioreactor for photosynthetic organism are light availability, CO_2 introduction, O_2 removal and sufficient mixing. In the following the main characteristic of the two technologies along with advantages and disadvantages are given.

1.4.1 Open cultivation systems

Open systems are reactors open to the environment. They can be divided into open vessels, natural water bodies, cascade systems and raceway ponds (*Figure 1.4*). Mechanical mixing is necessary to prevent biomass settling. These reactors have many disadvantages, including the lack of good monitoring and control systems for parameters like pH, temperature, mixing and light availability. Seasonal variations make it impossible to obtain replicable data. In addition, high water losses due to evaporation occur. Finally, one of the main drawbacks is contamination from different microorganisms present in the environment, which can lead to poor productivity. The advantages include the lower investment costs and easier maintenance with respect to closed photobioreactors (Drosg et al., 2015).

(a) *(b)*

Figure 1.4 *(a) Open cascade system (Drosg et al., 2015) (b) Raceway ponds*

1.4.2 Closed cultivation systems

There exist several types of closed photobioreactors, such as tubular reactors, laminar reactors, hanging plastic sleeves or fermenter-like tank reactors (*Figure 1.5*). Closed systems are much more expensive to build and difficult to operate and scale up, but crucial operating variables could be better controlled. Compared to open systems, they have higher

productivity, higher photosynthetic efficiencies and reduced contamination risks (Drosg et al., 2015). Usually, they are not suitable for wastewater treatment applications due to the fouling problem, and are mostly employed in the production of algal biomass for commercial purposes, since the maintenance of axenic culture is facilitated.

(a) *(b)*

Figure 1.5 *(a) Hanging plastic sleeves (b) Tubular photobioreactor at pilot-scale (Drosg et al., 2015)*

1.5 Renewable resources and biopolymer production

The term biomass means any organic matter that is available on a renewable or recurring basis, including dedicated energy crops and trees, agricultural food and feed crop residues, aquatic plants, wood and wood residues, animal wastes and other waste materials. The technical challenge of the exploitation of such renewable feedstock is the development of low energy, non-toxic and environmentally friendly pathways to convert the biomass to useful chemicals and fuels. In particular, in terms of carbon footprint, this process should not generate more carbon than is being removed from the atmosphere. The difference between C_{in} removed from the air, and C_{out} generated from the energy used, is defined as the carbon footprint ΔC. Ideally, by design all carbon footprints should be positive such that C_{in} is greater than C_{out}. This leads in a natural way to the reduction of global warming gasses, affecting current climate change. Advantages of using renewable resources are:

- to extend the lifetime of available crude oil;
- to mitigate the build up of greenhouse CO_2 in the atmosphere;
- feedstock supplies are domestic;
- feedstock is flexible, non-toxic, sustainable;
- to obtain oxygenated building blocks that avoid the oxygenation process, which usually involve toxic reagents.

Disadvantages include current economic circumstances (cost of products), seasonal supply, the requirement of space to growth, the wide range of materials to deal with and the ethical questions on the use of food resources for chemicals production. Significant advances have been made in the development of fuels, chemicals and materials totally or partially obtained from renewable feedstocks. For example, they include biodiesel from plant oils and algae,

bioethanol and butanol from sugars and lignocellulose, polymers, foams and thermosets from lignin and plant oils, lubricants, fibres and composites, solvents, specialty chemicals and surfactants, agrochemicals and pharmaceuticals. To determine the biobased content of solid, liquid and gaseous samples a standard test method using radiocarbon analysis can be applied (ASTM D6866). Polymers produced from renewable resources could be completely or only partially bio-based. Generally, the biodegradability is improved, but not always, the biopolymers are biodegradable. Whether a plastic is biomass or petroleum-based it is of a different question than whether it will biodegrade. In nature, all organic substances will eventually biodegrade given the conditions of time, temperature, moisture and oxygen. Times range from a couple of weeks to thousands of years. Biodegradable polymers need between 6 months and 2 years to biodegrade, instead conventional plastics like polystyrene (PS) and polyethylene (PE) need approximatively between 500-1000 years to degrade. On the other hand, compostable materials, regulated by ASTM D6400/EN13432 standards, undergo degradation by biological process during composting to yield CO_2, H_2O, inorganic compounds and biomass at a rate consistent with other known material (paper, grass, food scrapes). Furthermore, they do not leave any fragments in the residue, do not contain any heavy metals or toxins and will support plant life. A product that is compostable, must be biodegradable. However, the reverse logic is not necessarily true.

A wide range of feedstock is available to produce biopolymers. Bio-based plastics are mainly derived from sugar, starch, corn, wheat, sugarcane, potato, sugar beet, rice, plant oil, etc., the so-called first generation feedstock. Because of potential competition with food and animal feed, there have been advancements over the years in using lignocellulose feedstocks, that are not suitable for food or feed (i.e. wood, short-rotation crops, wheat straw, bagasse, corncobs, palmfruit bunches, switch grass). It can be either non-food crops (e.g. cellulose) or waste materials from first generation feedstock (e.g. waste vegetable oil). Starch plastics are one of the most important polymers in the bio-based polymer market. They have relatively poor physical properties that can be improved by the addition of various plasticizers or copolymers. The diversity of formulations developed different products as packaging film and foams, plant pots, tableware, toys. Actually, they are partially bio-based but fully biodegradable/compostable. In the second generation, the fossil based copolymers (polyvinyl alcohol, polycaprolactone, etc.) are to be replaced by renewable polymers (polylactic acid, polyhydroxyalkanoates, etc.) to obtain fully bio-based polymers.

One of the possibilities to avoid ethical conflicts and dodge a disadvantageous development on the resources market is the production of biomass by microorganisms, such as bacteria, microalgae and cyanobacteria. Bacteria efficiently convert different carbon sources into a diverse range of polymers with varying chemical and material properties. Although bacteria

synthesize only a few intracellular polymers, they can produce a wide range of extracellular polymeric compounds (Rehm, 2010).

Microalgae contain similar raw materials as traditional crops: high-quality oils, proteins, pigments as well as hydrocarbons and sugars. Culturing microalgae has several advantages over conventional farming such as high yields and the ability to grow in a range of environments. Algae do not need agricultural land therefore there is no competition for food or farmland. Moreover, the use of algae opens the possibility of utilizing CO_2, thus neutralizing greenhouse gas emissions from factories or power plants (Bugnicourt et al., 2014).

Table 1.2 shows some of the microbial biopolymers with their main characteristics. In the following sections, the main features of two biopolymers, cyanophycin and polyhydroxyalkanoates, are briefly discussed.

Table 1.2 *Some microbial polymers with their characteristic (adapted from Rehm, 2010)*

Polymer	**Main component**	**Producer**	**Industrial applications**
Alginate	Mannuronic acid and guluronic acid	*Pseudomonas* sp. *Azotobacter* sp.	Biomaterial (drug delivery)
Xanthan	Glucose, mannose and glucuronate	*Xanthomonas* sp.	Food additive
Dextran	Glucose	*Leuconostoc* sp *Streptococcus* sp.	Blood plasma extender and chromatography media
Cellulose	D-glucose	*Alphaproteo-bacteria* *Betaproteo-bacteria* *Gammaproteo-bacteria* *Gram-positive bacteria*	Food, diaphragms of acoustic transducers and wound dressing
Hyaluronic acid (HA)	Glucuronate and N-acetyl glucosamine	*Streptococcus* sp. *Pasteurella multocida*	Cosmetics, tissue repair and drug delivery
Cyanophycin granule polypeptide (CGP)	Aspartate and arginine	*Cyanobacteria* *Bacteria*	Dispersant, water softener
Polyhydroxyalkanoates (PHA)	(R)-3-hydroxy fatty acids	*Gram-negative bacteria* *Gram-positive bacteria* *Archea* *Cyanobacteria*	Bioplastic, drug delivery, medical applications

1.5.1 Cyanophycin

Cyanophycin or multi-L-arginyl-poly (L-aspartic acid) is a non-protein, non-ribosomally produced amino acid copolymer, composed of equimolar amounts of aspartic acid and arginine. It consists of a polyaspartic acid backbone and arginine residues, which are linked to the β-carboxyl group of each aspartic acid by their α-amino groups (*Figure 1.6*). The

molecular masses of the polymer range from 25 to 100 kDa (Allen and Smith, 1969). In water it is soluble under acidic (pH<2) or alkaline (pH>9) conditions, and insoluble at physiological pH (Lang et al., 1972; Allen and Weathers, 1980). Cyanophycin was first discovered by the Italian botanist Borzi in cyanobacteria, and initially it was described as a polymer that exclusively occurs in cyanobacteria (Borzi, 1887). This polypeptide is synthetized by several species of cyanobacteria, but has also been found in few heterotrophic bacteria, like *Acinetobacter sp*., *Bordetella bronchiseptica*, *Clostridium botulinum*, *Desulfitobacterium hafniense* (Aravind et al., 2016). Cyanophycin is produced under conditions of imbalanced growth, where nutrient starvation (e.g. phosphate or sulfate) occurs, together with adverse light intensities and low temperature (Simon, 1973; Obst et al., 2004). It is present as insoluble inclusions (optically opaque granules) in the cytoplasm and is thus commonly also referred to as the Cyanophycin Granule Polypeptide (CGP) (Lang et al., 1972; Allen and Weathers, 1980). It serves as an intracellular energy reservoir within cyanobacteria under nutrient limitations, hence a storage compound for carbon, nitrogen and energy (Simon and Weathers, 1976). CGP content is low in exponentially growing cells, but increases during stationary phase to amounts of 8-18% (w/w) of the cell dry mass (Aravind et al., 2016). In *Synechocystis sp*. PCC 6803 Trauttman et al. (2016) obtained 18 g of cyanophycin per g cell dry mass as a result of phosphate starvation. The synthesis of cyanophycin is directed by cyanophycin synthetase cphA that was identified in 44 prokaryotes by genetic analysis over the genomic sequences of 570 strains. Among them, different cphA genes have been cloned from *Anabaena variabilis*, *Synechocystis* species and *Acinetobacter baylyi*, and, by gene transfer, cyanophycin synthesis was introduced into various heterotrophic microorganisms like *E. coli*, *Ralstonia eutropha*, *P. putida*, yeast, etc. so that they accumulate cyanophycin in their cells up to 50% of cell dry mass (Aravind et al., 2016).

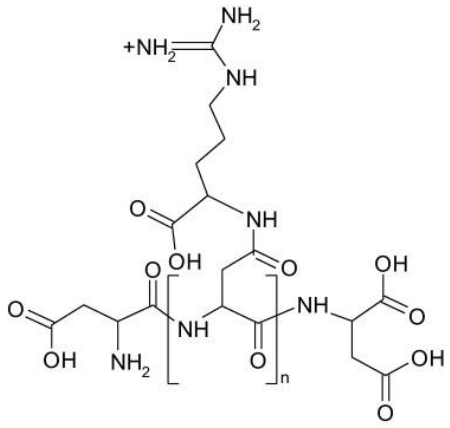


Figure 1.6 *Structure of cyanophycin*

The industrial application of cyanophycin is not present right now; however, it serves as a starting point for the synthesis of many other important chemicals. This is why cyanophycin

has recently attracted the attention of the scientific community as a biodegradable replacement for petrochemical-based industrial products. Cyanophycin can be hydrolysed to its constituent, amino acids, arginine and aspartic acid, to a derivative with reduced arginine content or even to poly(aspartic acid) (PASP). There are three main methods for the industrial production of PASP: thermal condensation, or catalysed polymerization, of aspartic acid, and thermal polymerization of maleic acid and ammonium hydroxide. PASP has numerous applications. As substitute for the polyacrylic acid, it is used as corrosion inhibitor in water treatment plants and low-temperature cooling tower. Due to its ability to chelate metal ions, it provides corrosion inhibition (Hasson et al., 2011). CGP's poly (aspartic acid) is used as material for hydrogels (Solaiman et al. 2011). As well it may act as a super-swelling material in diapers, feminine hygiene product and food packaging. It is used also as biodegradable detergent and dispersant for various applications (Hasson et al., 2011). Polyaspartates (PAA) are condensation polymers based on aspartic acid that would be alternatives to polycarboxylates. *Figure 1.7* shows how the reduction of PAA would lead to products such as 3-aminotetrahydrofuran and 2-amino-1,4-butanediol, close analogs of high volume chemicals currently used in industry, while arginine could be converted to 1,4-butanediamine used to synthetize nylon-4,6 with adipic acid as co-monomer.

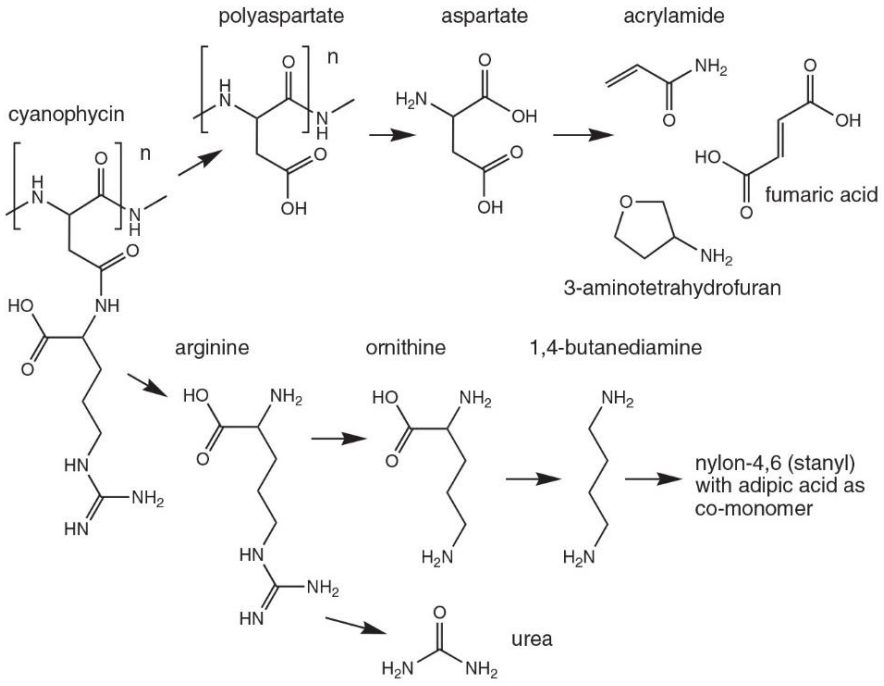
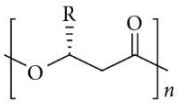


Figure 1.7 *Structure of cyanophycin and potential products derived from cyanophycin (van Beilen and Poirier 2008)*

Finally, dipeptides derived from CGP are valuable products useful in nutritional and biomedical applications. For example, arginine has numerous physiological roles in many cardiovascular, gastrointestinal and immune disorders (Sallam and Steinbüchel, 2010).

1.5.2 Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates is a family of naturally-occurring biodegradable biopolyester synthetized by various microorganism. They are produced in response to conditions of physiological stress and promote long-term survival of microorganism under nutrient-starvation conditions by acting as carbon and energy reserves. Depending on the total number of carbon atoms within a single monomer, PHA can be classified as short-chain length PHA (scl-PHA; 3-5 carbon atoms), medium-chain lentgh PHA (mcl-PHA; 6-14 carbon atoms) or long-chain length PHA (lcl-PHA; 15 or more carbon atoms). The most widespread and best characterized member of PHA is the poly-3-hydroxybutyrate (PHB) which is a homopolymer of 3-hydroxybutyrate. It was first discovered in bacteria by Maurice Lemoigne in 1925 (Bugnicourt et al., 2014). *Figure 1.8* shows the general structure of PHA and lists some examples of their structural derivatives.



Poly(3-hydroxyalkanoates) [PHA]

R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure 1.8 *General structure of polyhydroxyalkanoates (PHA) and examples of their structural derivatives*

The PHA bioaccumulation is widespread among the bacterial and archaeal domains with more than 70 genera of PHA-producing microbes. Bioaccumulated PHA is stored in the form of intracellular lipid granules. These microorganism produces PHA through different carbon catabolic pathways coupled with PHA anabolic pathways (*Figure 1.9*), thus successfully producing PHA from different carbon sources that includes saccharides (e.g. fructose, maltose, lactose, xylose, arabinose, etc.), n-alkanes (e.g. hexane, octane, dodecane, etc.), n-alkanoic acids (.g. acetic acid, propionic acid, butyric acid, valeric acid, lauric acid, oleic acid, etc.), n-alcohols (methanol, ethanol, octanol, glycerol, etc.) and gases (e.g. methane and carbon dioxide). Also wastestreams like waste frying oil, vinegar waste,

waste fat, food waste, agricultural waste, domestic wastewater, plant oil mill effluent, crude glycerol from biodiesel production, plastic waste, landfill gas, etc. are sources of carbon for the production of PHA (Amy Tan et al., 2014).

1.5.2.1 Production of PHA by Gram-negative bacteria

Most PHA-producing bacteria were found to be Gram-negative bacteria like *Azohydromonas*, *Burkholderia*, and *Cupriavidus*. *Azohydromonas lata* (ATCC 29714) is capable of producing between 50% and 88% DCW of poly(3-hydroxybutyrate) (P3HB) from various sugars including glucose, fructose and sucrose while *Burkholderia* sp. USM (JCM 15050) could synthesize up to 69 % DCW of P3HB from fatty acids. However, the presence of lipopolysaccharide (LPS) endotoxins in the bacteria's outer cell membrane is a problem because may co-purify with crude PHA polymer during the extraction process. LPS is a pyrogen that would render the PHA polymer unsuitable for biomedical applications. It could be removed, with repeated solvent extractions or with purification with activated charcoal, but in this way the overall cost of PHA production increases.

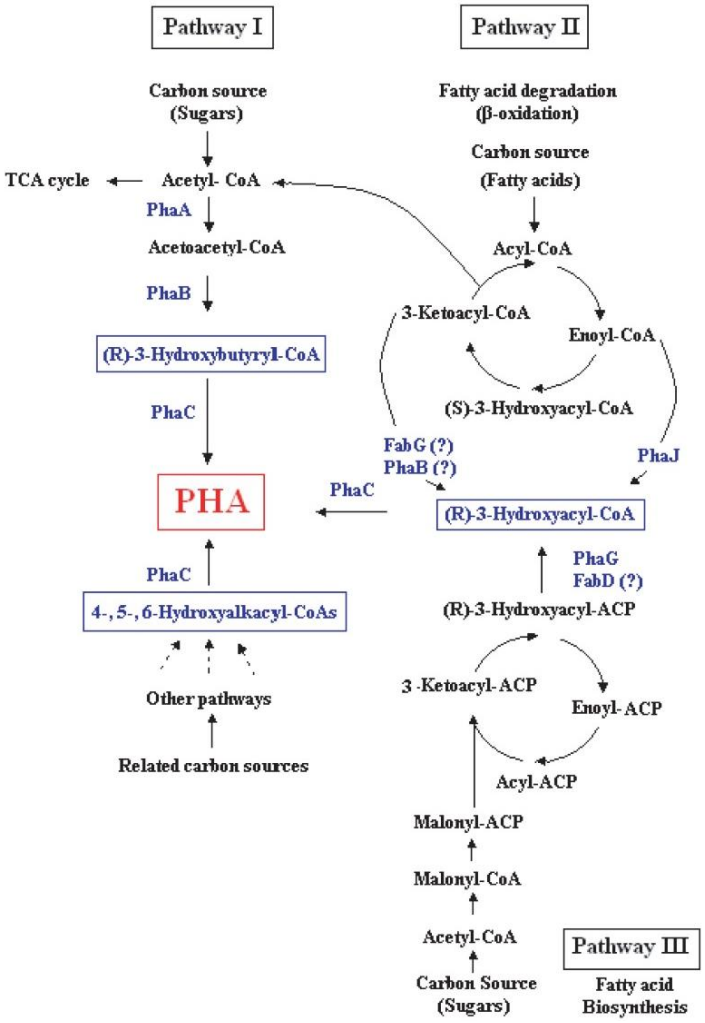


Figure 1.9 *PHA biosynthetic pathways (Philip,2007)*

1.5.2.2 Production of PHA by Gram-positive bacteria

PHA production in Gram-positive bacteria has been reported in genera *Bacillus*, *Caryophanon*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Microlunatus*, *Microcystis*, *Nocardia*, *Rhodococcus*, *Staphylococcus*, *Streptomyces* (Amy Tan et al., 2014). Compared to Gram-negative bacteria, Gram-positive bacteria were mostly found to produce scl-PHA and lower PHA contents, between about 2 and 50 % DCW. They do not produce LPS, but some species are known to produce lipidated macroamphiphiles including lipoglycans and lipoteichoic acids (LTA), which have immunogenic properties similar to LPS.

1.5.2.3 Production of PHA by Archaea

PHA is also found in archaea but limited to haloarchaeal species, specifically the genera *Haloferax*, *Halalkalicoccus*, *Haloarcula*, *Halobacterium*, *Halobiforma*, *Halococcus*, *Halopiger*, *Haloquadratum*, *Halorhabdus*, *Halorubrum*, *Halostagnicola*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, and *Natronorubrum* (Amy Tan et al., 2014). Haloarchaea require high salt concentrations for normal enzyme activity, growing at saturation conditions of up to 6 M NaCl. They accumulates PHA between 0.8 to 22.9% DCW from glucose, volatile fatty acids and more complex carbon sources such as starch, whey hydrolysate, vinasse and crude glycerol from biodiesel production. *Haloferax mediterranei* (DSM 1411), which requires 2 to 5 M NaCl for growth, can accumulate high PHA levels between 50 and 76% DCW. Very few contaminating organisms can survive in the hypersaline conditions required for the growth, thereby reducing the sterility requirements and its associated cost. Conversely, high salinity accelerates corrosion phenomena and causes greater use of chemicals. The recovery of PHA is relatively easy, because haloarchaea undergo cell lysis in distilled water and release PHA granules that can be recovered by low speed centrifugations.

1.5.2.4 Production of PHA by Cyanobacteria

Cyanobacteria are prokaryotes that have the ability to accumulate polyhydroxyalkanoates under photoautotrophic condition. For their growth and multiplication, they need some simple inorganic nutrients such as phosphate, nitrate, magnesium and calcium as macro and ferrous, manganese, zinc, cobalt and copper as micronutrients. The first cyanobacterial species for which the presence of PHB was reported is the N_2 fixing *Chlorogloea fritschii*, in the year 1966 (Balaji et al., 2013). The occurrence of PHB has been shown in many cyanobacterial species such as *Spirulina sp*., *Aphanothece sp*., *Gleothece sp*., *Synechococcus sp*. and *Synechocystis sp.*, but many of them were found to contain PHA at concentrations ranging from 0.04% to 10% of dry cell weight (*Figure 1.10*). Although Nishioka et al. (2001), Sharma and Mallick (2005) and Panda and Mallick (2007) demonstrated PHB accumulation of 55%, 43% and 38% DCW respectively in

S*ynechococcus* sp. MA19, *Nostoc muscorum* and *Synechocystis sp*. PCC 6803, metabolic engineering is being deeply explored to introduce new metabolic pathways to broaden the utilizable substrate range and to enhance PHB synthesis. Cyanobacteria were transformed with the genes encoding PHB synthesis (3-ketiolase, acetoacetyl-CoA reductase and PHB synthase) (Balaji et al., 2013).

Sharma et al. (2007) showed that PHB yield in cyanobacteria is a function of various encompassing cultural and nutritional conditions. Growth phase, light-dark cycles, temperature, pH, nutrient limitations (nitrogen and phosphorus), mixotrophy, chemoheterotrophy and gas exchange limitation are critical variables for enhanced PHB accumulation in cyanobacterial cell. Asada et al. (1999) pointed out that the main problems with the cultivation of cyanobacteria relate to lower growth rates compared to that of heterotrophic bacteria, self-shadowing problems, when the cell concentration is high and the higher energy costs required for treatments such as drying of cells.

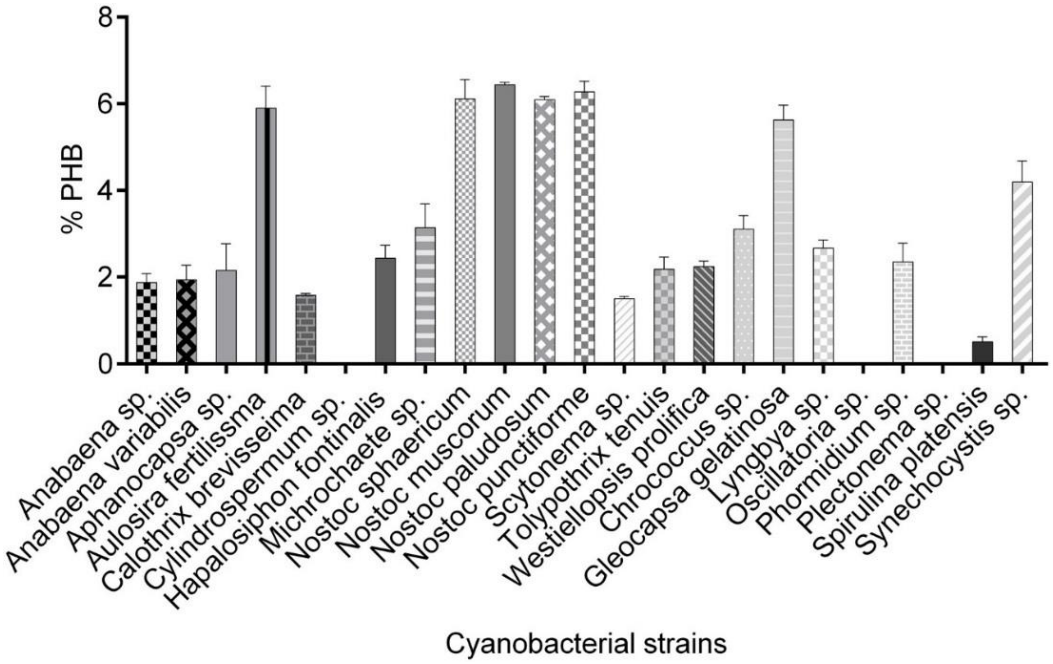


Figure 1.10 *Screening of cyanobacterial strains for PHB (%) (Ansari and Fatma, 2016)*

1.5.2.5 General properties of polyhydroxyalkanoates

PHA are thermoplastic polymers and they differ in their properties depending on the monomeric composition, which can be determined form gas chromatography (GC), liquid chromatography (LC) and nuclear magnetic resonance spectroscopy (NMR). PHA polymer's average molecular mass (*Mw*), molecular mass distribution (*Mn*), and polydispersity index (PDI=*Mw*/*Mn*) can be determined through gel permeation chromatography (GPC) system, calibrated with polystyrene standards. The *Mw* of PHA spans over a wide range from 50 kDa to 10000 kDa and, depending on *Mn* value, PDI could be between 1.1 and 6.0. *Table 1.3* summarizes the typical values of properties of PHA.

Table 1.3 *Range of typical properties of PHA*

Property	Values
Glass transition temperature (T_g)	-54-4°C
Melting temperature (T_m)	160-175°C
Thermodegradation temperature (T_d)	227-256°C
Crystallinity degree (X_{cr})	40-60%
Young's modulus (E)	1-2 GPa
Tensile strength (σ)	15-40 MPa
Elongation at break (ε)	1-15%
Water vapour transmission rate (WVTR)	2.36 g mm/m^2·day
Oxygen transmission rate (OTR)	55.15 cc mm/m^2·day

Thermal properties, glass transition temperature (T_g,), melting temperature (T_m) and thermodegradation temperature (T_d), are examined to determine the temperature conditions at which the polymer can be processed and can be obtained using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Crystallinity (X_{cr}) can be measured by structural analysis instrument including FTIR, DSC and X-ray diffraction. Mechanical properties can be identified following standardized test methods such as ASTM standards. Bugnicourt et al. (2014) points out that PHA also have the following features:

- insoluble in water;
- good ultra-violet resistance;
- poor resistance to acids and bases;
- soluble in chloroform and other chlorinated hydrocarbons;
- biocompatible, hence suitable for medical applications;
- sinks in water, facilitating its anaerobic biodegradation in sediments by biogeochemical mechanism;
- non toxic;
- less "sticky" than traditional polymers when melted.

