Ph.D. Program in Civil, Chemical and Environmental Engineering

Curriculum in Chemical, Materials and Process Engineering

Department of Civil, Chemical and Environmental Engineering

Polytechnic School, University of Genova, Italy.

WINERY WASTEWATER TREATMENT BY MICROALGAE CO-CULTURE FOR LOW-COST BIOMASS PRODUCTION IN A BIOREFINERY CONCEPT

Elena Spennati

WINERY WASTEWATER TREATMENT BY MICROALGAE CO-CULTURE FOR LOW-COST BIOMASS PRODUCTION IN A BIOREFINERY CONCEPT

BY

ELENA SPENNATI

Dissertation discussed in partial fulfillment of

the requirements for the Degree of

DOCTOR OF PHILOSOPHY

Civil, Chemical and Environmental Engineering

curriculum in Chemical, Materials and Process Engineering,

Department of Civil, Chemical and Environmental Engineering, University of Genoa, Italy

June, 2021

Advisers:
Prof. Guido Busca - Department of Civil, Chemical and Environmental engineering, University of Genoa
Prof. Attilio Converti - Department of Civil, Chemical and Environmental engineering, University of Genoa
Prof. Patrizia Perego - Department of Civil, Chemical and Environmental engineering, University of Genoa

External Reviewers:
Prof. Gianpiero Pataro - Department of Industrial engineering, University of Salerno
Prof. Raquel Pedrosa Bezerra – Department of Morphology and Animal Physiology, Universidade Federal Rural de Pernambuco

Examination Committee:
Prof. Marco Panizza – Department of Civil, Chemical and Environmental engineering, University of Genoa
Prof. Giancarlo Cravotto – Department of Drug Science and Technology, University of Turin
Prof. Ricardo Pinheiro de Souza Oliveira – School of Pharmaceutical Science, Universidade de São Paulo

Ph.D. program in Civil, Chemical and Environmental Engineering

Curriculum in Chemical, Materials and Process Engineering

Cycle XXXIII

Cover-design: Giulia Curletto (XXIX cycle) and Margherita Pongiglione (XXVIII cycle)

ABSTRACT

The growing industrialization, the high level of pollution, and the reduction of resources lead to the necessity to find an alternative way to handle the entire production chain from the raw materials to the finished products. The zero-waste strategy and the circular economy are the best solutions to remediate the dramatic conditions in which our planet pours. Moreover, the reduction of fossil fuels reserves and the continuously increasing demand for energy around the world has led to the necessity to find an eco-sustainable alternative to conventional fuels. In the last few years, biofuel production from different plant sources has been increasingly studied by researchers. The production of third-generation biofuels from raw materials that do not compete with food crops is attracting more and more attention. Third-generation biofuels can be produced from microalgal biomasses or from their intracellular components such as lipids. Moreover, their production, if compared to conventional biomasses, reduces land and water utilization along with the use of pesticides.

Microalgae are unicellular microorganisms able to grow under autotrophic, heterotrophic or mixotrophic conditions depending on the carbon source used in their metabolism as well as light conditions. They are composed mainly of lipids, proteins and carbohydrates, whose relative proportions depend in particular on the species and growth conditions. They are generally used for human or animal nutrition, or extraction of added-values components for chemical and pharmaceutical industries, but also for biofuel production.

In recent years, lipid, protein, and pigment extraction from microalgae has been widely studied for various applications such as the productions of biodiesel from the lipid fraction and of nutraceuticals and dyes from vitamins, proteins, and pigments. However, it is important to stress that the microalgae biorefinery concept is the only way to make microalgae competitive with products obtained from conventional sources, and that the use of microalgae to produce only biofuels or only nutraceuticals has not yet reached clear-cut economic feasibility. The biggest challenges are the relatively high cost of biomass production and the energy demand for the extraction and separation processes. Therefore, in order to make microalgae products economically viable and increase their marketability, it is necessary to reduce costs, for example by valorizing process residues as co-products. It is generally accepted that the sale of co-products will make the production of biofuels from microalgae economically feasible. Indeed, it was estimated that the residue of microalgal biomass after lipid extraction could be worth between 100 and 225 USD per ton and could yield co-products ranging in value from 0.95 to 2.43 USD per gallon of biodiesel produced. Moreover, the microalgae protein fraction has an economic value that ranges from 0.86 USD/kg as feed to 5.57 USD/kg as food.

Moreover, thanks to their capability of also metabolizing organic carbon, microalgae can in fact be grown in wastewaters, thus reducing the use of fresh water, the cost of growth medium, the energy consumption and, at the same time, the wastewater polluting impact. There are several studies in the literature focusing on the use of microalgae to treat wastewaters such as municipal and textile wastewaters, among others. In this contest, agri-food wastewaters are good candidates to be used as microalgae medium because they are rich in nutrients and the resulting biomass obtained after treatment could be used for the extraction of high added value components, such as protein and pigments. Between them, winery wastewaters (WWWs) which are released from different activities of the wine making process, namely tank washing, transfer, bottling and filtration, are suitable to be treated by microalgae. The polluting impact of WWWs is related to their high organic load (polyphenolic compounds, sugars, organic acids and esters), low pH (3–5), high content of suspended particles and large volumes (0.5–14 L per liter of wine produced). Owing to the release of organic compounds and inorganic ions, their disposal in land without adequate treatment can change the physicochemical properties of groundwater such as color, pH and electrical conductivity, among others.

Whit regard to the open issues recalled above, the research project has aimed to develop a biorefinery from microalgae. Winery wastewaters were used as growth medium both for microalgal biomass production and reduction of pollutant impact, the protein fraction was extracted from the biomass as high added-value component, and the residual biomass was submitted to pyrolysis process to produce biofuels. The topic addressed by this thesis is organized and subdivided into chapters as follows.

Chapter 1- Literature review on the biorefinery concepts, the application to microalgae production and the microalgae world situation concerning their metabolisms, growth system, industrial application (wastewaters treatment, extraction of high-added-value components and, biofuels production).

Chapter 2- The optimization of winery wastewaters concentration in microalgae growth medium to obtain both high microalgae concentration and productivity and good results in terms of reduction of pollutant impact. Three different winery wastewaters collected from different steps of the winemaking process were studied. The co-culture of *Chlorella vulgaris* and *Arthrospira platensis* was grown under continuous light and air supply. The optimized parameters obtained in the previous section were studied at different light conditions to identify the prevalent metabolism of the microalgae to consume the pollutant molecules present in the wastewaters. Moreover, the microalgae growth was performed into different photobioreactor configurations: tubular photobioreactor (TP),

column photobioreactor (CP) and, open pond (OP) to improve the biomass concentration and the pollutant impact removal efficiency.

Chapter 3- To perform a scale-up of the process the co-culture was grown in 20 L column photobioreactor, the growth medium under the condition optimized in the previous chapters was supplied continuously by a pump system.

Chapter 4- The extraction of high-added value components from microalgal biomass were investigated taking into account the biorefinery concept. The optimization of the protein extraction process from *A. platensis* by Ultrasound-Assisted Extraction was performed using Box-Behnken Design in which the effects of extraction time, solvent volume, and mass of *A. platensis* were investigated. Moreover, the extraction and purification of c-phycocyanin from *A. platensis* and the subsequent protein extraction on wet c-phycocyanin residue was performed. The protein extraction from the co-culture grown in the different photobioreactor configurations was carried out under