In conclusion, PHA thermally decomposes at temperature just above the melting point and exhibits low strain at break, so to increase the processability of the polymer and at the same time reduce the intrinsic brittleness, blend with other polymers or plasticizers is needed.

PHA can be degraded in both aerobic and anaerobic environments. Under aerobic conditions, degradation products are carbon dioxide and water, whereas anaerobic conditions produces carbon dioxide and methane. PHA are degraded upon exposure to soil, compost or marine sediment. Degradations occurs most rapidly in anaerobic sewage and slowest in sew water. Many microorganisms are able to degrade PHA, such as *Gram-negative* and *Gram-positive bacteria*, *Streptomyces* and *fungi*, *Firmicutes* and *Proteobacteria*. These microorganisms colonize on the surface of the polymer and secrete

enzymes, which break down the polymer into its molecular building blocks, called hydroxyacids, used as carbon source for biomass growth. Their degradation depends on a variety of factors such as microbial activity, moisture, temperature, pH of the environment, exposed surface area, molecular weight, nature and crystallinity of the polymer. PHA are compostable over a wide range of temperatures, even at a maximum of around 60°C with moisture levels at 55%, but the majority of the degrading microorganisms were isolated at ambient or mesophilic temperatures, and only few of them work at higher temperature (Balaji et al., 2013; Bugnicourt et al., 2014).

1.5.2.6 Industrial production of polyhydroxyalkanoates

Industrially, PHA are produced, extracted and purified form bacteria by optimising the conditions of microbial fermentation of glucose or other sugar. Researchers are working to obtain PHA using carbon sources wastes (non-food competiting sources), and with genetic engineering to increase the yield in PHA. *Table 1.4* shows the commercial polyhydroxyalkanoates production facilities (Bugnicourt et al., 2014).

Table 1.4 *Commercial PHA: names, producer, origin and products (Bugnicourt et al., 2014)*

Commercial name	**Producer**	**Country**	**Product**
Biomer	Biomer	Germany	Biomer P209 Biomer P226 Biomer P240
Minerv-PHA	Bio-on	Italy	MINERV-PHA™
Biogreen	Mitshubishi Gas	Japan	Biogreen
Biocycle	PHB Industrial	Brazil	BIOCYCLE 1000 BIOCYCLE 18BC-1 BIOCYCLE 189C-1 BIOCYCLE 189D-1
Ecogen	Tianan Biological Material Poly-one	China	ENMAT Y1000 ENMAT Y1000P ENMAT Y3000 ENMAT Y3000P
Mirel	Metabolix	USA	Mirel P4001 Mirel P4010 Mirel P5001 Mirel P5004 Mirel M2100 Mirel M2200 Mirel M4100
Nodax	P&G Chemicals	USA/Japan	Nodax ™
METABOLIX	Telles LLC	USA	Mvera™ B5011 Mvera™ B5010
Jiangsu Nantian	Jiangsu Nantian Group	China	P(3HB)

Goodfellow	Goodfellow Cambridge Ltd	UK	Polyhydroxyalkanoate - Biopolymer (PHA) Polyhydroxybutyrate/Polyhydroxyvalerate 12% - Biopolymer (PHB88/PHV12)
Tepha	Tepha Inc	USA	P(4HB)

Depending on the composition and resulting properties, PHA have many and wide-ranging potential applications. Due to their impermeability to water and air are considered very suitable for products such as bottles, films and fibers. PHA latex can be used to cover paper or cardboard to make water-resistant surfaces as opposed to the combination of cardboard with aluminium, which is currently used and is non-biodegradable. The gas barrier property is useful in applications for food packaging and for making plastic beverage bottles. It is also possible to use PHA to make the following articles, thanks to their piezoelectric nature: pressure sensors (for keyboards, stretch and acceleration measuring instruments, material testing, shock wave sensors, lighters, gas lighters), acoustics (microphone, ultrasonic detectors, sound pressure measuring instruments), oscillators (headphones, loudspeakers, for ultrasonic therapy and atomization of liquids) (Philip, 2007). The presence of C5-monomers results in a material easier to process and similar to polypropylene. Mcl-PHA typically are rubber-like materials. For medical application, PHA fibers are especially sought after to make swabs and dressing materials for surgery. In the cardiovascular area, pericardial patches, artery augments, cardiological stents, vascular grafts, heart valves, implants and tablets, sutures, dressings, dusting powders, prodrugs and microparticulate carriers are currently made of PHA (Philip, 2007). Shrivastav et al. (2013) show how PHA is used as novel drug delivery systems, in particular in the areas of cancer therapy and controlled delivery of drugs including steroids, vaccines and other biological molecules. Indeed, PHA possesses biodegradability and biocompatibility characteristic as drug carrier use.

1.6 A new process integrating the treatment of sludge reject water and the production of polyhydroxyalkanoates

In this process, the municipal wastewater treatment is integrated with the production of polyhydroxyalkanoates (*Figure 1.11*). Activated sludge from wastewater treatment plants (WWTP) is a source of PHA-storing microorganism that accumulate this polymer as a carbon and energy reservoir. The process is accomplished by a sequence of operation, which consists in the acidogenic fermentation to produce the volatile fatty acids (VFAs), the selection of the PHA-storing biomass, a batch process to maximize the accumulation of PHA in the microorganism, and finally the recovery of the polymer from the biomass. Next sections briefly discuss these steps.

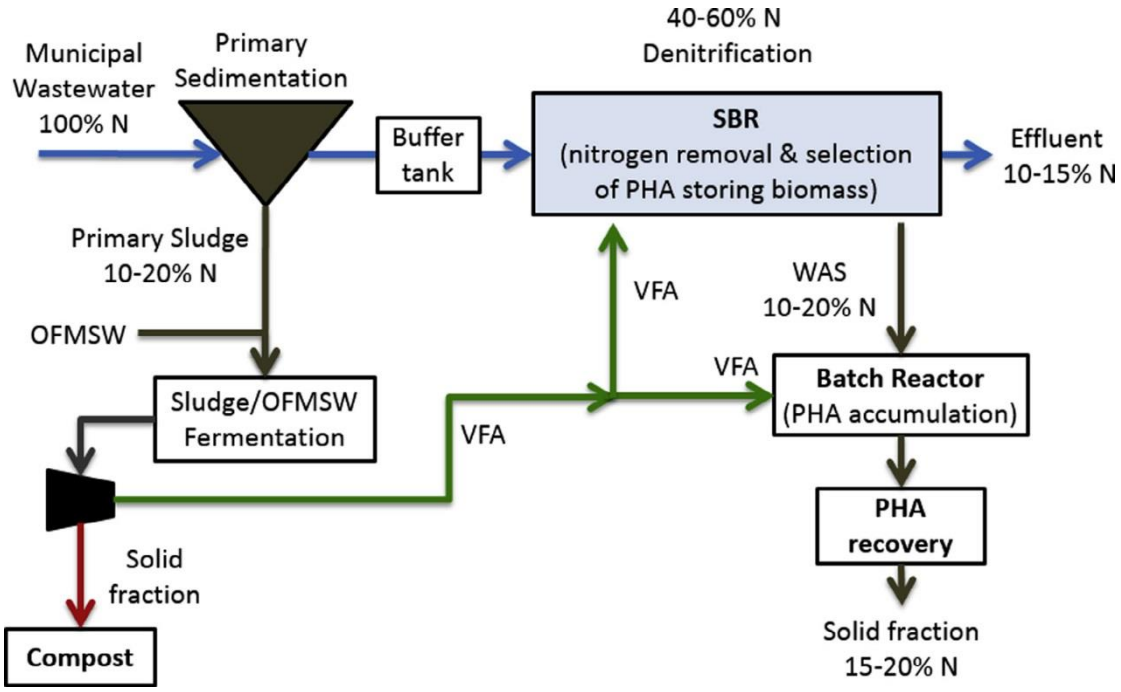


Figure 1.11 *Integrated scheme for nitrogen removal via nitrite and PHA production within WWTP (Basset et al., 2016)*

1.6.1 Cellulosic primary sludge fermentation

In this stage, the production of short chain fatty acids (SCFAs) from sewage sludge through alkaline fermentation is accomplished. Acetic acid (CH_3COOH) and propionic acid (CH_3CH_2COOH) are the most abundant acids in the sludge fermentation liquid, consisting of 60-70% of total SCFAs. In the fermentation unit, the temperature is maintained at 37°C (Longo et al., 2015), while pH is an important factor that controls hydrolysis and acidification. Alkaline conditions lead to more soluble protein and carbohydrate generation and to higher hydrolysis of particulate matter. At the same time, the methanogenic activity is inhibited resulting in enhanced SCFAs production in the fermentation liquid. The optimal pH value appears to be within the range 9-11 and can be maintained with the use of sodium hydroxide (NaOH) or calcium hydroxide ($Ca(OH)_2$) To increase the sustainability of the process and to reduce its cost, the pH can be adjusted with alkaline silicate minerals (wollastonite $CaSiO_3$). However, the application of high pH also result in a higher nutrients release in the liquid phase, with the ammonium reaching up to 500 mg N/L and the phosphate 80 mg P/L. It is important to limit the net release of nutrients during the fermentation, since these must be removed before moving to the next stage (Basset et al, 2016). After the solid and liquid separation is performed by centrifugation, ammonia and phosphorus are recovered from the supernatant as struvite, $(NH_4)MgPO_4 \cdot 6(H_2O)$ magnesium ammonium phosphate, by the addition of magnesium hydroxide $Mg(OH)_2$.

This process is sustainable only when the ammonium to phosphate molar ratio is properly balanced in the fermentation liquid in order to be removed as struvite (Longo et al., 2015).

1.6.2 Selection of PHA-storing biomass

This stage consists in a sequencing batch reactor (SBR) where the selection of PHA-storing biomass occurs simultaneously with the nitritation/denitritation process under an aerobic/anoxic, feast/famine regime (*Figure 1.12*). The oxidation of ammonium to nitrite occurs during aerobic-feast conditions and is followed by denitritation (nitrite is reduced to molecular nitrogen) during anoxic/famine conditions (Frison et al., 2015). The VFAs required for the feast phase are recovered from the liquid produced by the acidogenic fermentation. In this regime, they are continuously depleted, favouring the PHA storage. Instead, during the anoxic-famine phase, characterized by the absence of VFAs, internally stored PHA is the carbon source utilized for denitritation process. It is essential to establish the desired COD/N ratio (2.5 g COD g^{-1} N) for denitritation and the selection of the biomass, by controlling an external carbon source. This plays an important role because the presence of non-VFA COD contributes to the growth of non-PHA storing biomass, as an extra substrate for PHA storing organisms during famine phase and/or as storage substrate in terms of polysaccharides (Basset et al., 2016).

Figure 1.12 *Schematic representation of aerobic/anoxic, feast/famine regime in a sequencing batch reactor (Basset et al., 2016)*

1.6.3 PHA accumulation and extraction

The biomass collected under famine regime during sequencing batch reactor is used in the fed-batch reactor for the accumulation of PHA. The VFAs required are recovered from the liquid obtained during the acidogenic fermentation. The amount of fermentation liquid added corresponds to 50% of the initial volume. The efficiency of PHA accumulation is higher when the liquor is free of nutrients, which could promote biomass growth instead of polymer accumulation (Basset et al., 2016). This is why it is essential to limit the net release of nutrients during fermentation, in order to achieve lower concentration of $N\text{-}NH_4$ and $P\text{-}PO_4$ in the produced fermentation liquid. The biopolymer is extracted from lyophilized biomass by chloroform extraction. The percentage of PHA in the biomass is expressed with respect to total suspended solids (% g PHA/g TSS).

1.7 Aim of the thesis

Wastewater bio-treatment with microalgae is particularly attractive because of their photosynthetic capabilities, removing nutrients and converting sunlight energy into useful biomass. The aim of this thesis is to evaluate the possibility to apply microalgae and cyanobacteria for valuable compounds production process, based on wastewater exploitation. In particular, the possibility of integrating microalgae in a polyhydroxyalkanoates production process will be assessed. Different strategies will be explored: a first process will be proposed, where microalgae may be applied as an intermediate step between the acidogenic fermentation and the sequential batch reactor treatment (Longo et al., 2015; Basset et al., 2016), allowing the removal of nutrients, nitrogen and phosphorus. In this way, two goals might be achieved, thus decreasing the nutrients concentration, which could promote the biomass growth instead of the polymer accumulation during the following batch phase, and also recovering nutrients in an energy-rich algal biomass. A second process will be proposed, by directly cultivating cyanobacteria on acidogenic fermentation liquid to produce the polyhydroxyalkanoates. Indeed, if grown under appropriate conditions, some of these microorganisms are able to produce PHA and other high-value compounds, like cyanophycin. For this reason, the fermented liquid obtained after the acidogenic fermentation will be tested as culture medium for microalgal and cyanobacterial species in batch experiments.

Initially, a screening of different microalgal and cyanobacterial species will be required. Subsequently, the influence of factors such as light, CO_2 insufflation, and temperature on the growth of selected microorganisms will be analysed. At the same time, an assessment of nutrient consumption will be carried out. Particular attention will be given to polyhydroxyalkanoates and cyanophycin, and the protocols for the extraction and quantification of these components from cyanobacteria will be thoroughly investigated.

Chapter 2

Materials and methods

This chapter describes the materials and methods used to perform the experimental part of the work. In the first part, the species of microorganism, the culture media used and the experimental setup are specified. The second part explains the techniques for controlling the growth and all the analytical techniques for nutrients determination. Finally, the methods for extraction and quantification of polyhydroxyalkanoates and cyanophycin are reported.

2.1 Microalgae strains and cultivation

Many species of microalgae and cyanobacteria were investigated, because it was not known *a priori* which one could survive and live in the real medium used for the experiments. Four types of microalgae were used, *Chlorella protothecoides* 33.80 (SAG-Goettingen), *Chlorella vulgaris* (Emerson 3 - CNR Firenze), *Muriella zofingiensis* (CNR - Firenze) and *Scenedesmus obliquus* 276-7 (SAG - Goettingen). Maintenance and propagation of cultures were performed in freshwater media (BG11) (Rippka et al., 1979), at ambient temperature, under continuous agitation. Three were the cyanobacteria tested in the experiment, *Anabaena cylindrica* PCC 7122, *Synechococcus sp*. PCC 7002 and *Synechocystis sp*. PCC 6803. Also these three species were maintained in standard media, under continuous agitation, respectively in $BG11_0$, BG11 without Hepes and standard BG11 (compositions reported below).

2.2 Culture media

Different standard media were necessary to perform the experiment and to maintain all the species used, in addition to the real wastewater medium. All the standard media were prepared by mixing the component in demineralized water, then they were sterilized in autoclave for 20 minute at 121°C.

2.2.1 Real wastewater medium

The medium used was derived from the acidogenic fermentation of mixed primary and secondary sewage sludge (Longo, 2015). This process, performed at 37°C, yields a medium rich in short-chain volatile fatty acids (SCFAs). Acetic acid (CH_3COOH) and propionic acid (CH_3CH_2COOH) are the most abundant acids in the sludge fermentation liquid, consisting of 60-70% of total SCFAs. The pH is an important factor that controls the hydrolysis and acidification during sewage sludge fermentation. This process is performed

under alkaline conditions obtained thanks to the use of sodium or calcium hydroxide (NaOH, $Ca(OH)_2$) or with wollastonite ($CaSiO_3$), an alkaline silicate mineral. The application of high pH is also associated with a higher nutrients release in the liquid phase (Basset, 2016). *Table 2.1* shows the composition of the medium.

Table 2.1 *Characterization of the real medium used*

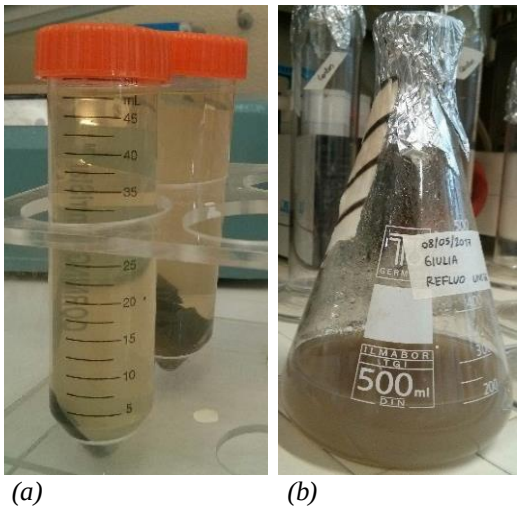
$N\text{-}NH_3$	**$N\text{-}NO_3^-$**	**$N\text{-}NO_2^-$**	**$P\text{-}PO_4^{3-}$**	**COD**
mg/L	mg/L	mg/L	mg/L	mg/L
606.2 ± 36.84	15.41 ± 0.853	-	98.38 ± 0.597	6742.6 ± 423.3

To allow a comparison, *Table 2.2* shows a common composition of urban wastewater (it was taken from the wastewater treatment plant of Montebello Vicentino (45°27'26"64 N, 11°23'4"20 E). It can be noticed how much higher are the concentration of ammonium, phosphates and COD in the medium used in our experiments.

Table 2.2 *Characterization of common urban wastewater*

$N\text{-}NH_3$	**$N\text{-}NO_3^-$**	**$N\text{-}NO_2^-$**	**$P\text{-}PO_4^{3-}$**	**COD**
mg/L	mg/L	mg/L	mg/L	mg/L
2.13	0.16	21.04	2.52	495.92

The medium was stored in 500 mL aliquots at a temperature of -18°C. Before use, it was necessary to centrifuge it (10000 rpm, 15 minutes) to remove most suspended solids. *Figure 2.1* shows the medium after the centrifugation step *(a)* and the medium obtained after sterilization in autoclave for 20 minutes at 121°C *(b)*.



(a) *(b)*

Figure 2.1 *(a) Real medium after the centrifugation (b) Sterilized medium*

2.2.2 BG11

This medium was used to maintain all the microalgae cultures. It is buffered to pH 8 thanks to the presence of the buffer HEPES. The composition were reported in the *Table 2.3*.

Table 2.3 *Composition of BG11 medium*

Component	Concentration (mg/L)
Na_2Mg EDTA	1
Ferric ammonium citrate	6
Citric acid · H_20	6
$CaCl_2 \cdot 2H_20$	36
$MgSO_4 \cdot 7H_20$	75
K_2HPO_4	30.5
H_3BO_3	2.86
$MnCl_2 \cdot 4H_20$	1.81
$ZnSO_4 \cdot 7H_20$	0.222
$CuSO_4 \cdot 5H_20$	0.079
$COCl_2 \cdot 6H_20$	0.050
$Na_2MoO_4 \cdot 2H_20$	0.391
Na_2CO_3	20
$NaNO_3$	1500
Hepes pH 8	1 M

2.2.3 BG11 500N/80P

This medium was prepared to verify if some of the microalgae species could suffer the composition of the real wastewater medium, so the recipe was modified to have a standard medium with the same amount of ammonia and phosphorus as in the real wastewater medium (§2.2.1). The sodium nitrate $NaNO_3$ was substituted with ammonium choride NH_4Cl to have almost 500 mg/L of nitrogen as ammonium, and more dipotassium hydrogenphosphate K_2HPO_4 was used to have 80 mg/L of phosphorus as orthophosphate.

2.2.4 BG11$_0$

This medium was necessary to maintain the culture of Anabaena cylindrica PCC 7122, but was also used to perform the experiment for the production of polyhydroxyalkanoates. In this case, the recipe of BG11 was modified eliminating the sodium nitrate $NaNO_3$ and the buffer HEPES and adding 0.4 g/L of sodium bicarbonate $NaHCO_3$.

2.2.5 BG11 P

This medium was used to perform all the experiments for the production of cyanophycin with the cyanobacteria *Synechocystis* sp. PCC 6803. The recipe of the standard BG11 medium was modified eliminating components containing phosphorus, e.g. the dipotassium hydrogenphosphate K_2HPO_4. To avoid starvation of potassium an equimolar quantity of potassium chloride KCl was used. The recipe was also modified using an equimolar concentration of ammonium chloride NH_4Cl instead of the sodium nitrate $NaNO_3$.

2.3 Experimental setup

Experiments were performed in batch reactors. Two types of reactors were used, bottle reactors and vertical flat-plate polycarbonate photobioreactors (PBR). The effect of light, temperature and CO_2 were studied in order to understand their influence on the growth rate. The reactor temperature was maintained constant thanks to a thermostated incubator or with the help of a thermostatic bath. Two different LED systems, a warm white panel and a lamp (Photon System Instruments, SN-SL 3500-22) provided light. It was measured with a photoradiometer (HD 2101.1 from Delta OHM) which quantifies the photosynthetically active radiation (PAR). PAR is considered as the wavelength range between 400 nm and 700 nm and is commonly quantified as photosynthetic photon flux (µmol photons m^{-2} s^{-1}) (Drosg, 2015). Additionally, a magnetic stirrer was used to prevent any deposition of biomass, thus ensuring a good mixing within the reactor.

2.3.1 Bottle reactors

These batch experiments were carried out in Quickfit® Drechsel Bottles (*Figure 2.2a*) with a volume of 250 mL and a diameter of 5 cm (*Figure 2.2a*). In some of the experiments a CO_2-air (5% v/v) mixture was fed to the reactor by bubbling it through an appropriate pipe.

2.3.2 Flat-plate photobioreactor (PBR)

Two flat-plate photobioreactors were used in batch mode (*Figure 2.2b*). They had a volume of 300 mL, a depth of 1.2 cm, and an irradiated surface measuring 11.5 cm (length) and 21.5 cm (width). A CO_2-air (5% v/v) mixture was fed to the reactor bubbling it at the bottom. This ensured additional mixing as well.

(a) *(b)*

Figure 2.2 *(a) Drechsel Bottles; (b) Flat-plate PBR*

2.4 Growth analysis

To verify the growth of the different species of microalgae and cyanobacteria, the value of the optical density was checked every day. In the experiments with microalgae, the cell concentration was measured every day counting the cells by an optical microscope. Instead, in the experiments with cyanobacteria the value of pigments, chlorophyll *a* and carotenoids, were measured and they were used as an index of growth. Finally, in the first and in the last day of all batch experiments dry cell weight was determined. Every day it was also verified that the pH was within the desired range.

2.4.1 Optical Density (OD)

To measure the value of the optical density a spectrophotometer (Spectronic UV-500® UV-visible) was used. The instrument includes two lamps, a deuterium one (for the wavelengths of the visible) and a tungsten one (for the UV wavelengths), aligned perpendicularly to the two seats for the cuvettes (with optical path of 1 cm). The desired wavelength was selected with a monochromator. The 750 nm wavelength was used because there was no absorption by the chlorophyll of the cells and, in this way, the measurement was not altered by the cell pigment composition. The sample had to be first properly diluted so that the measurement would be within range [0.1-1], within which there is a linear relationship between measured absorbance and cell concentration. It was also necessary to set the zero to remove the medium contribution to the absorption. The value of optical density is given by

$$OD = abs \cdot dil \tag{2.1}$$

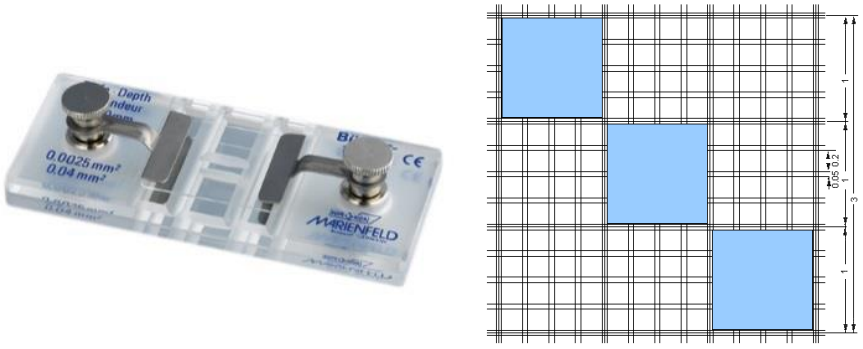
where *abs* is the absorbance measured and *dil* is the dilution factor used for the analysis.

2.4.2 Microalgal cell concentration

The cell concentration was determined by counting the cells at the optical microscope using the Bürker® chamber (*Figure 2.3a*). It consisted of a rectangular slide, with sides of 7.5 cm and 3.5 cm respectively, and a thickness of 4 mm. It has two cells of 3 x 3 mm, 0.1 mm deep, each one divided into nine squares of 1 mm, separated by a triple line (*Figure 2.3b*). Each one of these was divided in sixteen smaller squares, delineated by a double line. A culture sample was taken and diluted with a factor of 2 to 200 depending on the estimated concentration. The cell count was made only for three squares, along the diagonal, and the average value was considered. Cell concentration was calculated according to:

$$cell/mL = n \cdot dil \cdot 10^4 \tag{2.2}$$

where *n* is the average number of cells counted, *dil* is the dilution factor, and 10^4 is a conversion factor due to the volume of the chamber (0.1 µL).



(a) *(b)*

Figure 2.3 *(a) Bürker chamber (b) Schematic representation of one of the two cells*

2.4.3 Pigments extraction and quantification

The extraction and quantification of pigments, chlorophyll *a* and carotenoid was made thanks to the solvent N,N-dimethylformamide (DMF), which solubilizes the wall and the cell membranes by bringing the pigments into solution. 500 µL of sample was taken daily and centrifuged at 13000 rpm for 10 minutes. After the supernatant was removed, 1 mL of solvent was added. This was done in the dark, because once taken into solution, the chlorophyll is photosensitive. Samples were then stored in the dark in a freezer for at least 48 hours to ensure complete pigment extraction. After further centrifugation at 13000 rpm for 10 minutes, the absorption spectrum on the extract between 350 nm and 750 nm was performed using DMF as reference. Samples were diluted to have absorbance values within the range [0.1-1], within which there is a linear relationship between absorbance and concentration. The final concentration of chlorophyll *a* and carotenoid was determined by the following equations, modified from Wellburn (1994).

$$\frac{\mu g}{mL} Chl_a = (abs_{664} - abs_{750}) \cdot dil \cdot \varepsilon \cdot \frac{V_{DMF}}{V_{sample}} \quad (2.3)$$

$$\frac{\mu g}{mL} Car = [(abs_{461} - abs_{750}) - (abs_{664} - abs_{750}) \cdot 0.04] \cdot dil \cdot \varepsilon \cdot \frac{V_{DMF}}{V_{sample}} \quad (2.4)$$

where *abs* is the absorbance at specific wavelength, *dil* is the dilution factor and ε is the molar attenuation coefficient equal to 11.92 and 4 for chlorophyll *a* and carotenoid determination, respectively.

2.4.4 Dry cell weight (DCW)

Dry cell weight measurements were performed to determine the amount of biomass per unit volume. The method is based on the filtration of a known volume of sample by vacuum

flask. The filter was placed on a stainless steel Buchner funnel, fixed on the flask. Cellulose nitrate filters (Sartorius Stedim Biotech GmbH) with pore size of 0.45 µm were used for microalgae, instead acetate filters (GVS LIFE SCIENCES) with pore size of 0.22 µm were applied to cyanobacteria. Initially, the filter was placed in the oven for 15 minutes at 105°C to remove humidity. With the balance Atilon Acculab Sartorius group (sensitivity 10^{-4} g) the tare was weighed. After filtering the sample, the filter was placed for other two hours in the oven, after which it was weighed again. The difference between gross weight and tare, divided by the filtered volume, gave the biomass concentration:

$$DCW\ \left(\frac{g}{L}\right) = \frac{gross\ weight - tare}{sample\ volume} \tag{2.5}$$

It is possible to establish a correlation between dry weight and cell concentration, thus defining the weight per single cell. This is calculated by performing the ratio between the dry weight and the cell concentration *(Equation 2.6*).

$$weight\ per\ cell\ \left(\frac{pg}{cell}\right) = \frac{DCW}{cell/mL} \cdot\ 10^9 \tag{2.6}$$

Specifically, for *Chlorella protothecoides* 33.80 (SAG-Goettingen), weight per cell was calculated by preparing solutions at different concentrations and by averaging the calculated values. The obtained value was 16.38 ± 2.14 pg/cell.

2.4.5 Growth rate

The maximum growth rate is the one reached in the exponential phase, where there is no substrate limitation.

$$\frac{dC_x}{dt} = \mu_{max} \cdot C_x \tag{2.7}$$

where C_x is the cells concentration, t is the time and μ_{max} the growth rate constant. Integration of *Equation 2.7* between initial conditions (day 0) and final conditions returns the *Equation 2.8*.

$$\ln\left(\frac{C_x}{C_x^0}\right) = \mu_{max} \cdot (t - t^0) \tag{2.8}$$

For the calculation of the growth rate constant, only the first days, were fully representative of the exponential phase, and were taken into account.

2.4.6 pH measurement

Every day the pH was verified using a Hanna portable pH-meter (Code HI 9124) and it was eventually modified within the optimum range thanks to the use of solutions of sodium hydroxide NaOH or hydrochloric acid HCl.

2.5 Nutrients analysis

Nutrient analysis were carried out on the culture medium and on the sample obtained in the final day of the batch experiment. The sample was filtered using 0.45 µm filters for microalgae (Sartorius Stedim Biotech GmbH), and 0.22 µm filters for cyanobacteria (GVS LIFE SCIENCES). Standard procedures for wastewater based on colorimetric tests have been used (APHA-AWWA-WEF.1992). Specific nutrient was measured by turning it with specific reagent into a coloured compound. The concentration was calculated by measuring the absorbance with the spectrophotometer Spectronic UV-500® UV-visible at a specific wavelength. The correlation between measured absorbance and concentration is indicated in the specific kit, and is based on the Lambert Beer equation

$$abs = \varepsilon \cdot l \cdot c \tag{2.9}$$

where *abs* is the measured absorbance, ε the molar attenuation coefficient, l the optical path and c the concentration.

2.5.1 Chemical oxygen demand (COD)

The COD expresses the amount of oxygen originating from potassium dichromate $K_2Cr_2O_7$ that reacts with the oxidizable substances. The sample is oxidized with a hot sulfuric solution of potassium dichromate, with silver sulphate as catalyst. Chloride is masked with mercury sulphate. The concentration of unconsumed yellow $Cr_2O_7^{2-}$ ions or of green Cr^{3+} ions is determined photometrically. The method corresponds to DIN ISO 15705 and is analogous to EPA 410.4, APHA 5220 D and APHA D1252-06 B. The samples (2 mL) were properly diluted so that the absorbance falls within the range of the calibration curve. 2100 µL of reagents were added, then the samples remained for two hours at 148°C. After cooling for at least 30 minutes, the absorption at a wavelength of 445 nm was measured, with distilled water as reference. To avoid the interference of organic impurities present in the glass tubes in which the reaction takes place, these were previously washed with 15% sulfuric acid H_2SO_4. The calibration curve shown in the *Figure 2.4* was obtained with different solutions of potassium hydrogen phthalate with known COD values. The linear relation obtained is expressed by:

$$\frac{mg}{L}\ COD = -285.09 \cdot abs + 293.93 \qquad R^2 = 0.9926 \tag{2.10}$$

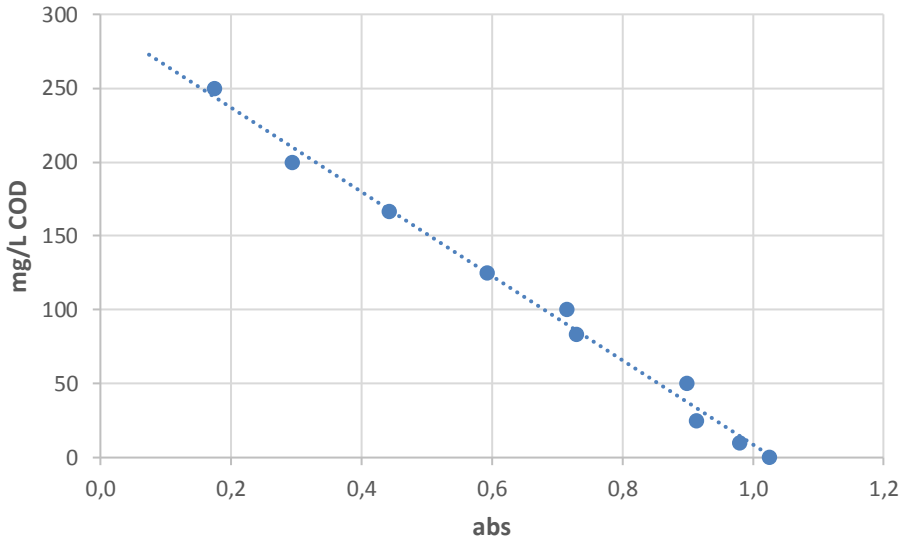


Figure 2.4 *COD calibration curve*

2.5.2 Ortophosphates

Innamorati et al. describe the method used for the detection of orthophosphates in Nova Thalassia vol. 11 (1990). It is based on the formation of a blue phosphomolybdic complex of the molybdenum blue group, whose concentration is measured by colorimetric with a spectrophotometer. One milliliter of reagent is composed of 500 µL of 5N sulfuric acid H_2SO_4, 100 µL of antimony potassium tartrate (1.36 g/L), 200 µL of ammonium molybdate tetrahydrate (30 g/L) and 200 µL of ascorbic acid (54 g/L). The reagent is not stable, so it must be freshly prepared for each analysis. 400 µL of reagent was added to 2 mL of diluted sample. After 10 minutes, the absorbance was measured at 705 nm wavelength. The calibration curve shown in *Figure 2.5* was obtained with different solutions at known concentrations of potassium dihydrogen phosphate.

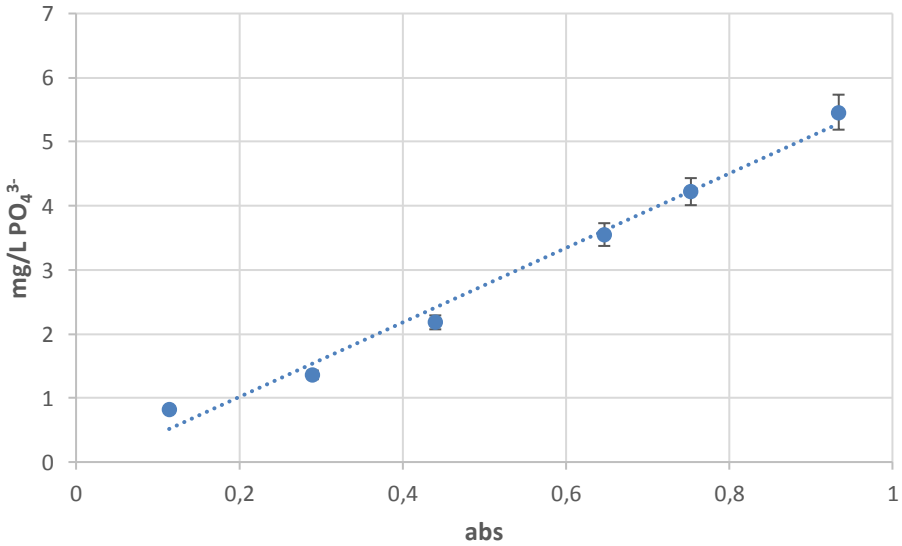


Figure 2.5 *Orthophospates calibration curve*

With the following linear relation, it is possible to calculate the concentration of ion PO_4^{3-}.

$$\frac{mg}{L} PO_4^{3-} = 5.811 \cdot abs - 0.1462 \qquad R^2 = 0.9866 \tag{2.11}$$

2.5.3 Ammonia

To measure ammonia, the Hydrocheck Spectratest diagnostic kit (Code 6201) was used. The Nessler reagent is added to 5 mL of properly diluted sample. This reagent forms a yellow-brown colloidal complex with ammonia under alkaline conditions. After 5 minutes, the absorbance is measured with the spectrophotometer at a wavelength of 445 nm. The calibration curve is obtained with various solutions of known concentration of ammonium chloride. *Figure 2.6* shows the linear relation between absorbance and concentration.

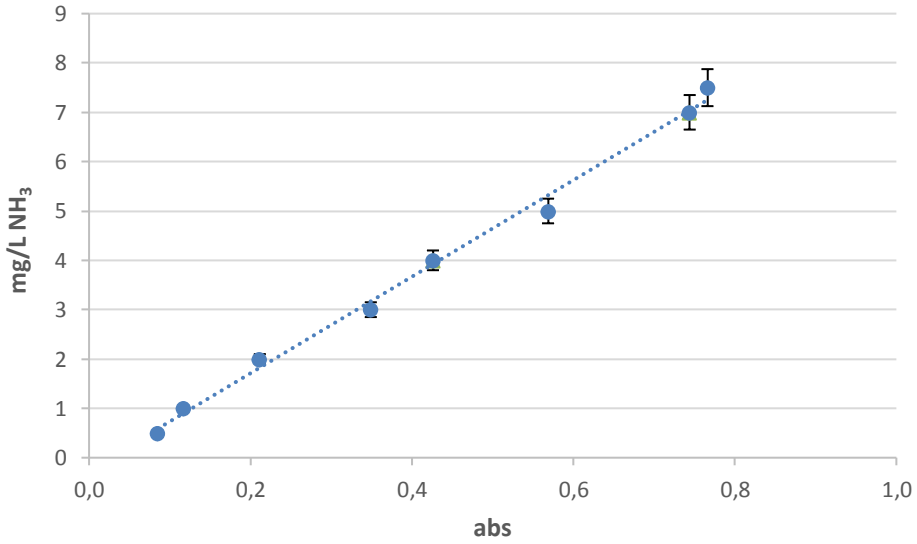


Figure 2.6 *Ammonia calibration curve*

The equation obtained is:

$$\frac{mg}{L} NH_3 = 9.702 \cdot abs - 0.1938 \qquad R^2 = 0.995 \tag{2.12}$$

2.5.4 Nitrate

The diagnostic kit used to measure nitrates was Hydrocheck Spectratest (Code 6223). In 3 mL of sample a pre-mixed reagent spoon was poured. After 10 minutes the absorbance was measured at 445 nm. The colorimetric reaction firstly reduces nitrates to nitrites, which reacts with sulfanilic acid forming a diazonium salt. The latter one, reacting with gentisic acid, produces the azo dye. The calibration curve shown in *Figure 2.7* was estimated using known nitrate concentration standard solutions.

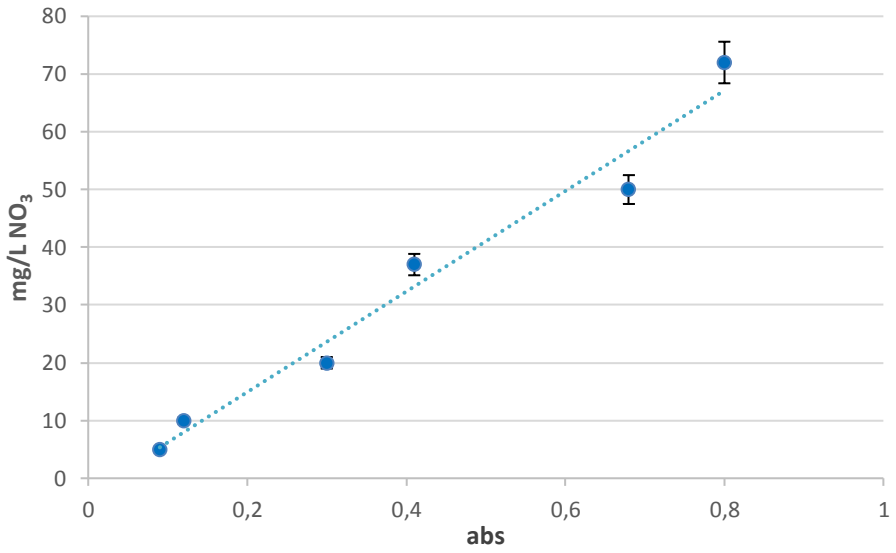


Figure 2.7 *Nitrates calibration curve*

The linear relation obtained is:

$$\frac{mg}{L}\ NO_3 = 88.99 \cdot abs - 3.323 \qquad R^2 = 0.9983 \tag{2.13}$$

2.5.5 Nitrite

For the determination of nitrites, the Hydrocheck Spectratest diagnostic kit (Code 6227) was used. The colorimetric reaction takes place in a 5 mL sample to which the reagent consisting of diazoto sulphanilic acid and naphthylamide is added. After 8 minutes the absorbance is measured at 520 nm. The calibration curve shown in *Figure 2.8* was obtained by standard solutions with known nitrite concentrations.

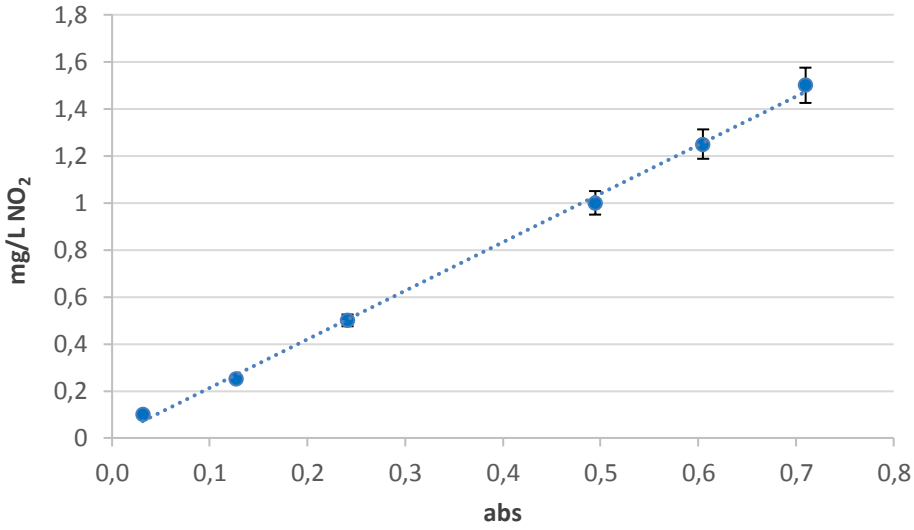


Figure 2.8 *Nitrite calibration curve*

The linear reaction between absorbance and concentration is:

$$\frac{mg}{L} NO_2 = 2.0653 \cdot abs + 0.0065 \qquad R^2 = 0.9983 \tag{2.14}$$

2.5.6 Total nitrogen

Total nitrogen measurement is required to obtain the amount of organic nitrogen present. The applied procedure is a digestion with potassium persulfate. The reagent is composed of sodium hydroxide NaOH (9 g/L) and potassium persulfate $K_2S_2O_8$ (40 g/L). The reaction is activated by heat. Actually, at 121°C the persulfate can hydrolyse to sulfuric acid and make an acid digestion, thus transforming all nitrogen into nitrate. After 24 hours, nitrate quantification is carried out according to the methodology described in §2.5.4. The calibration curve (*Figure 2.9*) was estimated from two solutions of known concentrations of sodium nitrate $NaNO_3$ and urea NH_2CONH_2.

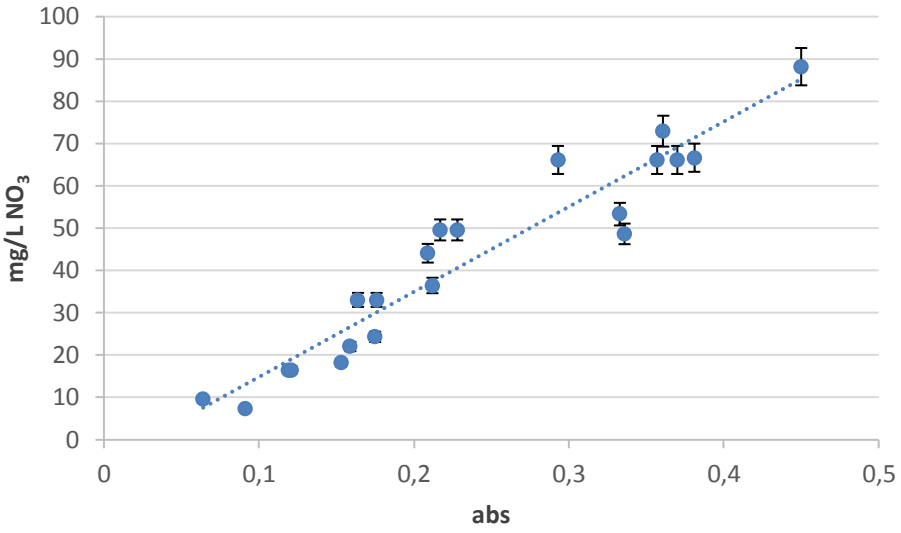


Figure 2.9 *Total nitrogen calibration curve*

The relation between absorbance and concentration is:

$$\frac{mg}{L} N_{tot} = 201.2 \cdot abs - 5.2856 \qquad R^2 = 0.9128 \tag{2.15}$$

2.6 Extraction and quantification of polyhydroxyalkanoates (PHA)

Various methods are available for the detection and analysis of intracellular microbial PHA. These methods are useful in identifying novel PHA-producing species or for routine monitoring of PHA production bioprocesses. Polyhydroxyalkanoates were extracted from dried cells using chloroform as solvent in a Soxhlet apparatus (Panda et al., 2006; Bhati et al., 2010; Ansari and Fatma, 2016). The biomass was dried in the oven for 24 hours at 80°C

and it was weighed. Then it was mixed with quartz powder and ground into a fine powder in a mortar pestle. The powder was put into a glass fiber thimble inside the main chamber of a Soxhlet extractor. The Soxhlet extractor (*Figure 2.10*) was placed into a flask containing the extraction solvent. This was heated, so that the chloroform vapour travelled up a distillation arm, and flooded into chamber housing the thimble of dried biomass. A condenser ensured that solvent vapour turns into liquid and dripped back down into the chamber housing the solid material. The chamber containing the biomass was slowly filled with warm solvent. Polyhydroxyalkanoates dissolved in the hot solvent. When the Soxhlet chamber was almost full, it was automatically emptied by a siphon side arm, with the chloroform running back down to the distillation flask. This solvent recirculation was repeated for about 30 hours. During each cycle, a portion of the volatile compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.

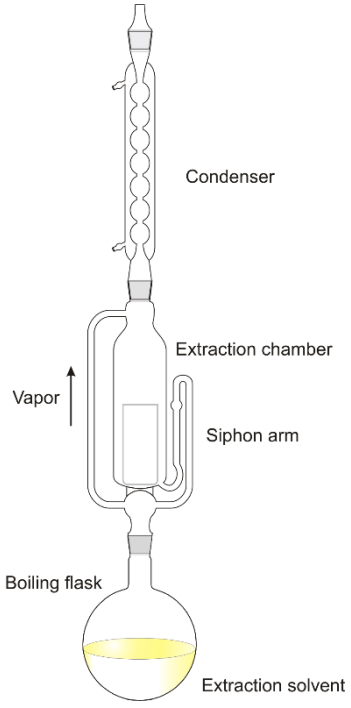


Figure 2.10 *Soxhlet extractor*

Commonly, the crotonic acid test was used as a method for the quantitative determination of PHA. In the crotonic acid test, PHA, after extraction, is dissolved in concentrated sulfuric acid and converted into crotonic acid, which has a strong UV absorption at 235 nm measurable with an UV spectrophotometer. While this method is a simple and fast way to quantify PHA, it tends to overestimate the PHA content due to the interference of other

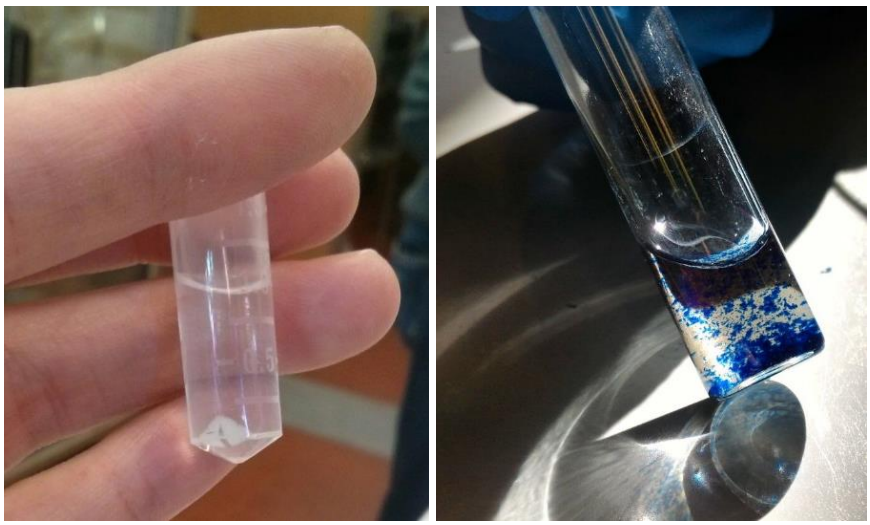
endogenous components, and is limited to the determination of poly-3-hydroxybutyrate (P3HB). For this reason, after 30 hours of extraction, a gravimetric quantification was carried out (Yellore and Desai, 1998; Panda et al., 2006; Basset et al., 2016). The polyhydroxyalkanoates solution was precipitated with three volumes of methanol (CH_3OH). Finally, gravimetric quantification was performed by filtration, achieved with a vacuum flask to which a stainless steel buchner funnel is attached. A fiberglass filter (Whatman GF/A) was placed on it. After the complete evaporation of the organic solvents, the filter was weighed. The difference between the gross weight and the filter tare divided by the initial weight of the extracted biomass provides the percentage of polyhydroxyalkanoates accumulated within the biomass:

$$\%\ PHA\ \left(\frac{g\ PHA}{g\ TSS}\right) = \frac{gross\ weight - tare}{initial\ biomass} \tag{2.16}$$

2.7 Extraction, purification and quantification of cyanophycin

Cyanophycin estraction was carried out as described in the protocols from Elbahloul et al. (2004) and Trautmann et al. (2016), with some modifications. A sample volume was centrifuged at 7500 rpm for 10 minute and the supernatant was discarded. The remaining pellet was dissolved in 1 mL absolute acetone and incubated at room temperature for 30 minutes in an orbital shaker to dissolve the cell wall lipids and to increase cell wall and membrane permeability. After a second centrifugation step (11000 rpm, 10 minutes) acetone supernatant was discharged again. The cells were washed twice with 50 mM Tris-HCl buffer (pH 7.5) to remove all soluble proteins and other compounds. Discarded the supernatant, the pellet was resuspended in 1.5 mL 0.1 M HCl, and incubated at room temperature for 30 minutes in an orbital shaker. A centrifugation step was performed to remove cell debris as pellet. The supernatant contained the solubilized cyanophycin. Addition of 500 mL of 0.1 M Tris-HCl set to pH 12 by sodium hydroxide (NaOH), and following incubation on ice led to the cyanophycin precipitation (*Figure 2.11a*). A further centrifugation step (14500 rpm, 15 min) was carried out to obtain a cyanophycin pellet. The described precipitation step was repeated once more to ensure a higher purity of the pellet. After disposal of the supernatant, the pellet was resuspended in 0.1 M HCl and a final centrifugation step was performed to remove insoluble proteins. Cyanophycin quantification was carried out according to Bradford (1976), with some modifications. The reagent was prepared dissolving Coomassie Brilliant Blue G-250 (100 mg) in 47 mL methanol CH_3OH (100%). To this solution 100 mL of 85% phosphoric acid H_3PO_4 was added. The resulting solution was diluted to a final volume of one liter. 2,5 mL of the reagent was added at 50 mL of the sample, and the absorbance was read after 2 minute at a wavelength of 595 nm. Since the protein-dye complex has a tendency to aggregate with

time, as shown in *Figure 2.11b*, it was recommended to read the absorbance between 5 and 20 minutes after the reagent addition.



(a) *(b)*

Figure 2.11 *(a) Precipitated cyanophycin (b) Aggregated protein-dye complex*

A calibration curve shown in *Figure 2.12* was made using bovine serum albumin (BSA) as standard.

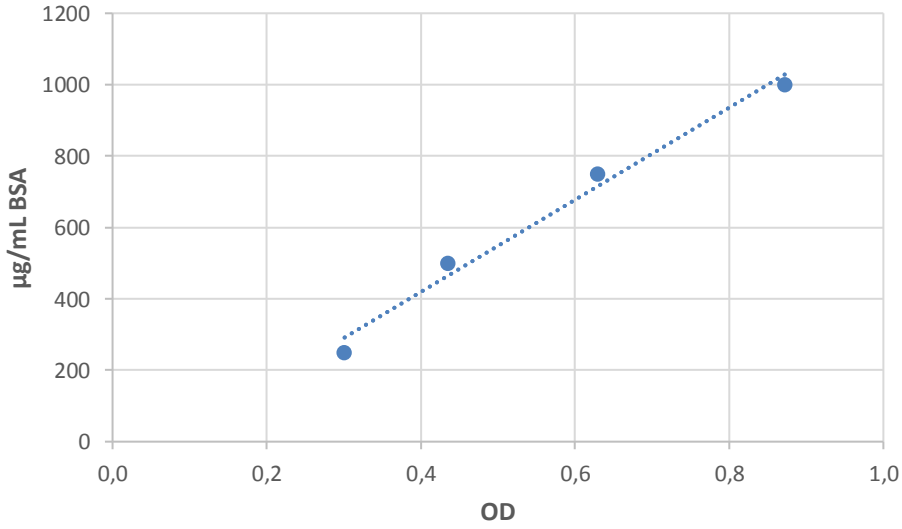


Figure 2.12 *Cyanophycin calibration curve*

The linear relation between absorbance and concentration is given by:

$$\frac{\mu g}{mL} = 1290.4 \cdot abs - 96.804 \qquad R^2 = 0.984 \tag{2.17}$$

where *abs* is the absorbance measured at 595 nm.

Chapter 3

Results and discussion

In this chapter, the results of batch experiments with microalgae and cyanobacteria are summarized and discussed. After describing initial experiments with standard media, the screening of microalgal species in the real wastewater is shown. Subsequently, experiments with *Chlorella protothecoides* are described and the effect of different light intensity is assessed. Afterward, the possibility of directly growing in the fermentation liquid a species of microorganism capable of synthesizing polyhydroxyalkanoates is evaluated. For this reason, the screening of three different cyanobacterial species is carried out. Experiments with *Synechocystis* sp. are shown, and the effect of carbon dioxide, temperature and different light intensity is discussed. In the final part, the experiments performed with standard media to validate and improve the protocols for the extraction of polyhydroxyalkanoates and cyanophycin from cyanobacteria are described.

3.1 Batch experiments with modified BG11

In the first series of experiments, four microalgal species were used: *Muriella zofingiensis* (MZ), *Scenedesmus obliquus* (SOB), *Chlorella vulgaris* (CV) and *Chlorella protothecoides* (CP). These were initially cultured in standard medium (BG11 500N/80P) modified so that to verify their growth performances in the presence of high concentrations of nitrogen (N-NH_3) and phosphorus (P-PO_4^{3-}). Specifically, when ammonia is dissolved in water (*Equation 3.1*) an equilibrium system between ammonia (NH_3) and ammonium (NH_4^+) is formed:

$$NH_4^+ + OH^- \xleftrightarrow{pK=9.25\ at\ 25°C} NH_3 + H_2O \quad (3.1)$$

The equilibrium between the two forms depends mainly on the pH, but also temperature has a significant effect. Free ammonia has harmful effect on microalgae growth in relatively low concentration (2mM), because it diffuses passively into cells, which have therefore little control over intracellular concentrations. Instead, ammonium is taken up by microalgae and cyanobacteria actively by transportation mechanisms, thus the intracellular concentrations can be controlled. The degree of toxicity seems to be related to the difference between the intracellular and extracellular (i.e. the medium) pH values (Markou et al., 2014). Ammonia toxicity should be taken into account when, as in this case, wastewaters rich in ammonia are used as cultivation medium. To avoid the negative effect

of the free ammonia, it is necessary to regulate the pH value, keeping it well below the value of pK.

Growth experiments were performed at 28°C, in bottles reactors under a continuous CO_2-air flow (5% v/v) and constant light intensity of 100 µmol photons $m^{-2}s^{-1}$. In *Figure 3.1* the growth curves for all the species are reported. None of them suffered the high concentrations of nutrients, but in all experiments, as mentioned early, a precise pH control was required. In fact, Markou et al. (2014) also noticed that when ammonium is used as nitrogen source the pH might drop due to the release of H^+ during assimilation. Indeed, in these experiments, the pH decreased with microalgae growth, up to a value of pH 3, if not controlled. For this reason, a solution of NaOH (5 g/L) was used. *Table 3.1* reports the growth rate constants. *S. oliquus* showed the fastest growth rate.

Table 3.1 *Growth rate of microalgal species in batch experiments in BG11 500N/80P*

Species	Growth rate (day^{-1})
MZ	1.061
SOB	1.272
CV	1.063
CP	1.085

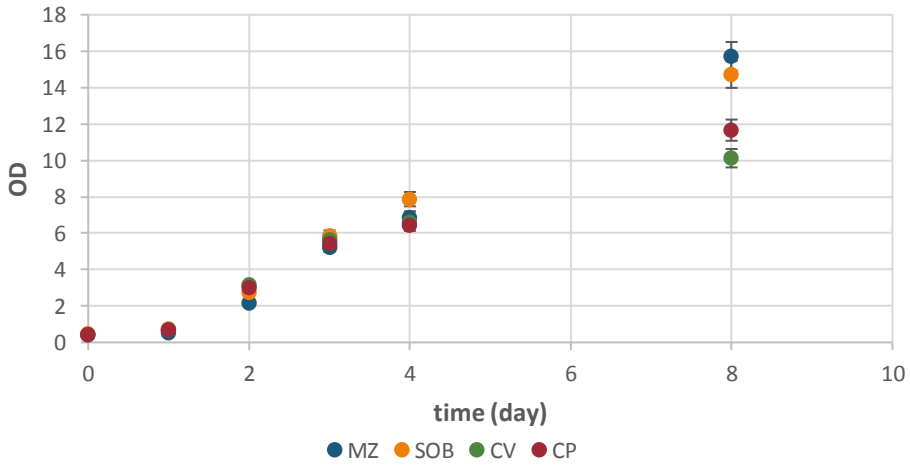


Figure 3.1 *Growth curves of M. zofingiensis (MZ), S. obliquus (SOB), C. vulgaris (CV) and C. protothecoides (CP) in BG11 500N/80P*

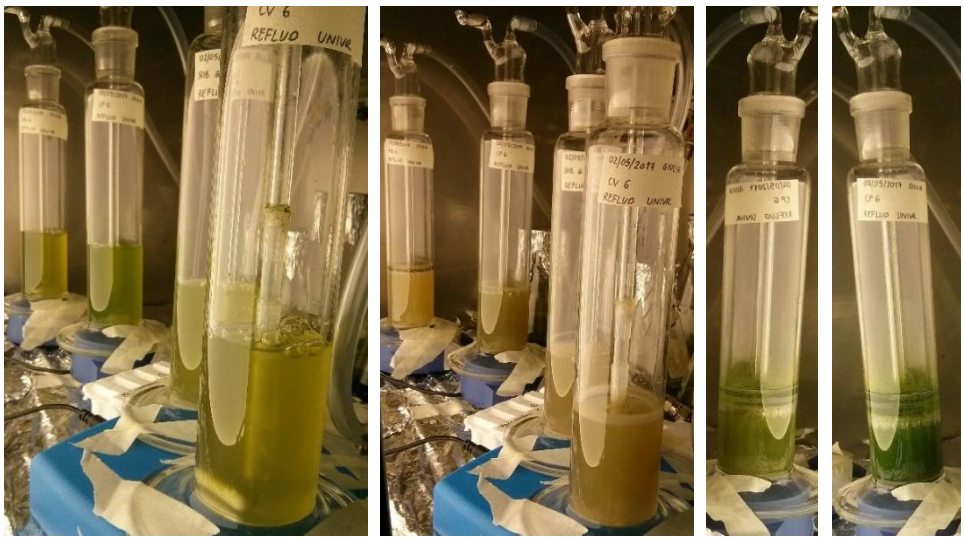
At the end of the growth curve, the remaining content of ammonium and phosphate in the medium was measured after filtration of the biomass, and results in terms of nutrient/biomass yields are shown in *Table 3.2* together with initial and final biomass concentrations. The values obtained for yields were consistent with experimental values found in literature. Sforza et al. (2015) for *S. obliquus* reported yields for phosphorus ranging from 0.01 to 0.045 $mg_P/mg_{biomass}$, while for nitrogen, values ranged from 0.075 to 0.25 $mg_N/mg_{biomass}$.

Table 3.2 *Initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$) for all species*

Species	C_{in} (g/L)	C_{fin} (g/L)	$Y_{P/X}$ ($mg_P/mg_{biomass}$)	$Y_{N/X}$ ($mg_N/mg_{biomass}$)
MZ	0.13	2.86	0.036	0.118
SOB	0.15	2.68	0.037	0.103
CV	0.16	2.2	0.044	0.129
CP	0.19	1.92	0.047	0.155

3.2 Screening of microalgal species in real wastewater

The four species of microalgae, proven to be able to resist in media with high ammonium concentration, were inoculated in the liquid obtained after the acidogenic fermentation (see §1.6.1). The aim of cultivating microalgae in this substrate is decreasing the nutrients content without consuming COD, so to make it available for subsequent bacterial PHA production. In fact, in this medium COD is mainly composed of volatile fatty acids, high value compounds, used in the following batch phase, where polymer accumulation in the biomass occurs, rather than biomass growth (see §1.6.3). For this reason, it was necessary to promote the photoautotrophic metabolism of microalgae, giving them an inorganic carbon source in excess, carbon dioxide, and light as source of energy. Sforza et al. (2012) proved that providing excess CO_2 stimulates microalgal photoautotrophic growth, maximizing photosynthetic metabolism. At the same time, this reduces the efficiency of organic substrate uptake, which remains in the culture medium. In these experiments bottle reactors were used. They were maintained at 28°C, at a constant light intensity of 100 µmol photons $m^{-2}s^{-1}$, and were fed with air enriched with excess CO_2 (5% v/v) bubbled through the culture. The four species of microalgae were inoculated into 100 mL of non-sterilized medium with an initial OD of 0.5 (*Figure 3.2a*).



(a) *(b)* *(c)* *(d)*

Figure 3.2 *Experiments with real medium and microalgae (a) day 0, four species (b) day 6, four species (c) day 13, C. protothecoides (d) day 15, C. protothecoides*

After 6 days *M. zofingiensis*, *S. obliquus* and *C. vulgaris* did not survive (*Figure 3.2b*). *C. protothecoides* needed 13 days to adapt to the new medium and to start to grow (*Figure 3.2c*). *Figure 3.3* reports the growth curve for *C. protothecoides*. The OD measured suggested that an increase of concentration occurred even in the early days, with a different trend with respect to cell counts. The medium was not sterilized, to be more consistent with actual industrial conditions, so these higher initial OD values possibly corresponded to the growth of other microorganisms within the reactor. After the 13th day, an exponential phase was observed, in terms of both optical density and cell concentration. For this reason, the growth rate constant of *Chlorella* has been calculated in relation to the cell count at this stage and it corresponded to 0.305 day^{-1}. Due to the long lag phase (13 days), *C. protothecoides* was continuously kept in this medium, and this culture was then used as preinoculum for all further experiments, in order to avoid long acclimation period in subsequent experimental runs.

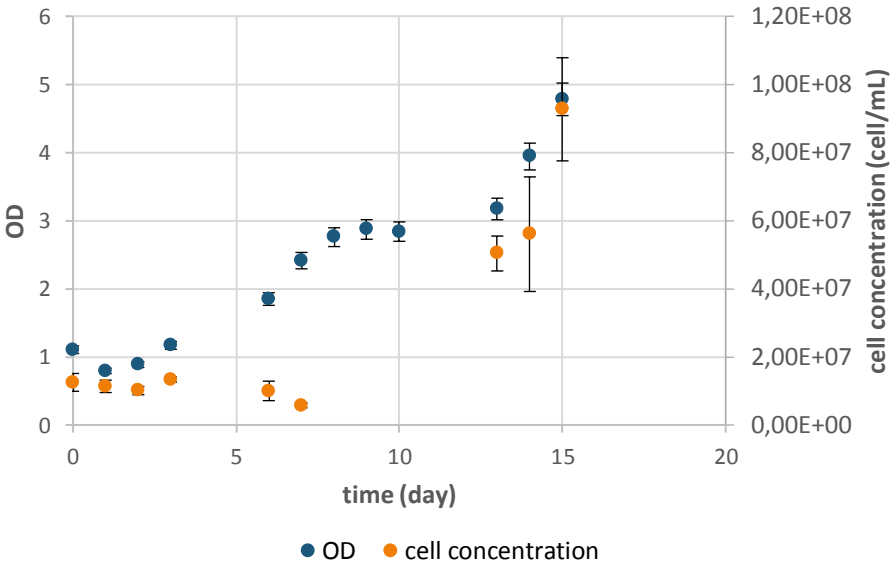


Figure 3.3 *Growth curve of C. protothecoides in the fermentation liquid*

Chlorella was reinoculated, using a sample of the culture preadapted in the medium. The growth curve obtained is shown in *Figure 3.4*. The lag phase had a significantly lower extent, and the calculated growth rate constant was 0.835 day^{-1}. After this acclimation, the growth of this species was found to be reproducible, suggesting that *C. protothecoides* adapted to the composition of this medium. In addition, the strong resistance capability of this species was confirmed, as it survived in wastewater for months, thus showing no competition with endogenous microflora.

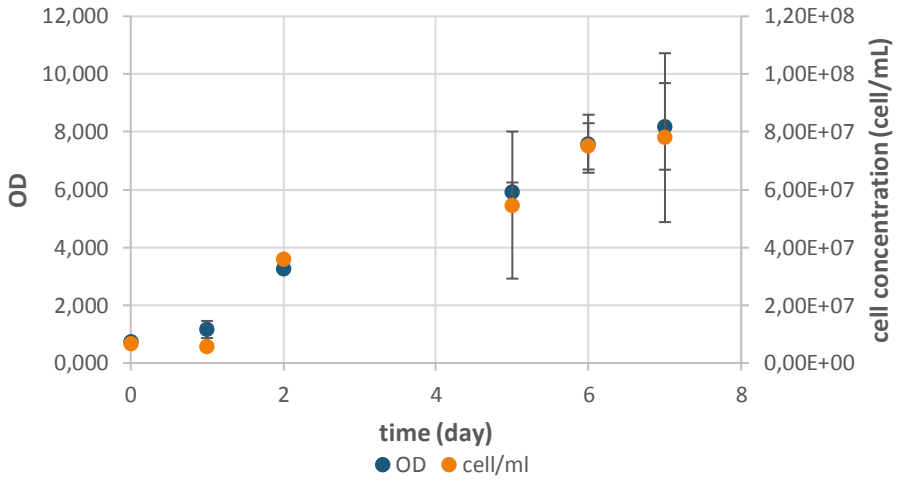


Figure 3.4 *Growth curve of C. protothecoides in the fermentation liquid with preadapted inoculum*

3.3 Experiments with *Chlorella protothecoides 33.80*

The specific growth rate of *C. protothecoides* in standard media is usually higher than that observed in real wastewater. Many factors might play a role on this, and some experiments were carried out to assess which variable could affect the growth rate, accordingly. Specifically, the medium was modified by the addition of micronutrients to verify a possible limitation, then it was diluted to verify possibly inhibition and sterilized to observe interaction with bacteria. Unless otherwise specified, all these experiments have been carried out under the same experimental conditions as defined in §3.2. Subsequently, the effect of different light intensity on the growth and on nutrient consumption will be analysed.

3.3.1 Experiments with micronutrients

To verify that there was no limitation of essential micronutrients for microalgae growth, these were added to the culture medium, according to the specific concentrations reported in *Table 3.3*.

Table 3.3 *Micronutrients added to the culture medium and their concentration*

Component	Concentration (mg/L)
Na_2Mg EDTA	1
Ferric ammonium citrate	6
Citric acid · H_20	6
$CaCl_2$ ·$2H_20$	36
$MgSO_4$·$7H_20$	75
H_3BO_3	2.86
$MnCl_2$ ·$4H_20$	1.81
$ZnSO_4$·$7H_20$	0.222
$CuSO_4$·$5H_20$	0.079
$COCl_2$·$6H_20$	0.050
Na_2MoO_4·$2H_20$	0.391

Thinking of a real wastewater treatment plant, adding micronutrients to the medium would be expensive, so it would be done only if these were essential for the growth of microalgae. It could be seen in *Figure 3.5* that the growth rate and final cell concentration reached when micronutrients were supplied was similar to the one obtained in the other experiments. Apparently in that medium all essential compounds were present in the sufficient amount and, for this reason, in the following experiments the medium was not modified.

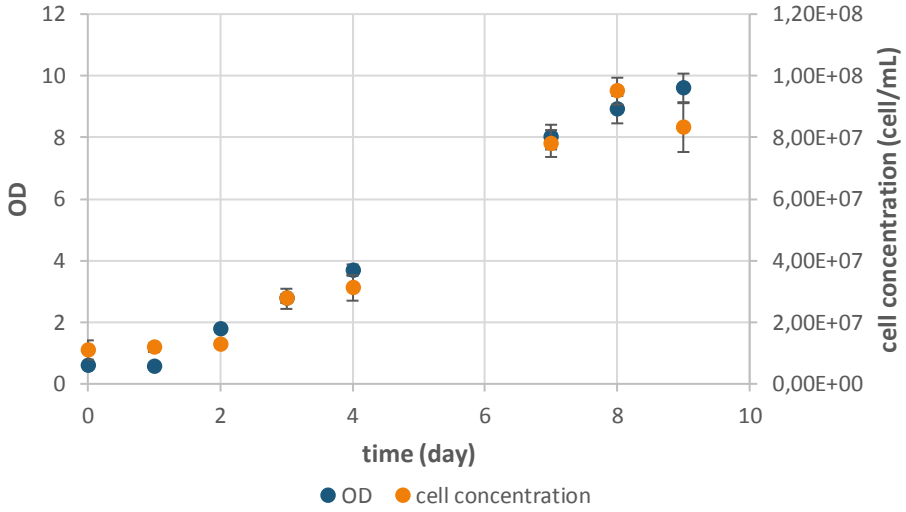


Figure 3.5 *Growth curve of C. prototheocoides in the fermentation liquid with the addition of micronutrients*

3.3.2 Experiments with diluted medium

In these experiments, the medium was diluted by 50% with demineralized water to verify if the concentration of some compounds present in the medium might inhibit the growth of *Chlorella*, reducing its growth rate. *Figure 3.6* shows the growth curve in term of both optical density and cell concentration.

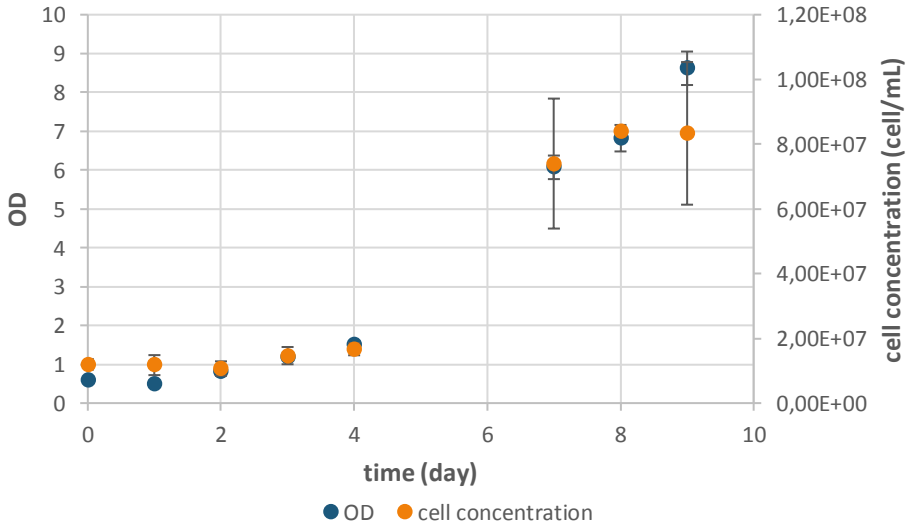


Figure 3.6 *Growth curves of C. prototheocoides in the fermentation liquid diluted at 50% with demineralized water*

The lag phase seemed to last only two days; however, the growth rate constant did not differ much from the one identified in the first growth curve, and it was equal to 0.285 day^{-1}. Starting from this culture, a new experiment was reinoculated in the same experimental conditions. In this case, the calculated growth rate constant was more than doubled compared to the previous one, and was equal to 0.728 $days^{-1}$. It was therefore essential to use a pre-inoculum already adapted to the specific experimental conditions to allow the microorganism to grow efficiently.

3.3.3 Experiments with sterilized medium

To verify the growth of an axenic culture of *C. protothecoides*, the medium was sterilized in autoclave for 20 minute at 121°C (*Figure 3.7a*) before being inoculated. The OD and cell count measurements decreased with time and, as can be seen from *Figure 3.7b*, *Chlorella* did not survive in these conditions. Another attempt was made, the wastewater was sterilized through centrifugation (10000 rpm for 15 minutes) and filtration with 0.2 µm filters.

(a) *(b)*

Figure 3.7 *(a) Sterilized real medium (b) Final day of batch experiment of Chlorella in sterilized medium*

The growth curve obtained is shown in *Figure 3.8*. Even in this case, the OD value decreased, confirming that in these experimental conditions *Chlorella* is not able to survive in the medium. These results suggest that some interactions between the microorganisms present in the non-sterilized medium and the microalgae occurred. Several studies have shown that consortia may positively affects physiology and metabolism of both algae and bacteria. The mechanism of the interaction is species specific as the microenvironment of each alga is different. Algae and bacteria interact in different modes, and mutualism,

commensalism and parasitism are some example (Ramanan et al., 2016). In these experiments, it was clear that the presence of the other microorganisms was essential for the survival of the microalgae. In particular, it appeared that a sequential growth (bacteria before, and microalgae after) occurred (see *Figure 3.3*), suggesting that probably bacteria transform some compounds of the wastewater that can be inhibitory for the microalgae alone. As a matter of fact, a consortium algae-bacteria could be used in the environmental technologies to obtain high-value products, as well as medium to high value chemicals.

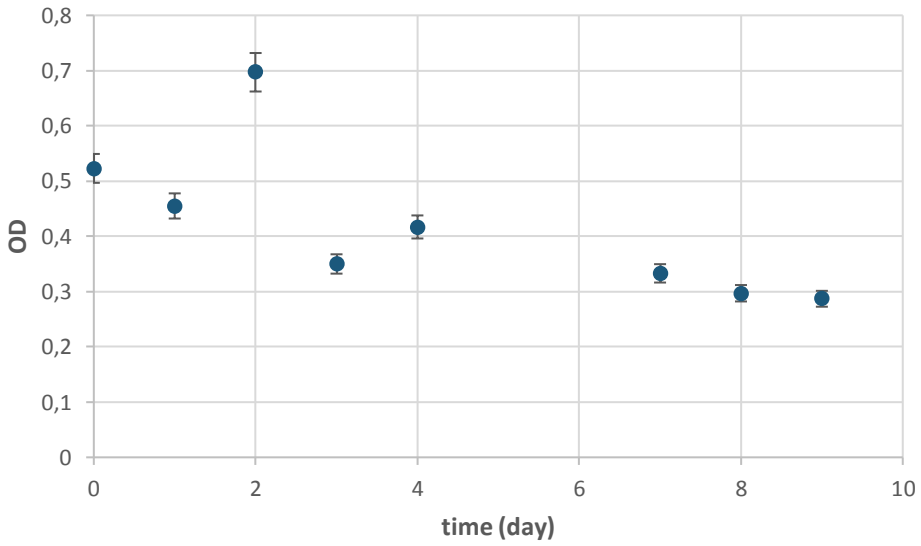


Figure 3.8 *Growth curve of Chlorella in flat panel reactor in sterilized medium*

3.3.4 Effect of light intensity

To analyse the effect of different light intensity on *Chlorella* growth and on nutrient consumption, some experiments have been performed in a different cultivation system, a flat plate photobioreactor. This type of reactor has the advantage of having high surface to volume ratio and the light path can be very short and evenly distributed across the reactor, minimizing the self-shading effect. Moreover, they have the flexibility as regards light capture angle and, depending on the agitation method used, a high mixing rate can be achieved along with low shear stress (Skjånes et al., 2016). The inoculum was always such as to give an initial OD of about 0.6 in 100 mL of non-sterilized medium. The reactor was maintained at a constant temperature of 28°C thanks to a thermostated incubator, and air enriched with CO_2 (5% v/v) was provided bubbling the reactor at the bottom. The reactor was exposed to three different light intensities: 100, 125 and 185 µmol photons $m^{-2}s^{-1}$ and *Figure 3.9* shows the three growth curves obtained. Although a pre-adapted inoculum was used, in the early days there was always a presence of a lag phase that last almost for three days.

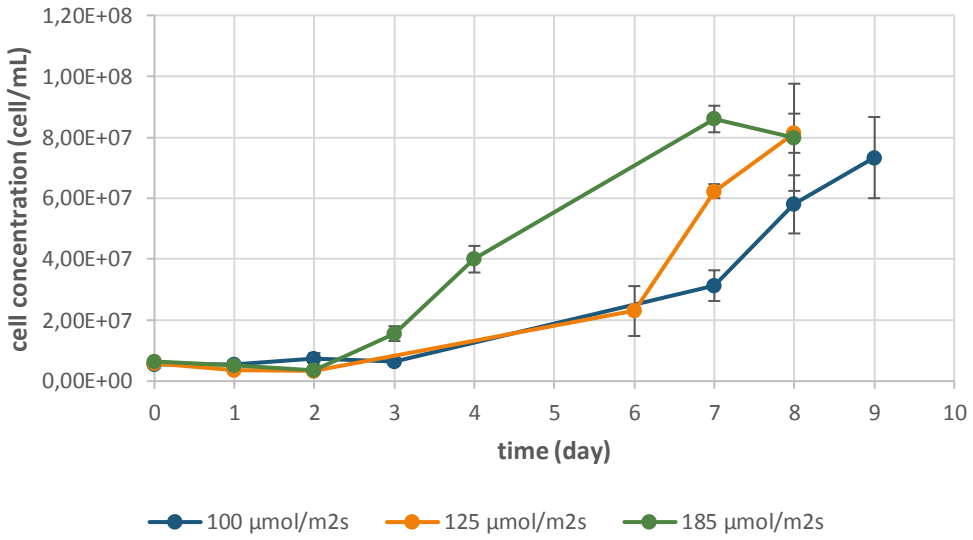


Figure 3.9 *Growth curves of C. prototheocoides with different light intensity*

In *Table 3.4* the calculated growth rates constants are reported. Looking at the values identified, there was a slightly increase in the growth rate when light was enhanced from 100 to 125 µmol photons $m^{-2}s^{-1}$, but when it was further increased to 185 µmol photons $m^{-2}s^{-1}$, *Chlorella* showed a considerable high growth rate constant, equal to 1.213 day^{-1}.

Table 3.4 *Growth rate of C. prototheocoides measured with different light intensity*

Light intensity (µmol photons $m^{-2}s^{-1}$)	Growth rate (day^{-1})
100	0.430
125	0.554
185	1.213

Ogbonna and Tanaka (2000) stated that the effect of light intensity on microalgal growth could be classified as four phases including:

- a lag phase where growth rate stays similar as the increase of light intensity;
- a limitation phase where growth rate rises with the increase of light intensity;
- a saturation phase where growth rate is independent from light intensity;
- an inhibition phase where the growth rate declines with the increase in light intensity.

Li et al. (2012) observed the effect of light for algae strain *C. prototheocoides*, and detected light limitation phase from 0 to 30 µmol photons $m^{-2}s^{-1}$, light saturation from 30 to 120 µmol photons $m^{-2}s^{-1}$, but identified a considerably enhanced growth rate when light was further increase to 200 µmol photons $m^{-2}s^{-1}$. However, light influenced not only the growth rate of microalgae, but also their biochemical composition. Although with higher light intensity a relevant growth rate was identified, the final cells concentration did not differ so much in the three experimental conditions, and on average it is equal to $(7.82\pm0.43)\cdot10^{7}$ cell/mL. Thus, it could not be assumed that biochemical composition of cells was changed.

The growth of *Chlorella* may be limited by several factors, for example, by nutrient or light limitation. However, the complexity of the system prevents from more precise assessments. At the same time, looking at *Table 3.5*, it could be noticed also that the higher the light intensity applied, the higher was the final biomass concentration measured. This was probably due to a higher availability of oxygen produced through photosynthesis by the microalgae, which promoted also the growth rate of the other endogenous microorganisms, causing a higher final concentration of the total biomass.

Table 3.5 *Initial and final biomass concentration at different light intensity*

Light intensity (µmol photons $m^{-2}s^{-1}$)	C_{in} (g/L)	C_{fin} (g/L)
100	0.08	1.75
125	0.08	2.59
185	0.09	3.12

On the last day of the experiments, an analysis on the filtrate was carried out to determine the residual contents of nutrients, following the procedures described in §2.5. The attention was focused on the nutrients to be removed, ammonium and phosphorus, and on the COD content, to be preserved. Student's t tests were applied to ascertain significant differences in nutrients contents in all the experimental conditions applied. The level of statistical significance was $p < 0.05$. *Figure 3.10* shows the initial and final phosphorus values measured. Phosphorus was almost totally consumed in all the experimental conditions, reaching the maximum of 96.25% of removal for the higher light intensity. Li at al. (2012) already demonstrated for *C. kessleri* that a higher light intensity, which resulted in higher biomass growth, led to higher phosphorus removal. However, the higher value of yield was obtained with the lower light intensity (see *Table 3.6*).

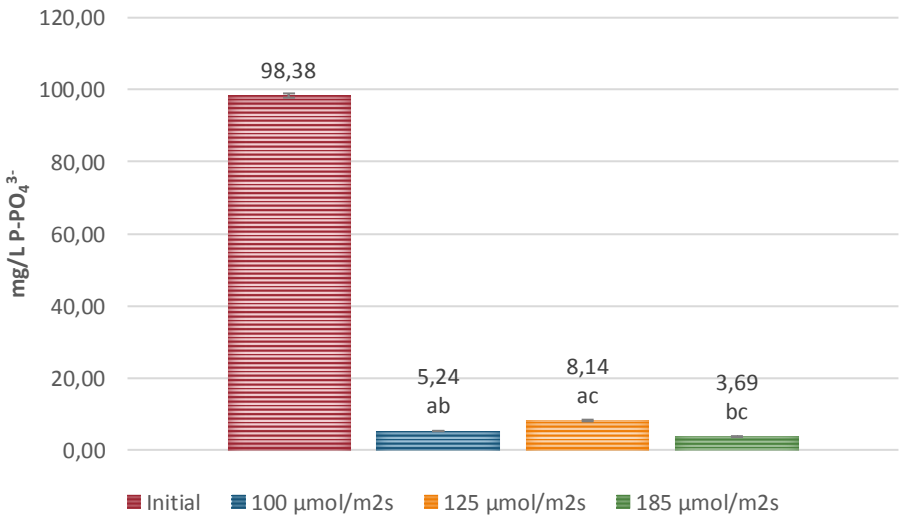


Figure 3.10 *Initial and final concentration of phosphorus at different light intensity*
The same letters correspond to statistically significant results

Table 3.6 *Phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$) obtained for different light intensity*

Light intensity (µmol photons $m^{-2}s^{-1}$)	$Y_{P/X}$ ($mg_P/mg_{biomass}$)	$Y_{N/X}$ ($mg_N/mg_{biomass}$)
100	0.056	0.159
125	0.036	0.185
185	0.031	0.099

Figure 3.11 shows the initial and final content of nitrogen as ammonium. Nitrogen was removed more when the flat plate PBR had a continuous illumination of 125 µmol photons $m^{-2}s^{-1}$, reaching a nitrogen/biomass yield of 0.185 $mg_N/mg_{biomass}$. At 185 µmol photons $m^{-2}s^{-1}$, the yield decreased extremely, nearly halving its value.

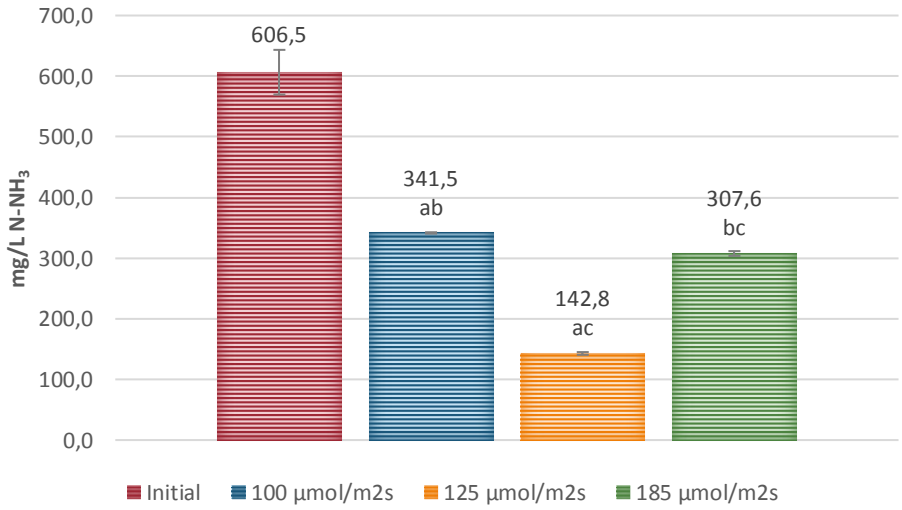


Figure 3.11 *Initial and final concentration of ammonium at different light intensity*
The same letters correspond to statistically significant results

In *Figure 3.12* the initial and final COD content are reported.

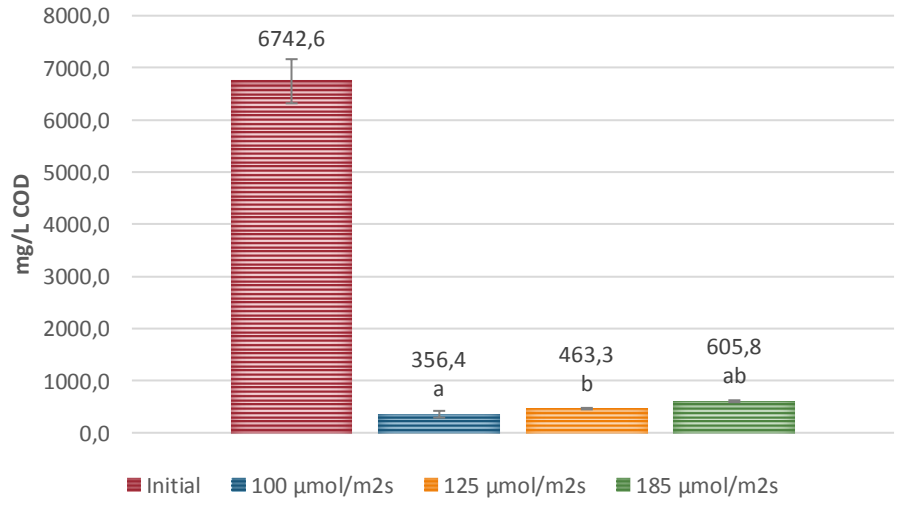


Figure 3.12 *Initial and final concentration of COD at different light intensity*
The same letters correspond to statistically significant results

At higher light intensity, less organic carbon was consumed. This was also described by Sforza et al. (2012), that observed an increased growth rate under CO_2 excess and also an inhibition of mixotrophic metabolism.

The analysis carried out demonstrated that the goal of removing ammonium and phosphorus without reducing the COD content in the medium was not achieved. Specifically, a remarkable phosphorus removal was obtained, which was almost total. Microalgae growth also reduced the ammonium concentration, but at the same time decreased the concentration of COD. *Table 3.7* shows the final concentration of COD and nitrogen as ammonium and the calculated COD/N ratio. Basset et al. (2016) stated that this ratio should have a value of 2.5 to obtain a complete denitritation and a correct biomass selection in the sequential batch reactor (see §1.6.2). The initial ratio in the medium was 11.12. The cultivation of microalgae in fermentation liquid reduced this ratio down to 1.97, thus approaching the desired value of 2.5. *Table 3.7* specifies also the concentration of the external carbon source to be used to reach the desired ratio. However, even if the ratio obtained was taken up to the desired value, much of the COD has been removed, causing an undesirable loss in terms of high-value compounds. Indeed, consuming COD in this medium means consuming volatile fatty acids, that are essential if this liquid has to be used in the subsequent sequential batch reactor and in the batch accumulation phase, to produce PHA from sludge reject water.

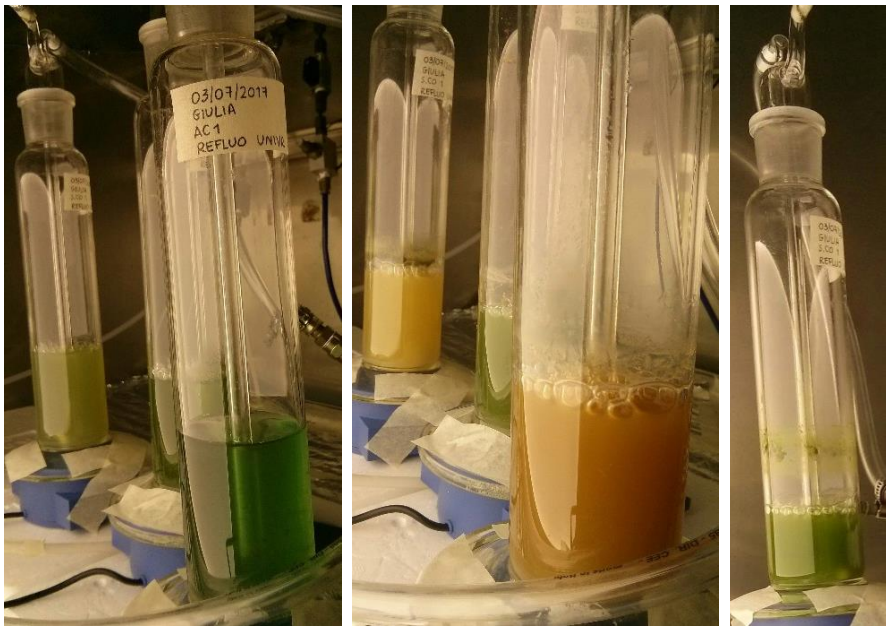
Table 3.7 *Final COD and nitrogen concentration, COD/N ratio and concentration of external carbon source to use to achieve a COD/N ratio equal to 2.5*

Light intensity (µmol photons $m^{-2}s^{-1}$)	**g/L COD**	**g/L N-NH_3**	**g COD/g N**	**g/L external carbon source**
100	0.356	0.342	1.04	0.497
125	0.463	0.143	3.24	-
185	0.606	0.308	1.97	0.163

3.4 Screening of cyanobacteria

In this section, the cultivation of cyanobacteria in the medium was analysed. Indeed, some of these microorganisms, under proper cultivation conditions, showed the ability to produce polyhydroxyalkanoates and other high-value compounds, like cyanophycin. Moreover, in this way the flowsheet of the process would also be simplified. The microorganisms would be cultivated directly in the liquid obtained after the acidogenic fermentation, and the sequential batch reactor and the accumulation phase would be removed. Cyanobacteria are the only prokaryotes that accumulate polyhydroxyalkanoates by oxygenic photosynthesis (Panda and Mallick, 2007; Ansari and Fatma, 2016). PHA accumulation is low under photoautotrophy, less than 10% (Ansari and Fatma, 2016). Enhanced

polyhydroxylkanoates accumulation has been observed in some cyanobacteria when grown mixotrophically with acetate. Wu et al. (2002) established that *Synechocystis* sp. PCC 6803 accumulated up to 15.2% under nitrogen-limited conditions in presence of sodium acetate, while Panda and Mallick (2007) demonstrated that combined effect of phosphorus deficiency and gas-exchange limitation in the presence of fructose and acetate boosted up to 38% (w/w) of dry cell weight in *Synechocystis* sp. PCC 6803. Three different species of cyanobacteria were tested in the fermentation liquid, *Anabaena cylindrica* PCC 7122, *Synechococcus sp*. PCC 7002 and *Synechocystis sp*. PCC 6803. These were inoculated in 50 mL of non-sterilized medium to have an initial OD of 0.5 (*Figure 3.13*).



(a) *(b)* *(c)*

Figure 3.13 *Experiments with real medium and cyanobacteria (a) day 0, three species (b) day 7, three species (c) day 10, Synechococcus sp. PCC 7002*

The bottle reactors, fed by a mixture air-CO_2 (5% v/v) are located in a thermostated incubator at a constant temperature of 28°C and at a constant light intensity of 100 μmol photons $m^{-2}s^{-1}$. *Anabaena* was the only one of the three microorganisms that did not survive, while the other two showed very different growth curves.

Synechococcus sp. PCC 7002 initially has a lag phase of 9 days, after which an exponential phase begins, which ends on the 21st day of growth. *Figure 3*.14 shows its growth curve in term of optical density. The OD values measured were incredibly high, reaching up to 31.76 on the 21th day of growth. Additionally, pigments analysis was carried out daily, and it was used as an index of growth to account for the growth of the cyanobacterial species only. *Figure 3.15* and *Figure 3.16* report growth curve for *Synechococcus* in terms of chlorophyll *a* and carotenoid contents.

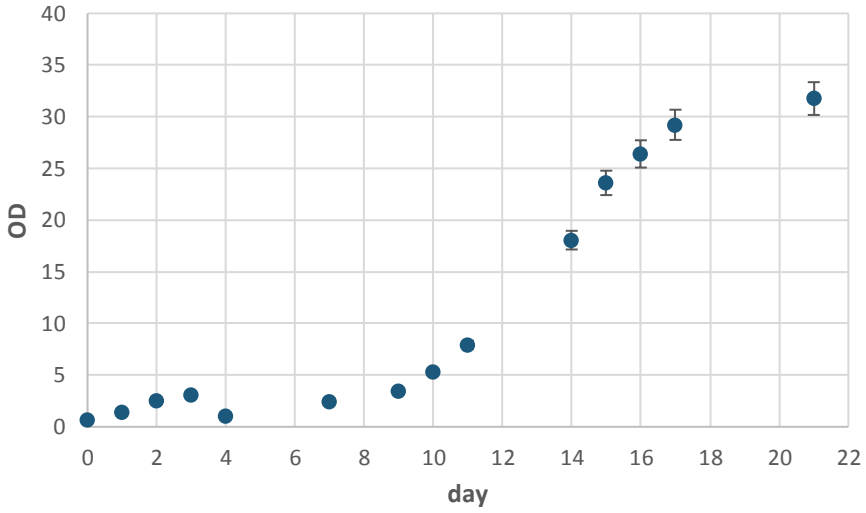


Figure 3.14 *Growth curve of Synechococccus sp. PCC 7002*

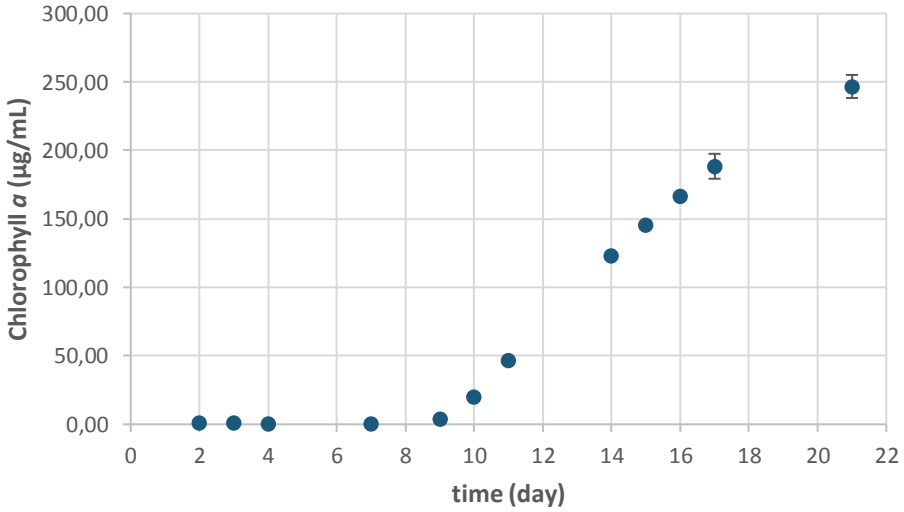


Figure 3.15 *Growth curve in terms of chlorophyll a of Synechococccus sp. PCC 7002*

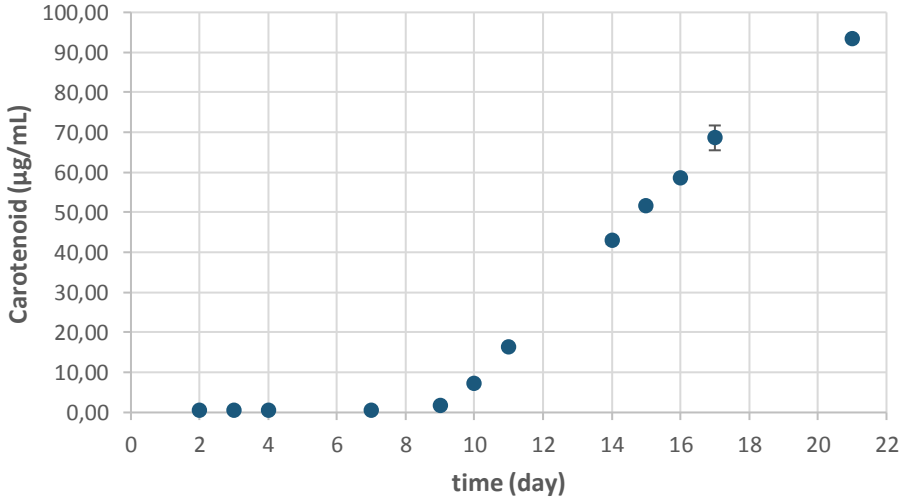


Figure 3.16 *Growth curve in terms of carotenoid of Synechococccus sp. PCC 7002*

It is clear that the lag phase for the cyanobacteria last 9 days, after which the exponential phase started. For this reason, the growth rate was calculated with respect to the content of chlorophyll *a*, and it was equal to 1.194 day^{-1}. Moreover, the biomass concentration also reached high values, and finally it was measured 7 g/L. Similar values for the growth rate were found in literature, together with the demonstration that *Synechococcus* sp. PCC7002 was able produce high amount of biomass, reaching final concentration of about 6 g/L (de Farias Silva et al., 2016).

On the 7th and on the last day of growth a sample of the culture was filtered and the nutrients analysis, phosphorus, nitrogen as ammonium and COD, were carried out. As can be seen from *Figure 3.17*, 92.36% of the phosphorus was consumed in the first seven days. This behaviour is known as luxury uptake, and is observed in microalgae as well as in cyanobacteria. They have the capability to sequester from the environment much more phosphorus than that immediately necessary for growth. These intracellular phosphorus reserves are accumulated as polyphosphate granules, and can be used as a phosphorus source when phosphate becomes depleted in the surrounding medium (Powell et al., 2009).

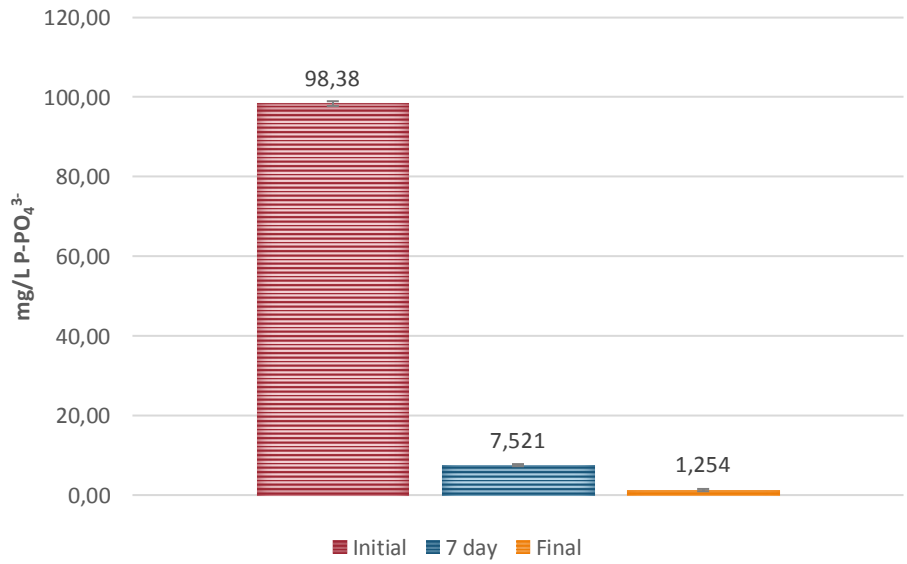


Figure 3.17 *Initial and final concentration of phosphorus*

On the other hand, *Figure 3.18* revealed that ammonia was consumed mainly after the seventh day. It is suggested that probably a sequential growth of bacteria and cyanobacteria occurred. In the first days, bacteria contributed to the removal of phosphorus, with the simultaneous luxury uptake by microalgae. On the other hand, bacteria initially were unable to consume ammonia probably due to the low oxygen concentration, which started to increase only during active growth of cyanobacteria (from the 7th day). *Figure 3.19* shows initial and final concentration of COD measured. Final concentration id slightly higher than 9th days sample, probably because a stationary phase of cyanobacterial growth was reached

and this commonly lead to a release of secondary metabolites that results in an increased COD in the medium.

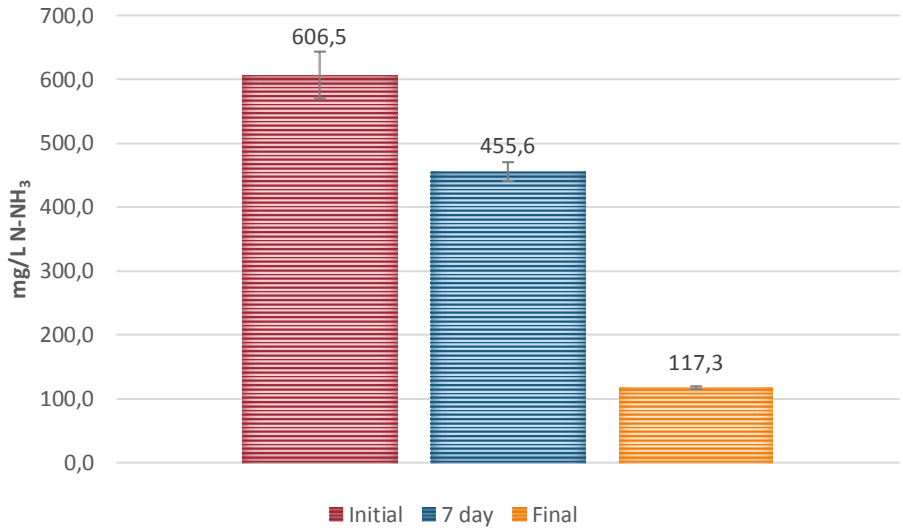


Figure 3.18 *Initial and final concentration of nitrogen*

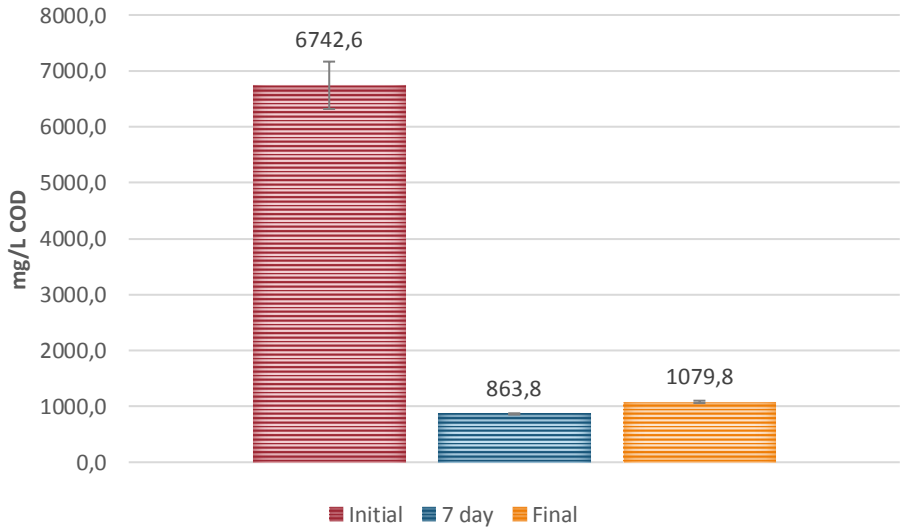


Figure 3.19 *Initial and final concentration of COD*

Table 3.8 shows initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$), and the values obtained perfectly matched ranges indicated in §3.1.

Table 3.8 *Initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$) and nitrogen/biomass yield ($Y_{N/X}$)*

C_{in} (g/L)	C_{fin} (g/L)	$Y_{P/X}$ ($mg_P/mg_{biomass}$)	$Y_{N/X}$ ($mg_N/mg_{biomass}$))
0.860	7.0	0.016	0.080

On the other hand, *Synechocystis* sp. PCC 6803 showed a very different growth curve, reported in *Figure 3.20*.

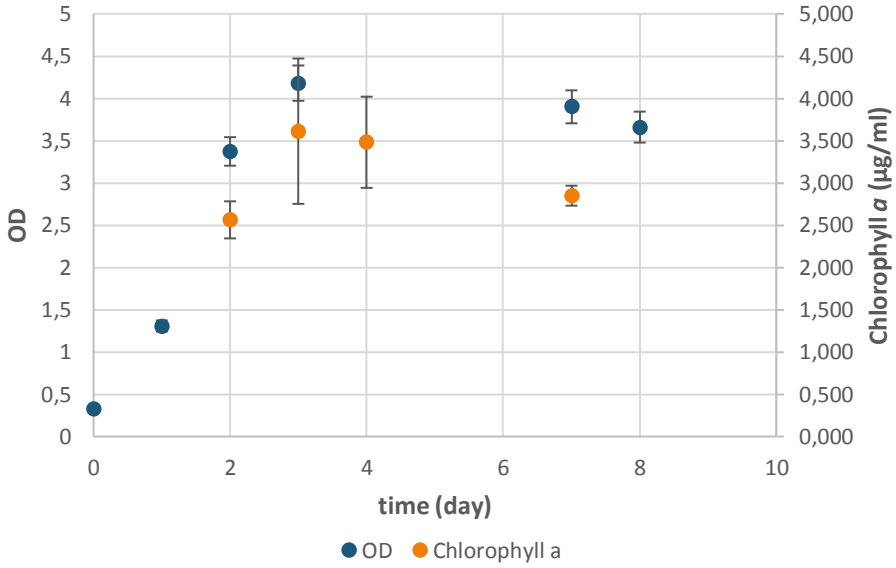


Figure 3.20 *Growth curve of Synechocystis sp. PCC 6803 in the fermentation liquid*

The main difference with *Synechococcus* is that there was no lag phase, even in the early days. Both the measured OD and the chlorophyll *a* content revealed an increasing trend from the first day, corresponding to the exponential phase of the growth curve. The measured growth rate constant was equal to 0.581 day^{-1}.

Both *Synechococcus* sp. and *Synechocystis* sp. did not shown any particular problems in acclimation to this highly concentrated medium. Consequently, both of them may have great potential in environmental technologies. Vijayakumar (2012) already stated that cyanobacteria are good candidates for wastewater treatment, due to their capability to use nitrogen compounds, ammonia and phosphate and to remove metal ions such as chromium, cobalt, copper and zinc. Moreover, the production of cyanobacterial biomass is abundant and can be used as a source for high value products. An example was polyhydroxyalkanoates, and Balaji et al. (2013) reported that the occurrence of PHB has been demonstrated for several cyanobacterial species, including *Synechococcus* sp. and *Synechocystis* sp.. However, given the results obtained in the first growth curves, the species *Synechocystis* sp. PCC 6803 was chosen, as it demonstrated to have a versatile carbon metabolisms, growing under photoautotrophic, mixotrophic and heterotrophic conditions. Moreover, *Synechocystis* metabolism has unique responses to environmental stresses (e.g., cold-stress, hyperosmotic stress and salt stress). In unfavourable environments, it shows activation of alternate pathways for the acquisition of carbon and nitrogen and for the reduction of photosynthesis efficiencies (Yu et al., 2013).

3.5 Experiments with *Synechocystis* sp. PCC 6803

The selected cyanobacterial species was inoculated with an initial OD of about 0.5-0.7 in 100 mL of the liquid obtained after the acidogenic fermentation. Bottle reactors were used, and the effect of carbon dioxide, temperature and different light intensity on growth and on nutrients consumption has been analysed. Pigments analysis was carried out daily, and it was used as an index to account for the growth of the cyanobacterial species only. For this reason, the growth rate was calculated with respect to the content of chlorophyll *a*. Student's t tests were applied to ascertain significant differences in nutrients contents in all the experimental conditions employed. The level of statistical significance was $p < 0.05$. All the experiments were performed in at least two independent biological replicates.

3.5.1 Effect of CO_2

Initially, the effect of CO_2 was evaluated. In one experimental conditions, no air was bubbled inside the reactor, in the other one reactors were provided with a mixture of air-CO_2 (5% v/v) bubbled through the culture. In both cases, reactors were perfectly mixed with a magnetic stirrer, and were maintained at a constant temperature of 28°C and under a constant light intensity of 100 µmol photons $m^{-2}s^{-1}$. Growth curves obtained are shown in *Figure 3.21*. The final OD reached when CO_2 was provided was greater than the other one, but *Synechocystis* needed more time to reach the stationary phase. This seemed to be reasonable, because in that case microorganisms had available both types of carbon, the organic one and the inorganic one.

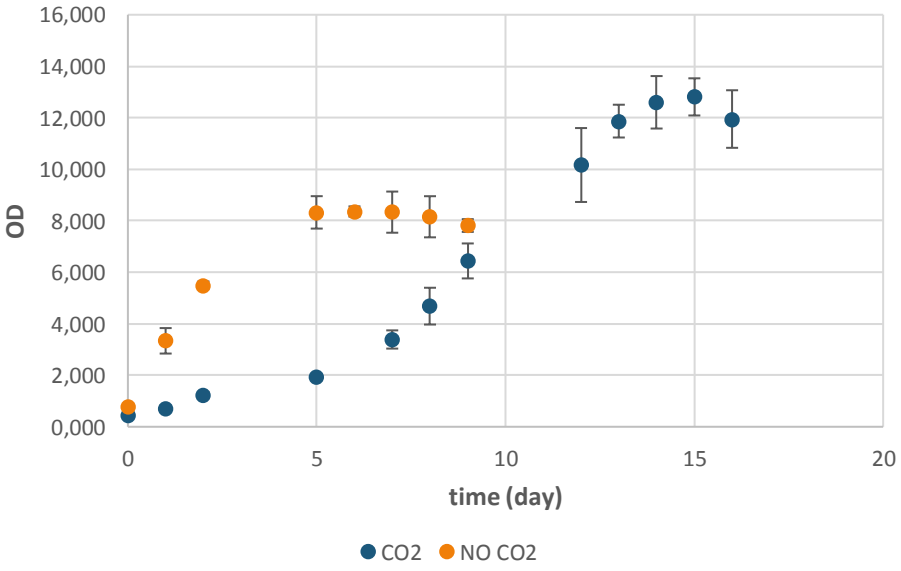


Figure 3.21 *Growth curve of Synechocystis sp. PCC 6803 with/without CO_2*

However, looking at the figures showing the trend of chlorophyll *a* (*Figure 3.22*) and carotenoid (*Figure 3.23*), it could be seen that *Synechocystis* grew much more when CO_2 was not supplied, reaching a higher final contents of both pigments.

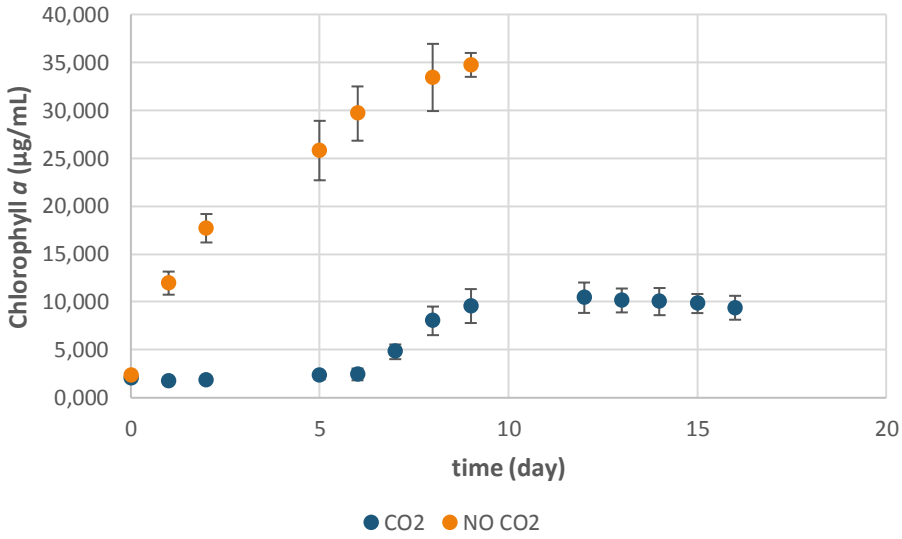


Figure 3.22 *Growth curve in terms of chlorophyll a of Synechocystis sp. PCC 6803 with/without CO_2*

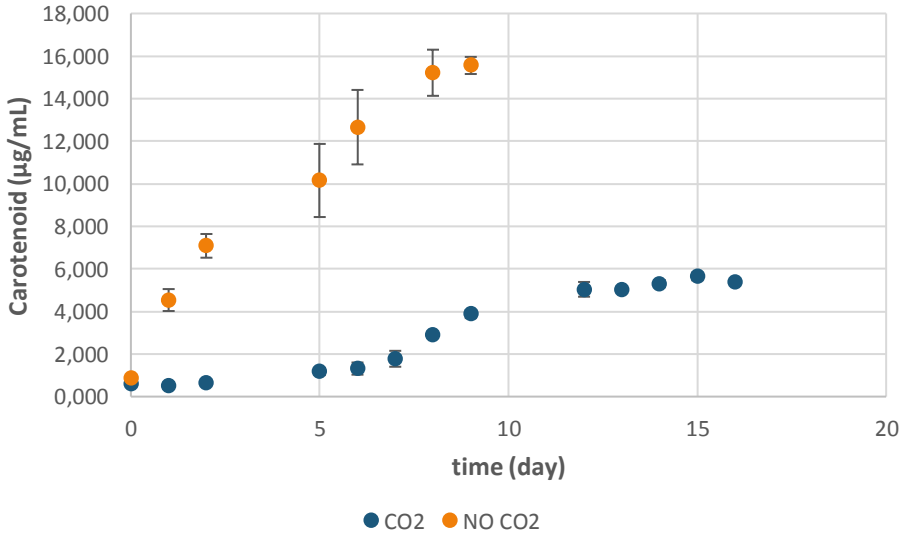


Figure 3.23 *Growth curve in terms of carotenoid of Synechocystis sp. PCC 6803 with/without CO_2*

The growth rate constant calculated when the culture was supplied with CO_2 was similar to that previously measured (§3.4), and was equal to 0.6 day^{-1}. On the opposite, when organic carbon was not supplied, the growth rate measured was significantly greater, and it was equal to 1.02 day^{-1} (*Figure 3.24*). Obviously, the pH in the two experimental conditions was different. In the presence of CO_2 it varied between 7-7.5 while in the absence of CO_2 it was always measured within range 8-8.5. It could be concluded that the

latter experimental condition was more favourable to the growth of *Synecocystis* in that specific medium. Yu et al. (2013) confirmed the capability of this cyanobacteria to grow either photoautotrophically or photoheterotrophically. Moreover, they noticed that *Synechocystis* may co-metabolize different organic acids, including pyruvate, acetate and succinate, when inorganic carbon was insufficient in the medium.

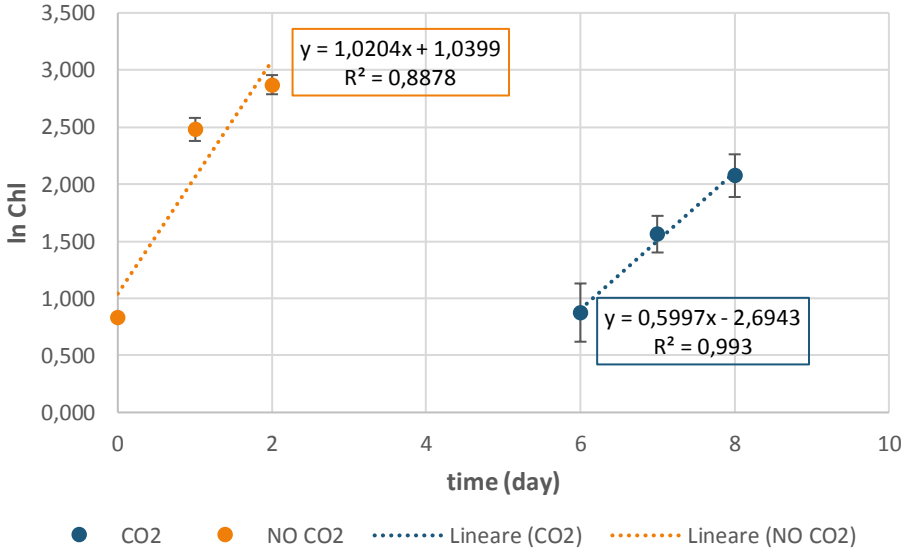


Figure 3.24 *Growth rate of Synechocystis sp. PCC 6803 with/without CO_2*

Figure 3.25 and *Figure 3.26* show, respectively, the initial and final concentration of phosphorus and ammonium measured in both experimental conditions.

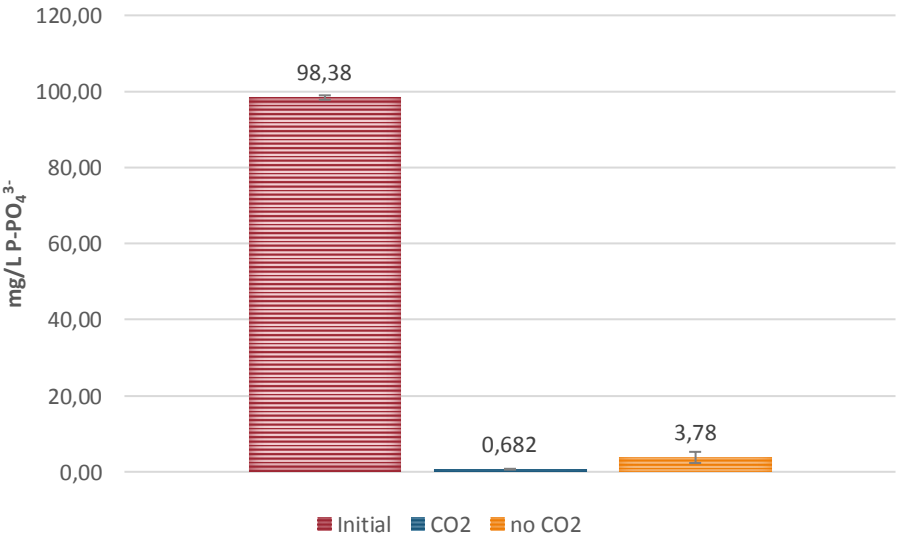


Figure 3.25 *Initial and final concentration of phosphorus with/without CO_2*

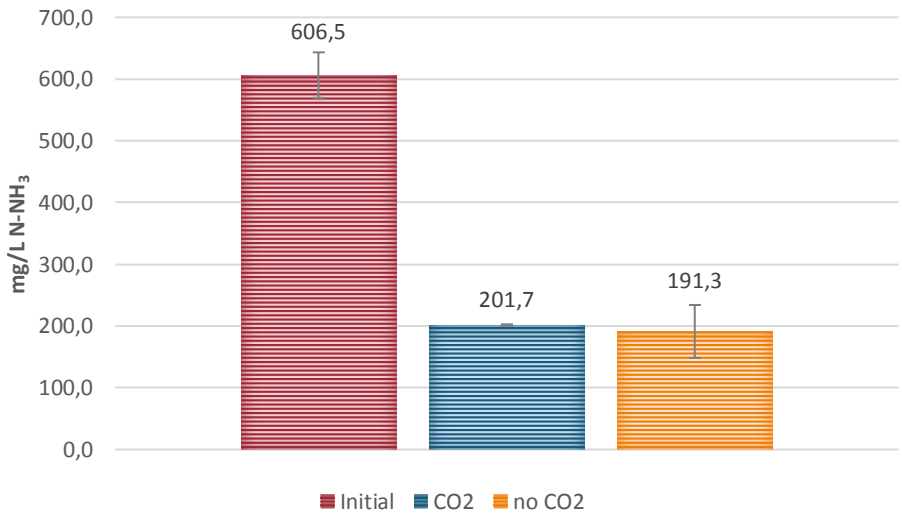


Figure 3.26 *Initial and final concentration of ammonium with/without CO_2*

Statistical analysis that revealed for both nutrients there was no significant difference between the data obtained. *Table 3.9* shows initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$).

Table 3.9 *Initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$) with/without CO_2*

	C_{in} (g/L)	**C_{fin} (g/L)**	**$Y_{P/X}$ ($mg_P/mg_{biomass}$)**	**$Y_{N/X}$ ($mg_N/mg_{biomass}$)**
CO_2	0.22±0.001	2.76±0.113	0.038	0.159
No CO_2	0.6±0.141	3.63±1.117	0.031	0.137

Figure 3.27 shows initial and final concentration of COD measured in both experimental conditions. There was no significant difference between the measured values, so that on average 76.77% of COD was removed by the microorganisms.

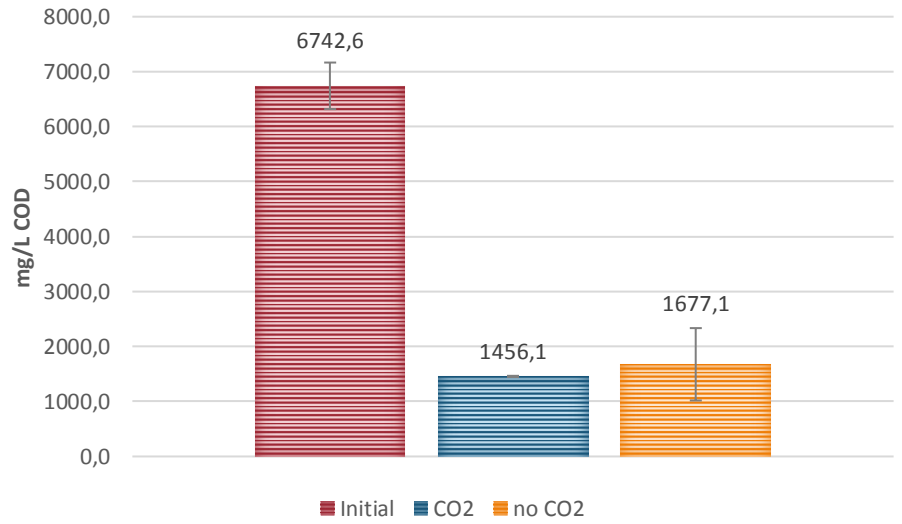


Figure 3.27 *Initial and final concentration of COD with/without CO_2*

The final biomass concentration was larger when CO_2 was not supplied (*Table 3.9*), but also the final contents of chlorophyll *a* and carotenoid was larger (*Figure 3.22* and *Figure 3.23*). Conversely, the OD measured seemed to indicate a higher concentration when inorganic carbon was provided (*Figure 3.21*). It was concluded that, when CO_2 was not supplied, the growth of cyanobacteria was favoured with respect to the one of other microorganisms, and, consequently, the consumption of the COD, the only source of carbon, might be mostly attributed to them. Moreover, Panda and Mallick (2007) observed that gas exchange limitation boosted up the accumulation of PHB. In this condition, an alkaline pH (8-8.5) was reached in the medium, but Panda et al. (2006) showed that at pH 8.5 PHB maximum accumulation was reached. For these reasons, all the subsequent experiments were performed without bubbling the mixture air-CO_2 through the culture.

3.5.2 Effect of temperature

The effect of three temperatures, 28°C, 32°C and 37°C, on the growth rate and on the consumption of nutrients was evaluated. Reactors at constant light intensity of 100 µmol photons $m^{-2}s^{-1}$ were maintained at the selected temperature with a thermostatic bath. Growth curves obtained are shown in *Figure 3.28*. The final OD measured was similar at 28°C and 32°C, instead at 37°C the final concentration reached was lower. *Figure 3.29* and *Figure 3.30*, reporting the growth curves in terms of pigments contents, also confirmed this trend.

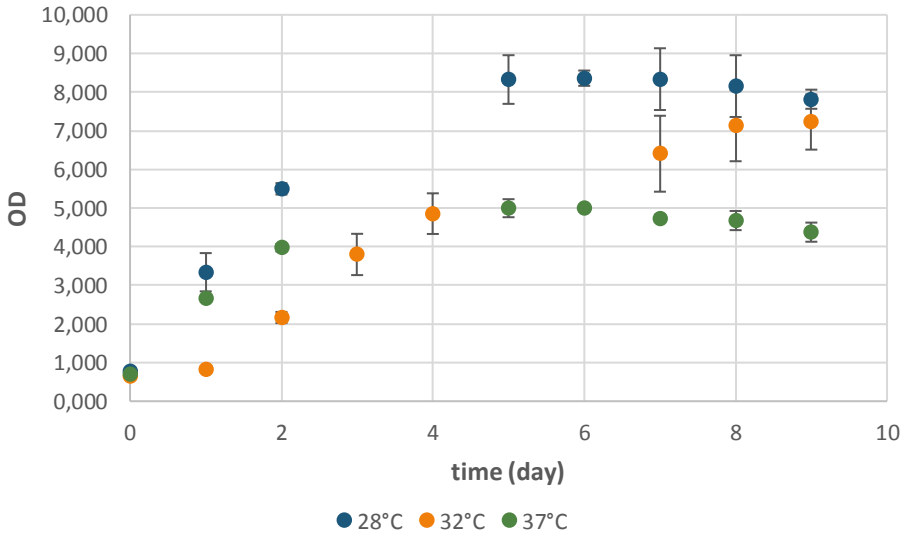


Figure 3.28 *Growth curve of Synechocystis sp. PCC 6803 at different temperatures*

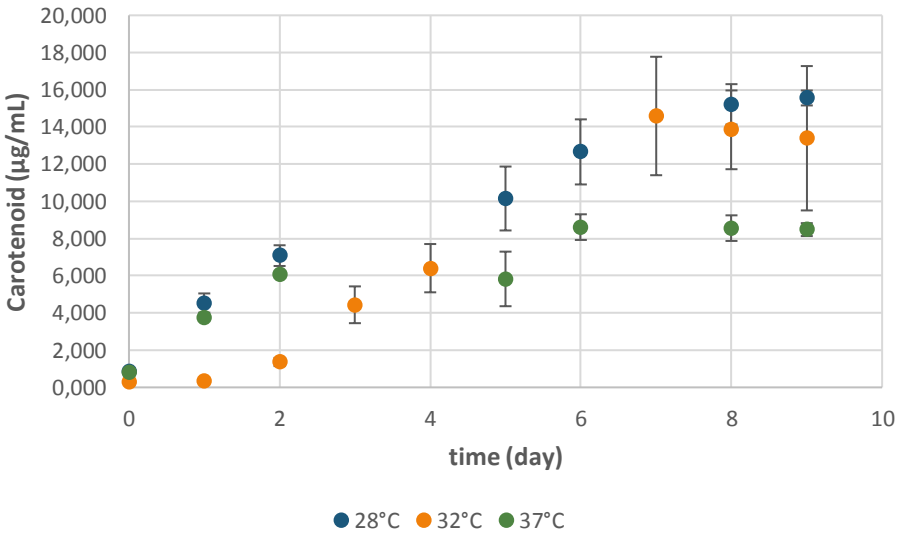


Figure 3.29 *Growth curve in terms of chlorophyll a of Synechocystis sp. PCC 6803 at different temperatures*

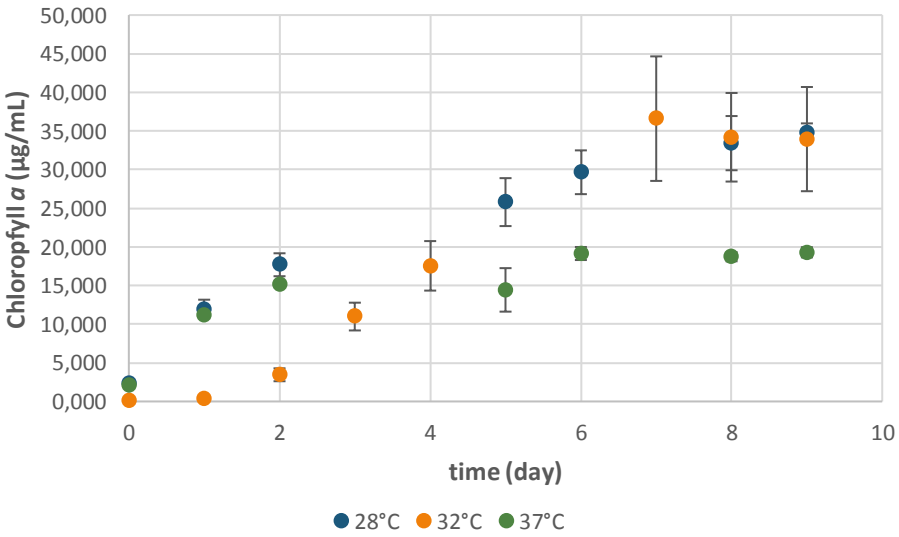


Figure 3.30 *Growth curve in terms of carotenoid of Synechocystis sp. PCC 6803 at different temperatures*

The main difference between the three temperatures investigated can be seen in *Figure 3.31*. The growth rate at 28°C and 37°C was almost the same, but at 32°C, it was significantly greater and equal to 1.77 day^{-1}. This result confirms the one by Sheng et al. (2011), who demonstrated that, compared with an optimal temperature of 30-33°C, a higher temperature (44°C) and a lower temperature (22°C) severely inhibited the specific growth rate, the biomass production rate, nutrient utilization rates and lipid production rate of *Synechocystis* sp. PCC 6803.

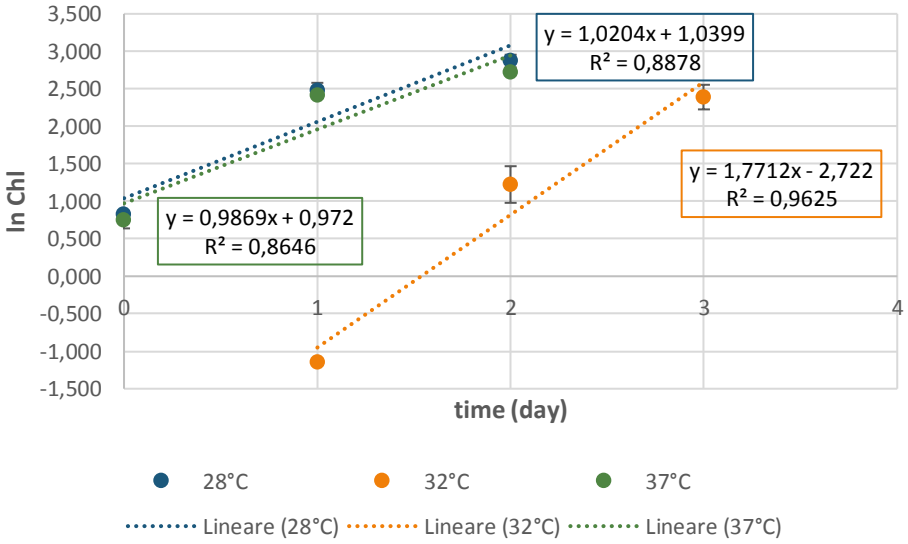


Figure 3.31 *Growth rate in terms of carotenoid of Synechocystis sp. PCC 6803 at different temperatures*

Looking at the initial and final concentration of phosphorus measured in the three conditions (see *Figure 3.32*), the statistical analysis revealed that there was no significant difference between the data obtained and on average 95.85% of phosphorus was removed by microorganisms.

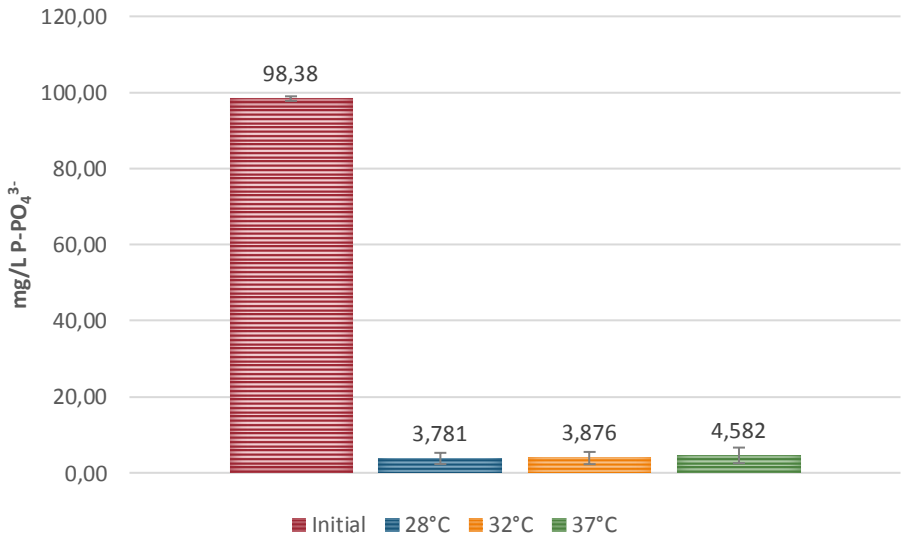


Figure 3.32 *Initial and final concentration of phosphorus at different temperature*

On the other hand, considering the measured concentrations of ammonium (see *Figure 3.33*), it could be noticed that at the highest temperature the removal of nitrogen decreased. Sheng et al. (2011) proposed that, since the nutrient concentration was not limiting, the lower nutrients utilization rate detected at higher temperature were due to temperature stress.

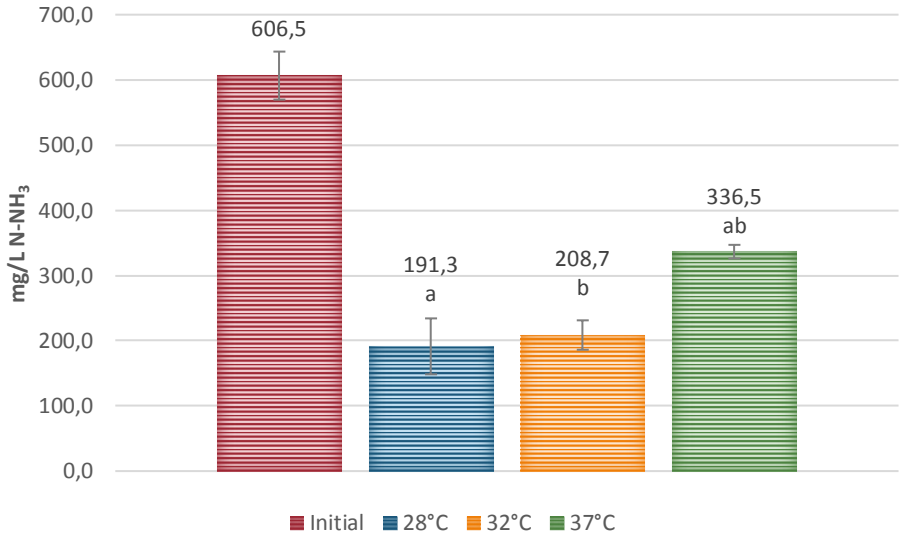


Figure 3.33 *Initial and final concentration of ammonium at different temperature*

Same letters correspond to statistically significant results

Figure 3.34 shows initial and final concentration of COD measured in all experimental conditions. There was no significant difference between the measured values and on average, 69.0% of COD was removed by microorganisms.

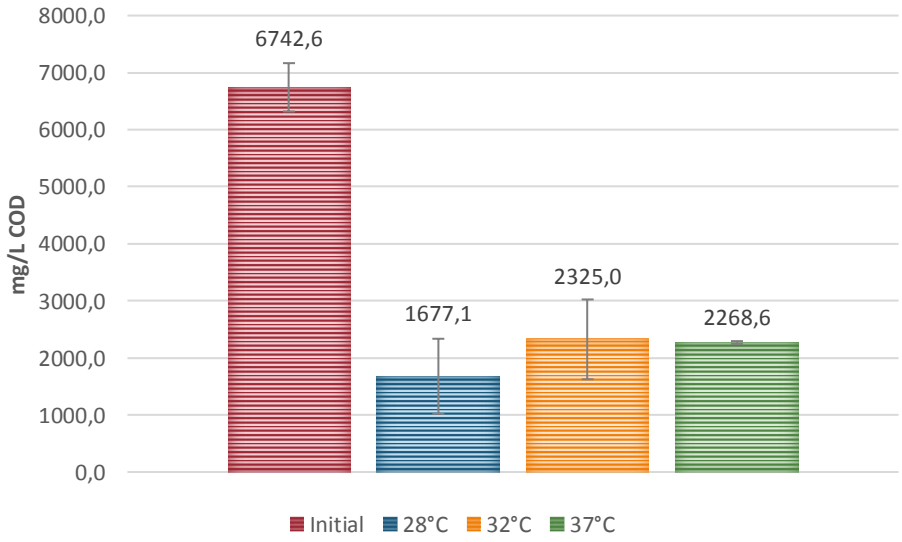


Figure 3.34 *Initial and final concentration of COD at different temperature*

In *Table 3.10* the initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$) are reported. Yield values obtained at 37°C were larger compared to the others, but this was due to the fact that at that temperature the final biomass obtained was lower. Between 28°C and 32°C, no significant difference can be noted, in

terms of both nutrient removal and biomass production. Moreover Panda et al. (2006) confirmed that this is the preferred temperature range for the PHB accumulation. However, all subsequent experiments were performed at 32°C, because the growth rate measured at this temperature was noticeably higher. In fact, in literature a similar value was achieved cultivating *Synechocystis* sp. in an ideal PBR (Yu et al., 2013).

Table 3.10 *Initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$) at different temperature*

	C_{in} (g/L)	C_{fin} (g/L)	$Y_{P/X}$ ($mg_P/mg_{biomass}$)	$Y_{N/X}$ ($mg_N/mg_{biomass}$)
28°C	0.6±0.141	3.63±1.117	0.031	0.137
32°C	0.23±0.071	3.06±0.028	0.033	0.141
37°C	0.49±0.014	2.2±0.057	0.055	0.158

3.5.3 Effect of light

Finally, the effect of different light intensities on the growth rate and on nutrients consumption was evaluated. Experiments were performed in bottle reactors, without providing air-CO_2, and at a constant temperature of 32°C. Three light intensities were tested, 100, 250 and 50 µmol photons $m^{-2}s^{-1}$. Growth curves obtained are displayed in *Figure 3.35*.

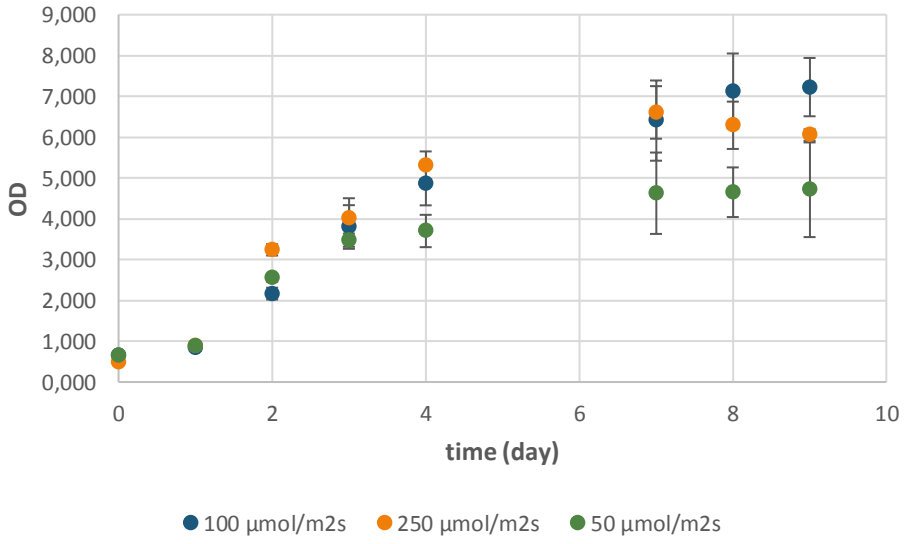


Figure 3.35 *Growth curve of Synechocystis sp. PCC 6803 at light intensity*

The largest concentration of biomass was obtained at 100 µmol photons $m^{-2}s^{-1}$, while the lower was at 50 µmol photons $m^{-2}s^{-1}$. *Figures 3.36* and *Figure 3.37* shows the growth curves in terms of pigments to account only for the growth of the cyanobacterial species. It could be seen that the maximum chlorophyll *a* and carotenoid contents were identified with 100 µmol photons $m^{-2}s^{-1}$.

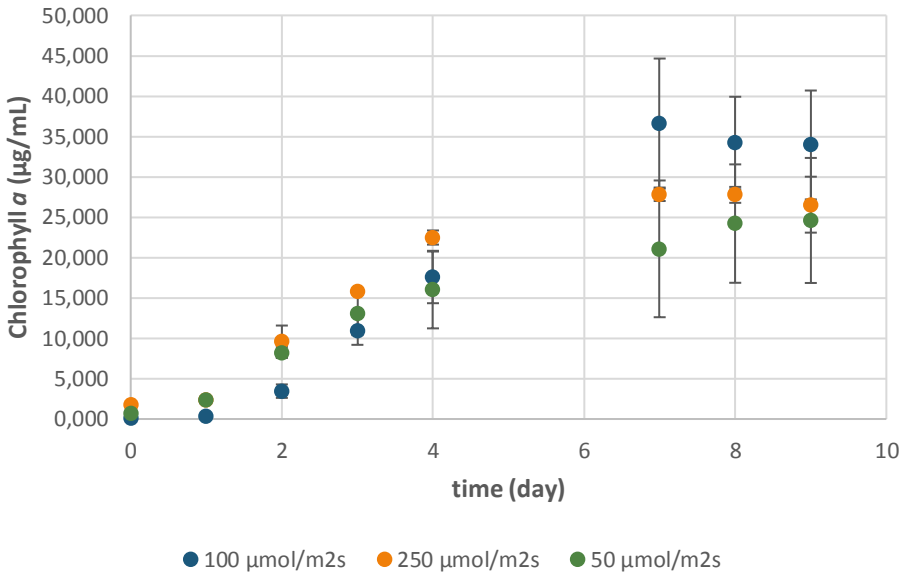


Figure 3.36 *Growth curve in terms of chlorophyll a of Synechocystis sp. PCC 6803 at light intensity*

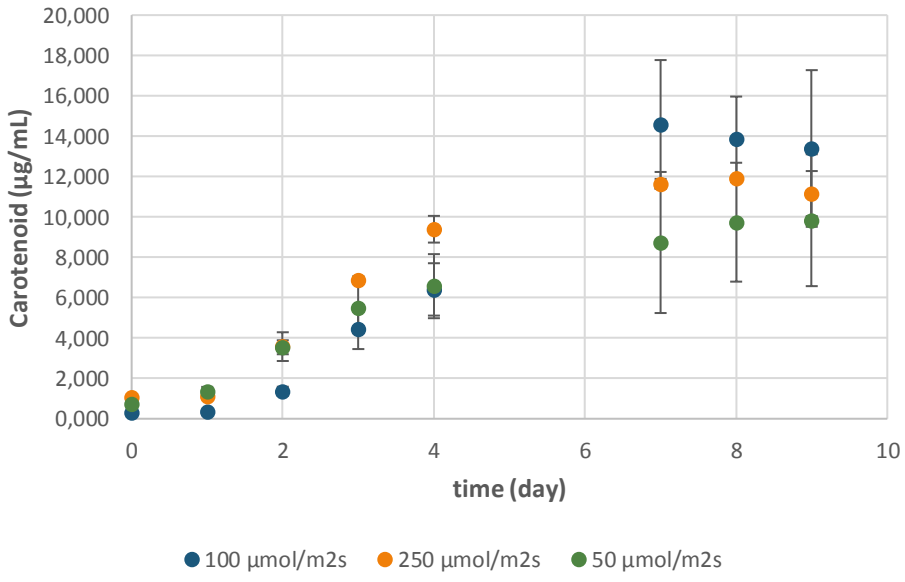


Figure 3.37 *Growth curve in terms of carotenoid of Synechocystis sp. PCC 6803 at light intensity*

Growth rate constant calculation (*Figure 3.38*) proved that the highest value was found at 100 µmol photons $m^{-2}s^{-1}$ (1.77 day^{-1}). At 50 µmol photons $m^{-2}s^{-1}$ it decreased to 0.848 day^{-1}. Anyway, this value was higher than the one found at 100 µmol photons $m^{-2}s^{-1}$ when the culture was supplied with CO_2 (0.6 day^{-1}), confirming that the presence/absence of inorganic carbon sources was crucial for the growth of *Synechocystis*.

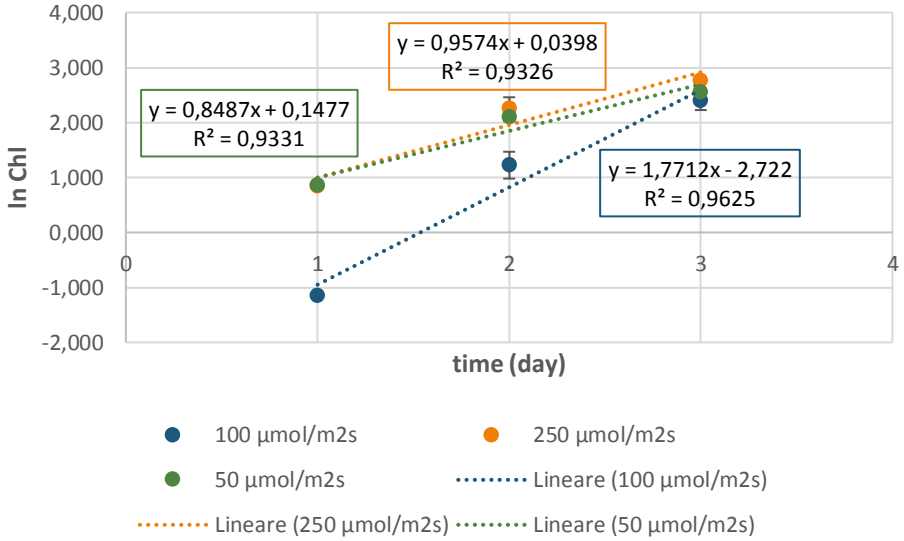


Figure 3.38 *Growth rate in terms of carotenoid of Synechocystis sp. PCC 6803 at light intensity*

On the last day of growth, a sample of the culture was filtered and the nutrients analysis (phosphorus, nitrogen as ammonium and COD) were carried out. As can be seen from *Figure 3.39*, at 100 and 250 µmol photons $m^{-2}s^{-1}$ no significant difference was found in the removal of phosphorus, instead at 50 µmol photons $m^{-2}s^{-1}$ it was lower. This was consistent with the fact the growth of microorganisms and so the final biomass measured was lower.

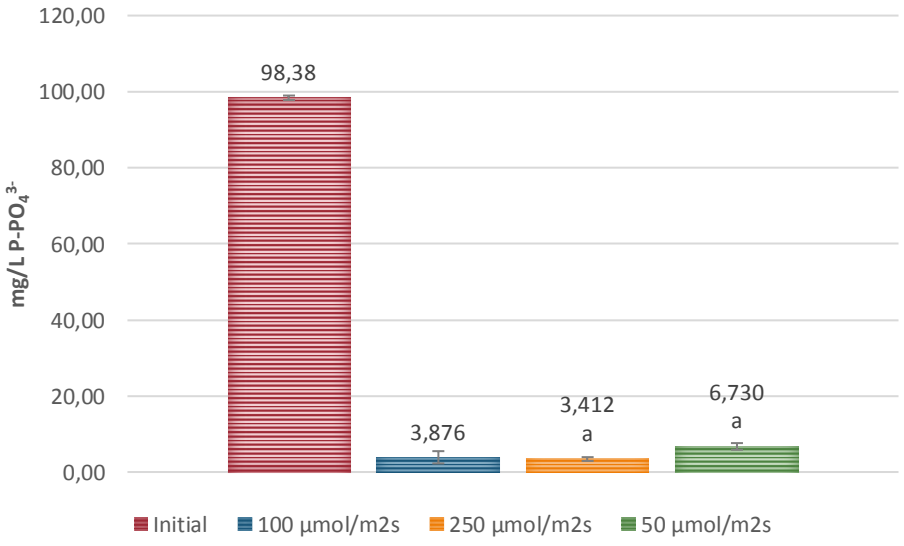


Figure 3.39 *Initial and final concentration of phosphorus at different light intensity*
Same letters correspond to statistically significant results

A similar trend was found for ammonium (*Figure 3.40*). At 100 and 250 µmol photons $m^{-2}s^{-1}$ no significant difference was found in the removal of ammonium, instead at 50 µmol photons $m^{-2}s^{-1}$ its consumption was lower.

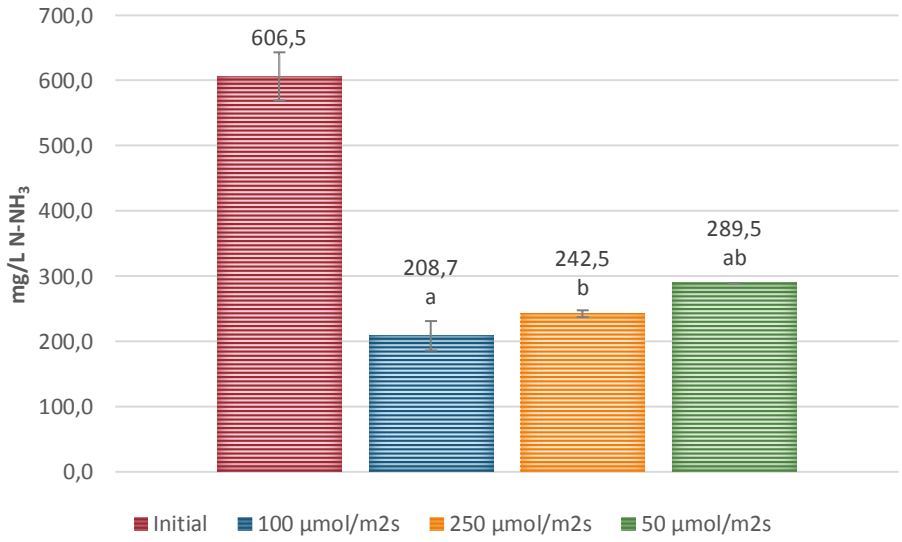


Figure 3.40 *Initial and final concentration of ammonium at different light intensity Same letters correspond to statistically significant results*

Figure 3.41 shows initial and final concentration of COD measured in all experimental conditions. There was no significant difference between the measured values and on average 67.46% of COD was removed by microorganisms.

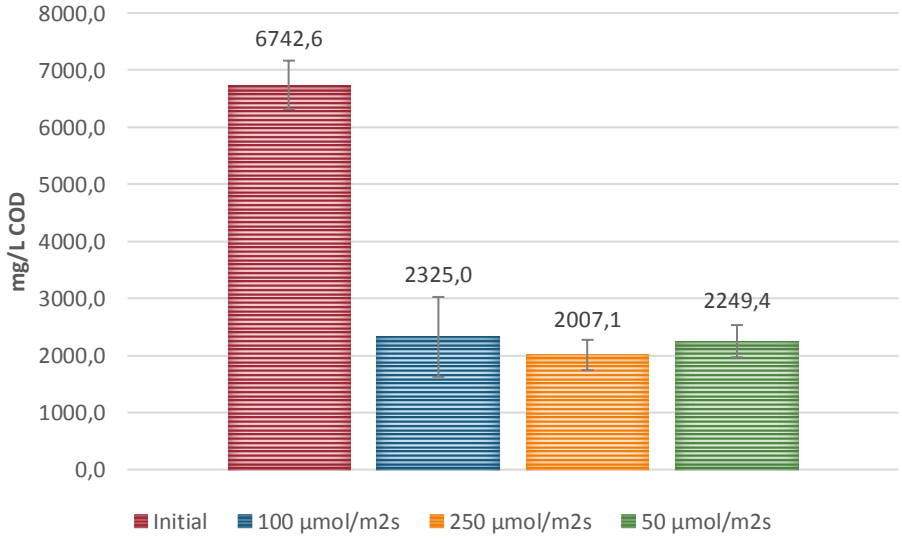


Figure 3.41 *Initial and final concentration of COD at different light intensity*

Results in terms of nutrient/biomass are summarized in *Table 3.11* together with initial and final biomass concentrations. The values obtained for yields are entirely consistent with experimental values found in literature. At 50 and 250 µmol photons $m^{-2}s^{-1}$ the nutrient/biomass yield calculated was larger with respect to the experiments at 100 µmol photons $m^{-2}s^{-1}$. In fact, a good phosphorus and ammonium removal was achieved also if the final biomass produced in those cases was lower.

Table 3.11 *Initial and final biomass concentration, phosphorus/biomass yield ($Y_{P/X}$), nitrogen/biomass yield ($Y_{N/X}$) at different light intensity*

Light intensity (µmol photons $m^{-2}s^{-1}$)	C_{in} (g/L)	C_{fin} (g/L)	$Y_{P/X}$ ($mg_P/mg_{biomass}$)	$Y_{N/X}$ ($mg_N/mg_{biomass}$)
100	0.23±0.071	3.06±0.028	0.033	0.141
250	0.14±0.028	2.38±0.339	0.042	0.163
50	0.38±0.085	2.45±0.099	0.044	0..153

The analysis carried out demonstrated that the best conditions for the growth of the cyanobacteria *Synechocystis* sp. PCC6803 in a batch reactor were the absence of inorganic carbon, a temperature of 32°C and a constant light intensity of 100 µmol photons $m^{-2}s^{-1}$. In that way the growth rate, the biomass concentration and the pigments contents were maximized. At the same time, the growth of the other microorganisms was reduced. Moreover, *Synechocystis* proved to have a great potential in wastewater treatment, showing the ability to survive and grow even if cultivated in a highly concentrated and non-sterilized medium. Sheng et al. (2011) highlighted that this cyanobacteria has excellent potential for large-scale biomass production thanks to its fast growth rate, naturally high lipid content, ability to be genetically transformed and robustness towards a wide range of environmental conditions.

3.6 Validation of the extraction and quantification process of polyhydroxyalkanoates

In this section, the experiments carried out to validate the polyhydroxyalkanoates extraction and quantification method are discussed. Several protocols have been found in literature, and they differ in both the extraction and the quantification steps according also to the microorganisms used. Yan et al. (2010) determined the PHA content in *Bacillus cereus* after chloroform extraction and gravimetric quantification. Bhati et al. (2010) detected PHA using gaschromatography, after an extraction of PHA from *Nostoc muscorum* and *Synecoscystis* sp. PCC6803 in hot chloroform. Jau et al. (2005) carried out methanolysis reaction on freeze-dried cells of *Spirulina platensis*, followed by quantification using gas chromatographic analysis. Fradinho and Domingos (2013) digested the lyophilized biomass of a consortium bacteria-algae with chloroform, methanol and sulphuric acid, while quantification was done using gaschromatography coupled to a flame ionization detector. Panda et al. (2006) and Ansari and Fatma (2016) performed a removal of pigments before the extraction in hot chloroform, and a spectrophotometric assay was then performed. Basset et al. (2016) proposed a gravimetric quantification, after lyophilisation, extraction with chloroform and precipitation with methanol. Amy Tan et al (2014) and Ansari and Fatma (2016) reported other quantification methods, like the Fourier transform infrared spectroscopy (FTIR), the proton nuclear magnetic resonance (^{1}H-NMR) and the transmission electron microscopy (TEM). In summary, chloroform extraction is the most

used method to extract PHA from freeze-dried microorganisms cells, although the protocols differ in the contact time. Moreover, the removal of pigments done by some authors seems to be related to the subsequent quantification method used. In fact, with the spectrophotometric assay it was an essential biomass pre-treatment. However, although this quantification method is easy to perform, it is limited to the P3HB determination and result can be interfered by other endogenous components, resulting in an overestimation of P3HB content. For these reason, the set up of extraction protocol was based on Basset et al. (2016), which is currently used and consists in an extraction of dry biomass in hot chloroform, followed by a precipitation with methanol and by a gravimetric quantification. However, no satisfactory results were obtained with *Synecocystis* in our work, probably due to specific characteristic of the structure of this strain. This is confirmed by the observation of Sudesh et al. (2002), suggesting that although this solvent extraction is routinely used, it is not efficient in extracting PHA from *Synechocystis* sp. PCC 6803 cells. In fact, thanks to Nile blue A staining they observed that many PHA granules still remained intact in the cells. For these reason other tests have been done, this time trying two different biomass pre-treatment. In one case, the biomass was mixed with quarz powder and ground into a fine powder in a mortar pestle; in the other one, freeze-dried cells were disrupted using a multi-beads shocker instrument. Unfortunately, even with pretreatments, no satisfactory extraction was obtained. Basset et al. (2016) performed the extraction in three hours, while other authors used longer procedure. Yellore and Desai (1998) carried out an extraction of the polymer from *Methylobacterium* sp. ZP24 in 36 hours using hot chloroform. Instead, Ansari and Fatma (2016) made the extraction using the Soxhlet apparatus and, after testing different duration of the extraction time, they demonstrated that best results were obtained after 30 hours. Therefore, we decided to follow this last extraction protocol in the subsequent tests. *Figure 3.42* shows the Soxhlet apparatus used.

Figure 3.42 *Soxhlet apparatus used for the polyhydroxyalkanoates extraction*

The precipitation step was carried out by many authors (Panda et al., 2006; Bhati et al., 2010; Ansari and Fatma, 2016) following the procedure of Yellore and Desai (1998), in where it was achieved using two volumes of cold diethyl ether. The method of Basset et al. (2016), using three volumes of methanol, was preferred. With these modifications to the protocol, experimental tests gave positive results, and *Synechocystis* sp. PCC 6803 grown for 6 days in $BG11_0$ (*Figure 3.43*), i.e. in nitrogen-starved conditions (§2.2.4), revealed after gravimetric quantification a PHA content of 2.99% of cell dry weight. This value was entirely consistent with data found in literature as Wu et al. (2001) in the same conditions measured an intracellular level of PHB of 3% DCW after 6 days, rising to 4.1% DCW at the 7th day.

Figure 3.43 *Synechocystis sp. PCC 6803 grow in $BG11_0$*

3.7 Validation of the extraction, purification and quantification process of cyanophycin

In this section, the experiments carried out to validate and improve the extraction, purification and quantification process of cyanophycin from cyanobacteria are reported. Several protocols have been found in literature, and quantification of cyanophycin could be done in several ways, for example by amino acid analysis after complete hydrolysis with HCl and by NMR spectroscopy (Aravind et al., 2016). However, the spectrophotometric method based on the Sakaguchi reaction or on the Bradford reaction is the most used one. With regard to the extraction and purification phase, attention is focused on the protocol of Elbahoul et al. (2004) and on the modifications proposed by Trautmann et al. (2016). Both authors proposed the removal of the pigments in the first step of the extraction process, and both of them used acetone, but Elbahoul et al. (2004) worked with lyophilized samples,

while Trautmann et al. (2016) used centrifuged samples. Experiments with the two types of samples taken from the same culture showed that best results were obtained with the centrifuged sample. In fact, the removal of pigments from dry samples proved to be problematic, probably due to residual moisture problems that prevented the solvent from permeating completely inside the cells. As a result, it was necessary to identify the correct volume of the sample to be analysed. Obviously, this also depends on the content of cyanophycin accumulated in the biomass, but here the goal was to check if there was a minimum volume to be used to minimize the measurement errors. In the experiments carried out, different volumes, taken from the same culture, should give the same result. Initially, the volumes tested were 5, 10, 15 mL of sample, and the results obtained provide consistent values among them as can be seen from *Table 3.12*.

Table 3.12 *Cyanophycin % (w/w) measured in different sample volume of the same culture*

	5 mL	**10 mL**	**15 mL**	**Average**
Experiment 1	8.96±0.044	8.51±1.15	8.26±0.82	8.58±0.36
Experiment 2	8.27±0.3	6.54±0.091	7.11±0.17	7.31±0.88

Then, the sample volume was reduced to 2 mL, but as shown in *Table 3.13*, the content of cyanophycin was always underestimated compared to values measured with 5 mL of sample, which was therefore identified as the minimum volume needed for a proper evaluation.

Table 3.13 *Cyanophycin % (w/w) measured in different sample volume of the same culture*

	2 mL	**5 mL**
Experiment 3	3.44±0.3	6.89±0.52
Experiment 4	6.72±1.06	10.13±0.25

Other differences between the two protocols applied, were the pH of the precipitation buffer, and its volume. Elbahoul et al. (2004) procedure was chosen, which used 0.1 M Tris-HCl set to pH 12 by NaOH. As for quantification, both reactions of Sakaguchi and Bradford were taken into account. Over the years, Sakaguchi's reaction has been modified by several authors (Izumi, 1964, Messineo, 1966, Sastry and Tummuru, 1984; Ke and Haselkorn, 2013), although the version of Messineo was applied more often as it does not give interference problems due to presence of other amino acids. Trautmann et al. (2016) followed this quantification techniques, while Elbahoul et al. (2004) preferred the one proposed by Bradford. This last method was chosen to quantify peptides, based on a calibration curve with bovine serum albumin as a standard. Specifically, we evaluated the assay proposed by Sedmark and Grossberg (1977). The protein reagent in this case was prepared as a 0.06% solution of Coomassie Brilliant Blue G250 in 0.6 N HCl. The reagent

has a brownish-orange colour that turns to an intense blue colour when it reacts with proteins. Although the quantification proved to be effective, this method led to high measurement errors. Conversely, it was demonstrated the possibility to substitute the reagent ethanol, expected by the Bradford method (Bradford, 1976), with methanol, in the right concentration. Other minor optimization (not reported) were carried out, and best results, shown in *Table 3.14*, were obtained culturing *Synechocystis* sp. PCC 6803 in BG11 P (*Figure 3.44*), i.e. in phosphorus-starved conditions (§2.2.5).

Table 3.14 *Cyanophycin % (w/w) measured in different sample*

Dry cell weight (g/L)	Cyanophycin % (w/w)
1.0	11.34±0.89
1.2	11.47±0.39
1.36	14.57±1.33
1.46	11.24±0.3
1.54	11.58±1.25

Figure 3.44 *Synechocystis sp. PCC 6803 grown in BG11 P*

A preliminary assessment of the possible presence of cyanophycin accumulated in *Synechocystis* sp. PCC 6803 grown in the fermentation liquid was done. *Figure 3.45* shows the precipitated cyanophycin, extracted from the cyanobacteria. However, in this case, the final dry weight identified not only the cyanobacteria, but referred to the total biomass, so results could not be reported by weight. The value obtained was 0.196 µg/mL of cyanophycin, an assessment which is only indicative that *Synechocystis* sp. PCC 6803

could accumulate this compound in that medium, because no optimization of conditions of growth to further the accumulation was done.

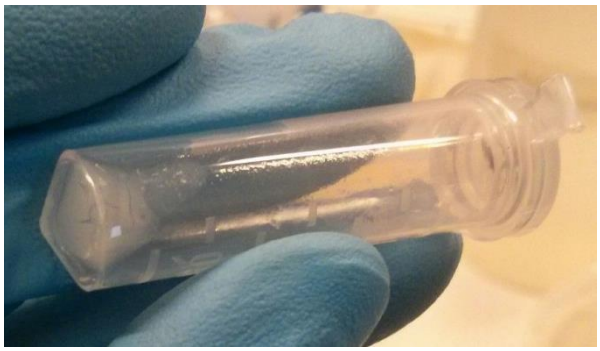


Figure 3.45 *Precipitated cyanophycin obtained from Synechocystis sp. PCC 6803 grown in the fermentation liquid*

Chapter 4

Preliminary process assessment

In this chapter, an analysis on different proposals of process scheme for the production of polyhydroxyalkanoates is presented. The feasibility of the process is evaluated through mass and energy balances, considering also the nutrient removal and the limits on outlet pollutant concentrations imposed by territorial law, so as to simultaneously carry out phycoremediation and production of high value added compounds. The starting point is the process scheme for the production of polyhydroxyalkanoates from sludge reject water. It is discussed the feasibility of the application of an intermediate step with microalgae for the removal of nutrients, as well as the possibility to produce polyhydroxyalkanoates directly in the fermentation liquid by means of cyanobacteria. Finally, the possibility to obtain other valuable side products from the same process is assessed.

4.1 Mass balances of the primary sedimentation and of the fermentation reactor

To perform the calculations, a waste water treatment plant with an inlet flow rate of 14400 m^3/day was considered, corresponding to a population served of 60000 P.E. (Q_{24}). To evaluate the daily flowrate of the stream to be treated, a rough calculation was based on data from Montebello WWTP, Vicenza. Experimental data showed that the COD inlet concentration is quite variable, but as a first calculation, a value equal to 1 g/L was taken, which is consistent with urban wastewaters of the Veneto region. After primary sedimentation, the supernatant has a COD concentration of about 0.2 g/L. Yin et al. (2016) stated that the yield of acidogenic fermentation was equal to 0.6 kg COD^{VFA}/kg COD. By knowing the concentration of the fermentation liquid to be obtained (6.743 g COD^{VFA}/L, experimental), through mass balances on the primary sedimentation and on the fermenter (*Figure 4.1*), it was possible to calculate the flow rate of the fermentation liquid which resulted to be 1044 m^3/day. COD concentration and flow rate of the streams are reported in *Table 4.1*.

Table 4.1 *COD concentration and flow rate*

Stream	Flow rate (m^3/day)	COD concentration (g/L)
Q_{24}	14400	1
Supernatant	13356	0.2
Sewage sludge	1044	11.24
Fermentation liquid	1044	6.743

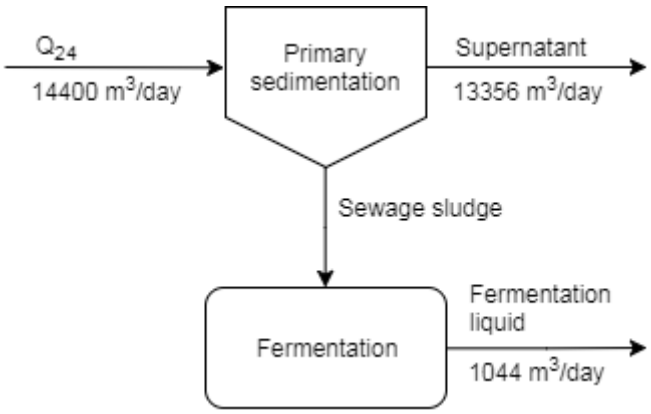


Figure 4.1 *Primary sedimentation and fermentation reactor*

The complete characterization of the fermentation liquid is reported in *Table 4.2*, while the limits of pollutant concentration at the outlet, imposed by territorial law for a plant for 10000-100000 inhabitants are summarized in *Table 4.3*. For the calculations, given the medium composition, total nitrogen was considered composed by ammonia only.

Table 4.2 *Characterization of the fermentation liquid*

$N\text{-}NH_3$ **mg/L**	**$N\text{-}NO_3^-$** **mg/L**	**$N\text{-}NO_2^-$** **mg/L**	**$P\text{-}PO_4^{3-}$** **mg/L**	**COD^{VFA}** **mg/L**
606.2 ± 36.84	15.41 ± 0.853	-	98.38 ± 0.597	6742.6 ± 423.3

Table 4.3 *Limits of pollutant concentration for active population 10000-100000 habitants (D.Lgs. 152/06)*

Total P	**Total N**	**COD**
mg/L	mg/L	mg/L
≤ 2	≤ 15	≤ 125

4.2 A new process integrating the treatment of sludge reject water and the production of polyhydroxyalkanoates

In the first process scheme polyhydroxyalkanoates are produced by mixed cultures of PHA-storing microorganisms, using acidogenic fermentation to obtain volatile fatty acids from sewage sludge (*Figure 4.2*) (Basset et al., 2016). Frison et al. (2015) showed that to obtain 1 kg PHA/day, 16.5 kg COD^{VFA}/day are needed. Specifically, 12.1 kg COD^{VFA}/day was used for the biomass growth during selection of the PHA-storing biomass (SBR), whereas 4.5 kg COD^{VFA}/day were needed for the accumulation phase. Considering the flow rate and the COD concentration entering the process (*Table 4.2*), 7040 kg COD^{VFA}/day were obtained after the acidogenic fermentation, which corresponded to a production of 429.4 kg PHA/day.

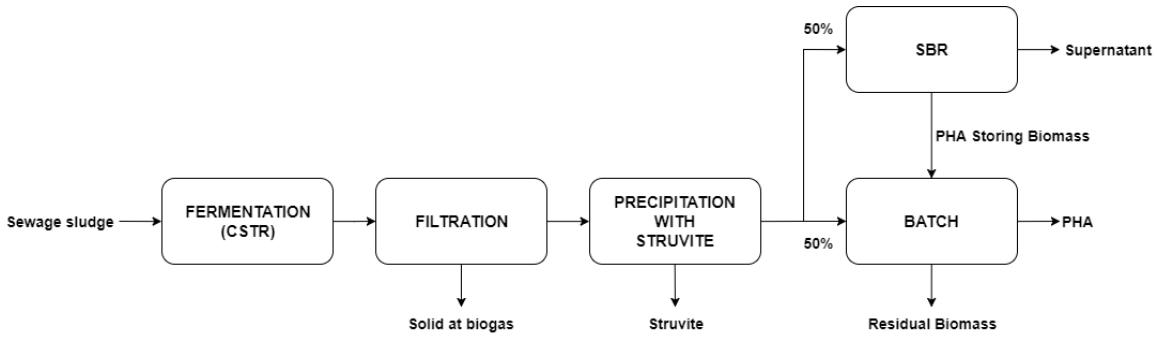


Figure 4.2 *Block flow diagram of the production process of PHA from sludge reject water*

4.3 Integration of the microalgal species *Chlorella protothecoides* 33.80

In this process scheme an intermediate step of cultivation of microalgae is included (*Figure 4.3*). The goal is the removal of nutrients, nitrogen and phosphorus, obtaining a valuable microalgal biomass. Moreover, this has to be achieved without consuming COD (in this liquid composed by volatile fatty acids) which is needed in the following batch phase to further polymer accumulation. For this reason microalgae are cultivated in the fermentation liquid, enhancing the photoautotrophic metabolism.

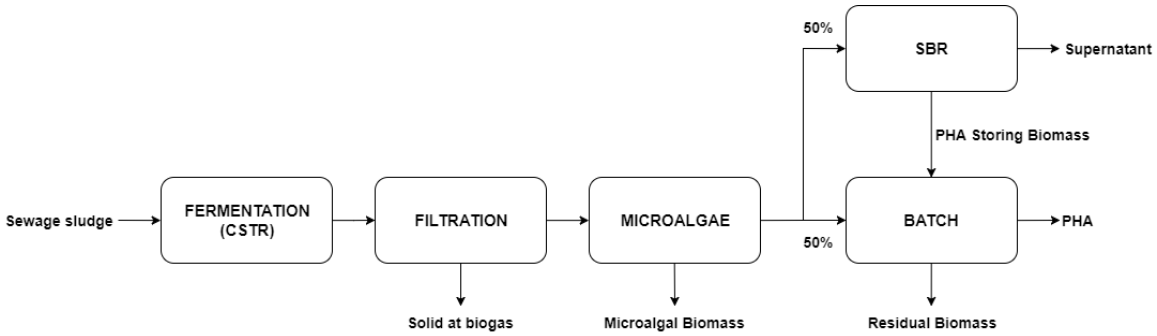


Figure 4.3 *Block flow diagram of the production process of PHA from sludge reject water with the integration of the microalgae treatment step*

Considering the concentration of nutrients in the inlet stream (nitrogen and phosphorus) together with the law limits (*Table 4.3*), a first design of the reactor for the microalgae cultivation is proposed. It is based on nitrogen mass balance, because the calculated N:P ratio (13.6:1) is imbalanced for nitrogen with respect to the one calculated by Sforza et al.(2017). In fact, the internal composition of microalgae may be different with respect to the universal Redfield C:N:P ratio (106:16:1), not only due to the specific algal strain considered, but also as a consequence of the operating conditions, such as nitrogen and phosphorus concentration and/or light limitation. To account for these aspects, based on experimental results and the microalgae stoichiometric equation from the literature (Boelee et al., 2014), Sforza et al. (2017), proposed a new version of the autotrophic algal growth stoichiometry:

$$CO_2 + 0.22NH_4^+ + 0.013H_2PO_4^- + 0.59H_2O \rightarrow 1.171O_2 + 0.207H^+ + CH_{1.88}N_{0.22}O_{0.3}P_{0.013} \quad (4.1)$$

where N:P ratio was equal to 16.9:1, specifically adapted to *Chlorella protothecoides* species. Process parameters retrieved from the literature and used in the design calculations are summarized in *Table 4.4*.

Table 4.4 *Process parameters used in the reactor design calculation*

Parameter	**Symbol**	**Value**	**Unit of measure**	**Data source**
Annual solar irradiation	ASI	4500	MJm^2y^{-1}	PVGIS Solar Irradiation Data
Photosynthetic efficiency	ε	0.07	dimensionless	Sforza et al., 2014
Low Heating Value of microalgae	LHV	20	$MJkg_X^{-1}$	Ramos Tercero et al., 2013
Maximum specific growth rate of microalgae	μ_{max}	0.87	day^{-1}	Sforza et al., 2014
N half-saturation constant	k_N	23.4	mg_PL^{-1}	Sforza et al., 2014

The reactor was assumed to be a continuous stirred tank reactor (CSTR), at steady state conditions. The mass balance of component *i* is expressed as:

$$0 = C_i^{in} - C_i^{out} + r_i \cdot \tau \quad (4.2)$$

where τ is the residence time, defined as the ratio of the reactor volume to the inlet volumetric flowrate. The nutrient consumption rate r_i is represented by the Monod kinetic, and it is linked to the biomass production rate through the nutrient/biomass yield ($Y_{i/X}$) according to

$$r_i = -Y_{i/X} \cdot \frac{\mu_{max} \cdot C_i^{out}}{k_i + C_i^{out}} \cdot C_X^{out} \quad (4.3)$$

The biomass concentration in the reactor ($C_X{}^{out}$) is calculated multiplying the biomass/nutrient yield ($Y_{X/i}$) by the difference between inlet and outlet nutrient concentration, as the decay term of the biomass is assumed as negligible:

$$C_X^{out} = Y_{X/i} \cdot \left(C_i^{in} - C_i^{out}\right) \quad (4.4)$$

Nitrogen inlet concentration is the one reported in *Table 4.2* (referring to ammonia only) whereas its outlet concentration is the one imposed by the law limit (*Table 4.3*). Nutrient/biomass yields were assumed as the average values of those measured in §3.3.4,

and were equal to 0.147 $mg_N/mg_{biomass}$ for nitrogen and 0.041 $mg_P/mg_{biomass}$ for phosphorus. Accordingly, the value obtained for the biomass concentration inside the reactor (*Equation 4.4*) is 4.03 g/L, and the hydraulic retention time (τ) calculated from *Equation 4.2* is equal to 2.94 day. By arranging the terms of *Equation 4.4* it is possible to calculate the phosphorus outlet concentration, that was totally consumed. Then, the volumetric productivity of biomass can be calculated as:

$$P_X = \frac{C_X^{out}}{\tau} \tag{4.5}$$

Concerning the energy balance, the biomass production depends on the incident solar energy. In fact, 20 MJ of energy from solar irradiation are necessary to produce 1 kg of biomass, according to the LHV of microalgae. Considering a photosynthetic efficiency (η) of 7% (Sforza et al., 2014), a maximum areal productivity is calculated as:

$$P_{m,A} = \frac{ASI \cdot \eta}{LHV_{microalgae}} \tag{4.6}$$

where *ASI* is the annual solar irradiation (data from PGVIS for north Italy- Padova). Finally, the height of the reactor can be calculated by:

$$H = \frac{P_{m,A}}{P_X} \tag{4.7}$$

On the base of the volumetric flow rate of fermentation liquid calculated in §4.1, the surface needed by the reactor can be evaluated:

$$S = \frac{Q \cdot \tau}{H} \tag{4.8}$$

The results obtained for the reactor design are shown in *Table 4.5*.

Table 4.5 *Design results obtained*

Parameter	Symbol	Value	Unit of measure
Biomass concentration inside the reactor	C_x^{out}	4.03	g_X/L
Total P concentration at outlet	C_P^{out}	0	g_P/L
Hydraulic retention time (HRT)	τ	2.94	day
Volumetric biomass productivity	P_X	1.37	g/L day
Reactor height	H	0.032	m
Reactor surface	S	9.74	ha
Per-capita reactor surface	s	1.6	m^2/E.I.

The hydraulic retention time calculated is larger than those usually observed in conventional activated sludge wastewater treatment (3-7 hours) (Metcalf and Eddy, 2003), but in this case the pollutant inlet concentration is much higher, more or less 20 times higher with respect to conventional wastewaters. In these growth conditions, however, *Chlorella* can remove all ammonia and phosphorus, even if their concentration is very high. Then, considering the algal stoichiometric equation in autotrophic conditions (*Equation 4.1*), the volumetric biomass productivity (P_X) and the molecular weight of biomass (22.2 $g_{biomass}$/mole), the oxygen produced by microalgae can be calculated, which is equal to 7095 kg O_2/day. This holds under a theoretical hypothesis of 100% autotrophic growth conditions for microalgae.

On the other hand, this produced oxygen is made available to aerobic degradation of organic matter performed by heterotrophic bacteria and other microorganisms living in the non-sterilized medium, and may lead to the consumption of the organic substrate, i.e. the volatile fatty acids. Experimental data shown in §3.3.4 confirmed that part of the organic carbon present in the medium was consumed during the cultivation of microalgae. However, this is in contrast with the aim of this intermediate step, where the removal of nutrients must be achieved without consuming COD. At the same time, the values of yields calculated in §3.3.4 proved the great potential of *Chlorella* in wastewater treatment, although in this case the reactor requires a very small height, and large areas. This problem could be overcome by increasing the depth of the tank (H), which obviously reduces the area required, but also enhances the light attenuation and consequently the mixotrophic behavior of microalgae. This implies that microalgae are favored in the assimilation of organic carbon. At the same time, there is a reduction in the produced oxygen, which is calculated based on experimental data (Grapiglia, 2017) for three values of reactor depth (*Table 4.6*).

Table 4.6 *Produced oxygen reduction under mixotrophic conditions for different values of reactor depth*

Reactor depth (m)	Produced oxygen reduction (%)
0.2	6.7
0.5	53.9
0.7	71.2

The produced oxygen reduction can be transformed into percentage of biomass produced under heterotrophic conditions, to evaluate the COD consumption due to microalgal heterotrophic metabolism. The heterotrophic stoichiometric growth equation used is the one proposed by Sforza et al. (2017):

$$0.22CH_3COO^- + 0.013H_2PO_4^- + 0.83CH_{1.943}N_{0.266}O_{0.569} + 0.1O_2 + 0.233H^+ \rightarrow CH_{1.88}N_{0.22}O_{0.3}P_{0.013} + 0.27CO_2 + 0.325H_2O \quad (4.9)$$

The fractional oxygen reduction obtained (*Table 4.6*) was multiplied by the moles of oxygen according to *Equation 4.1*, to account for the part of oxygen produced in autotrophic conditions that is in turn used by microalgae to growth under heterotrophic conditions. Then, considering *Equation 4.9* the moles of biomass produced through heterotrophic metabolism is calculated. The percentage of mixotrophy was expressed as the ratio between the moles of biomass produced under heterotrophic metabolism and the total moles of biomass produced. Consequently, the value of the volumetric biomass productivity (P_X) included the part produced both in the autotrophic and in the heterotrophic metabolism. By looking at the microalgal biomass composition ($CN_{1.88}N_{0.22}O_{0.3}P_{0.013}$), the productivity was expressed as carbon contained in the biomass. Then, it was accounted for the organic carbon actually fixed in the biomass (0.78 $g_{C,M}/g_C$), experimentally determined by Sforza et al. (2017). The value obtained had to be converted in terms of COD. The conversion factor was determined taking into account the composition of the fermentation liquid. It was assumed to be equally composed of acetic acid and propionic acid, and for each component carbon content was given by the ratio of molar weights. Through simple calculations, total organic carbon (TOC) was calculated equal to 2.99 g_C/L. Since the initial COD concentration in the wastewater was 6.743 g/L, the correction factor was estimated as 2.256 g_{COD}/g_C. Finally, applying the conversion factor, and considering the hydraulic residence time, the value of COD consumed by the microalgae in the heterotrophic metabolism was calculated. Moreover, the availability of oxygen for other microorganism was evaluated. It was given by the difference between the oxygen produced by microalgae during autotrophic metabolism (*Equation 4.1*), and the one consumed during heterotrophic metabolism (*Equation 4.9*). The oxygen production was calculated considering only the biomass produced in autotrophic metabolism, whereas the consumption was evaluated accounting only for the biomass produced under heterotrophic metabolism. Results obtained for three different reactor depth were summarized in *Table 4.7*.

Table 4.7 *Results obtained considering annual solar irradiation and three reactor depths (flow rate 1044 m^3/day)*

Reactor depth (m)	**0.2**	**0.5**	**0.7**
Reactor surface (ha)	1.54	0.61	0.44
Per-capita reactor surface (m^2)	0.26	0.10	0.07
Produced oxygen reduction (%)	6.7	53.9	71.2
Mixotrophy percentage in terms of heterotrophic biomass produced (%)	44	86.3	89.3
COD consumption by microalgae (g_{COD}/L)	2.77	5.44	5.63
COD left in the fermentation liquid (g_{COD}/L)	3.97	1.30	1.11
Net oxygen production by microalgae (kg O_2/day)	3709.5	447.4	218.8

As can be noticed, increasing the reactor depth allows a strong reduction of the area required, but the higher the depth, and the higher is the exploitation of mixotrophic metabolism by microalgae due to light limitation. This in turn leads to the reduction of the oxygen available for other microorganisms, but also to a higher consumption of the COD by microalgae. Moreover these results were obtained considering the annual solar irradiation (4500 $MJm^{-2}y^{-1}$), so the effect of different solar irradiations on model results was also evaluated. Summer solar irradiance was assumed 2153 $MJm^{-2}y^{-1}$, whereas winter solar irradiance was set equal to 522 $MJm^{-2}y^{-1}$ (PVGIS Solar Irradiation Data). Results obtained are reported in *Table 4.8* and *Table 4.9* respectively. As can be seen, during summer the mixotrophic behavior of microalgae is lowered by the higher light availability, except in the case with reactor depth of 0.7 m where almost all the microalgal biomass is produced through heterotrophic metabolism. Instead during winter mixotrophic growth of microalgae is enhanced with respect to summer, due to low light availability, but there is no oxygen availability for other microorganisms, because all the oxygen produced during autotrophic metabolism is consumed by microalgae during heterotrophic metabolism.

Table 4.8 *Results obtained considering summer solar irradiation and three reactor depths (flow rate 1044 m^3/day)*

Reactor depth (m)	**0.2**	**0.5**	**0.7**
Reactor surface (ha)	1.54	0.61	0.44
Per-capita reactor surface (m^2)	0.26	0.10	0.07
Produced oxygen reduction (%)	45	92	100
Mixotrophy percentage in terms of heterotrophic biomass produced (%)	84	91.5	92.1
COD consumption by microalgae (g_{COD}/L)	5.30	5.77	5.81
COD left in the fermentation liquid (g_{COD}/L)	1.44	0.97	0.93
Net oxygen production by microalgae (kg O_2/day)	622.4	48.2	0

Table 4.9 *Results obtained considering winter solar irradiation and three reactor depth (flow rate 1044 m^3/day)*

Reactor depth (m)	**0.2**	**0.5**	**0.7**
Reactor surface (ha)	1.54	0.61	0.44
Per-capita reactor surface (m^2)	0.26	0.10	0.07
Produced oxygen reduction (%)	100	100	100
Mixotrophy percentage in terms of heterotrophic biomass produced (%)	92.1	92.1	92.1
COD consumption by microalgae (g_{COD}/L)	5.81	5.81	5.81
COD left in the fermentation liquid (g_{COD}/L)	0.93	0.93	0.93
Net oxygen production by microalgae (kg O_2/day)	0	0	0

Thus, even though from an environmental point of view microalgae can be used to remove pollutants from this waste stream, the final goal of reducing N and P without reducing COD is hard to be achieved. Of course, to avoid the consumption of COD by microalgae, their photoautotrophic metabolism can be promoted. However, this implies the use of large areas

with a possibly unrealistic reactor depth. In addition, photoautotrophic mechanism involves the production of large quantities of oxygen that is available to other microorganisms, living within the medium and using organic carbon for their metabolism. This might be avoided only by sterilization of the waste stream, which is unrealistic in real systems. Increasing the depth of the reactor leads to a reduction of the area required and to a reduction of the oxygen produced by microalgae, but, in this case, the lower light availability promotes the mixotrophic behavior of microalgae, which consumes organic carbon of the medium. Anyway, it is clear that photosynthetic and valuable algal biomass can be produced in such a medium, thus suggesting that the production of algal biomass itself is promising even in this complex waste stream.

4.4 Production of polyhydroxyalkanoates by the cyanobacterial species *Synechocystis* sp. PCC 6803

As demonstrated in the previous section, microalgae cannot be used as an intermediate step for bacterial PHA production, but the cultivation of microalgae in such a medium is promising, so that another process scheme can be proposed. In this scenario, polyhydroxyalkanoates can be obtained by cyanobacteria directly cultivated in the liquid obtained after the acidogenic fermentation. As can be seen in *Figure 4.4*, this process is much simpler and direct with respect to §4.1. In fact, no selection of the biomass would be necessary, neither the batch accumulation phase. The production can be performed in a continuous systems, after a preliminary inoculation of cyanobacteria, that remains in the system if the residence time is kept higher that its wash out value. It was demonstrated in §3.5 the capability of *Synechocystis* sp. PCC6803 to grow in this medium, and the effect of CO_2, temperature and light on the growth rate and on nutrient consumption was evaluated. Experiments were carried out in batch mode, and the productivity reached was equal to 0.35 g/Lday. However, Gris et al. (2017) demonstrated the capability of this cyanobacterial species to reach a productivity of 2 g/Lday if cultivated in continuous systems. To understand the PHA production achievable, quantification tests were carried out on the cyanobacterial biomass. However, the low percentage reached (1% w/w) was measured before the protocol optimization (§3.6), so it is not representative of the real capability to accumulate polyhydroxyalkanoates of *Synecocystis* sp. in this medium. Therefore future investigation and optimization is needed. In literature Ansari and Fatma (2016) showed that, under photoautotrophic conditions, PHA accumulation is low. Enhanced polyhydroxylkanoates accumulation has been observed in some cyanobacteria when grown mixotrophically with acetate. Panda and Mallick (2007) demonstrated that combined effect of phosphorus deficiency and gas-exchange limitation in the presence of fructose and acetate boosted up to 38% (w/w) of dry cell weight in *Synechocystis* sp. PCC 6803. Wu et

al. (2002) established that *Synechocystis* sp. PCC 6803 accumulated up to 15.2% (w/w) under nitrogen-limited conditions in the presence of sodium acetate. Assuming to obtain 10 $g_{PHA}/g_{biomass}$, with a biomass volumetric productivity of 2000 $gm^{-3}day^{-1}$, 200 $g_{PHA}m^{-3}day^{-1}$ can be obtained. Considering a flow rate of 1044 m^3/day, this results in the production of 208.8 kg_{PHA}/day.

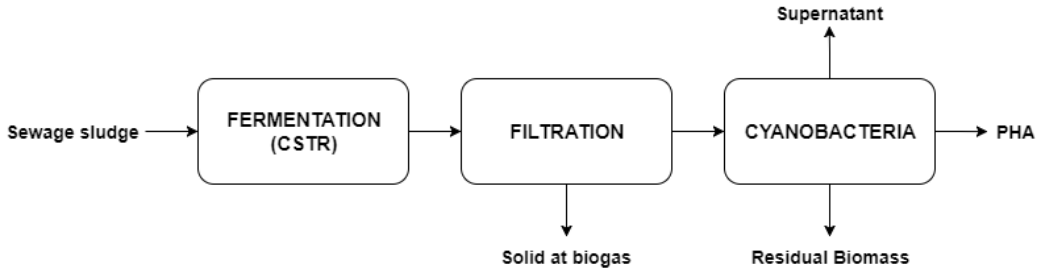


Figure 4.4 *Block flow diagram of the production process of PHA from sludge reject water by cyanobacteria*

Furthermore, using cyanobacteria in wastewater treatment has receiving increasing attention thanks to their great potential to take up nutrients such as ammonium, nitrate, orthophosphate and heavy metals (Vijayakumar, 2012). In §3.5, the nutrient/biomass yields for phosphorus and nitrogen were calculated, and the average values resulted equal to 0.04 $mg_P/mg_{biomass}$ and 0.150 $mg_N/mg_{biomass}$. Considering a volumetric biomass productivity of 2g/L day, the initial concentration of nutrients (*Table 4.2*) and the volumetric flow rate calculated in §4.1, it is possible to quantify the nutrients removed by cyanobacteria in this wastewater treatment process. Results are summarized in *Table 4.10*.

Table 4.10 *Nutrient removal achieved with cultivation of Synechocystis sp. PCC 6803 in wastewater (flow rate 1044 m^3/day)*

Nutrient	**$Y_{i/X}$ ($mg_i/mg_{biomass}$)**	**Nutrients removed (kg/day)**	**Nutrients left (kg/day)**	**% removal**
Phosphorus	0.04	83.5	18.8	81.6
Ammonia	0.150	313.2	320.5	50.6

Synechocystis sp. PCC 6803 demonstrated its potential in phycoremediation, achieving a good reduction of both phosphorus and nitrogen contents.

4.5 Production of polyhydroxyalkanoates and side products by the cyanobacterial species *Synechocystis* sp. PCC 6803

In this process scheme, it was also considered the possibility to obtain other high-value compounds from the cyanobacterial biomass of *Synechocystis* sp. cultured in the acidogenic

fermentation liquid (see the block flow diagram in the *Figure 4.5*). In literature, it is well known the capability of *Synechocystis* to produce cyanophycin and pigments. Trauttmann et al. (2016) measured 18 g of cyanophycin per g of cell dry mass. The presence of cyanophycin accumulated in the biomass when using the fermentation liquid as a culture medium has been experimentally verified. This assessment is only indicative that *Synechocystis* can accumulate this compound in that medium, because no optimization of conditions of growth was done. However, it was experimentally measured an accumulation of 11-14 % in that species (§3.7). Assuming to obtain 11 g of cyanophycin per g of cell dry mass, with a biomass volumetric productivity of 2000 g $m^{-3}day^{-1}$, 220 g $m^{-3}day^{-1}$ can obtained. Considering a flow rate of 1044 m^3/day, 230 kg/day of cyanophicyn were produced. Moreover, Gris et al. (2017) reported the capability of this specie to produce pigments, and measured an average phycocyanin productivity of 50 mg/L day. With the same flow rate of fermentation liquid of 1044 m^3/day, 52.2 kg/day of phycocyanin would be obtained in our case.

In summary, in view of simultaneous production of high added values products and pollutants removal (see *Table 4.10*), the exploitation of cyanobacteria looks quite promising.

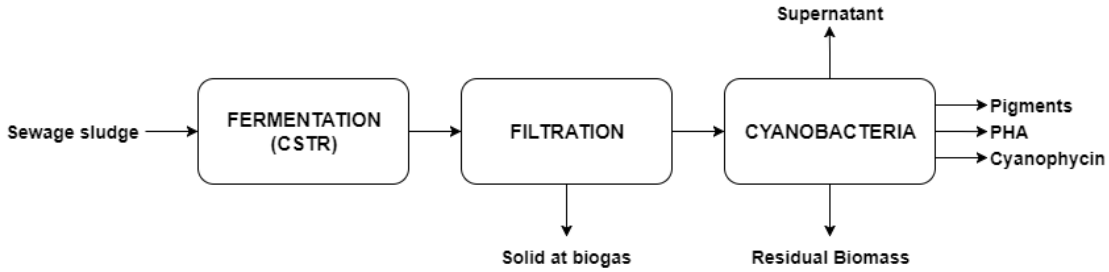


Figure 4.5 *Block flow diagram of the production process of PHA and side products from sludge reject water by cyanobacteria*

Conclusions

In this thesis, the application of microalgae and cyanobacteria in the production process of polyhydroxyalkanoates integrated with wastewater treatment was addressed. Initially it was shown the impossibility to apply the cultivation of microalgae as an intermediate step between the acidogenic fermentation and the sequential batch reactor (Longo et al., 2015; Basset et al., 2016) for the removal of nutrients, nitrogen and phosphorus, without consuming COD. In fact, in the experimental part of this thesis, microalgae resulted to carry out mixotrophy, thus consuming part of the COD. Even though photoautotrophic metabolism may be stimulated by providing CO_2 in excess or high light intensity, this may result in an increased net photosynthetic oxygen production, which can be exploited by other endogenous heterotrophic microorganism for aerobic degradation of COD. Accordingly, it seems practically unfeasible to avoid COD consumption in real plants. Anyway, the great potential of *C. protothecoides* in wastewater treatment was confirmed and a remarkable removal of both phosphorus and nitrogen, was achieved although the medium was highly concentrated. Measured nutrient/biomass yield was equal to 0.056 $mg_P/mg_{biomass}$ for phosphorus and 0.159 $mg_N/mg_{biomass}$ for nitrogen, respectively.

It was proved that two cyanobacterial species could be cultivated in that medium to directly produce polyhydroxyalkanoates. *Synechococcus* sp. PCC 7002 demonstrated its potential for phycoremediation, achieving an almost complete removal of phosphorus (98%), and a good reduction of both nitrogen (81%) and COD (84%) contents. Moreover, a relevant biomass concentration was obtained (7 g/L). *Synechocystis* sp. PCC 6803 did not show any particular problem in acclimation to this medium. The effect of CO_2 insufflation, temperature and light on the growth and on nutrients consumption was evaluated. It was shown that *Synechocystis* grow efficiently without bubbling of air enriched with CO_2, confirming the capability of this cyanobacteria to perform both photoautotrophic and photoheterotrophic metabolism. A relevant growth rate was measured (1.77 day^{-1}) and a batch productivity of 0.3 $gLday^{-1}$ day was calculated. The protocols for the extraction of polyhydroxyalkanoates accumulated in cyanobacteria was developed. The production and the accumulation of another high value compound, cyanophycin, was assessed, and the protocol for its extraction, purification and quantification, was improved. A preliminary development of a process scheme, where polyhydroxyalkanoates are produced by *Synechocystis* directly cultivated in the wastewater, was proposed, considering also the possibility to obtain simultaneously high-value side products, specifically cyanophycin and pigments.

In summary, the exploitation of this cyanobacterial species seems promising in view of the simultaneous production of high added values products and pollutants removal from wastewater. Biomass productivity in continuous system has to be verified as well as pigments productivity in that wastewater. Moreover, more investigation are needed to find the growth conditions which ensure to reach an optimum in the productivity of both biopolymer, polyhydroxyalkanoates and cyanophycin.

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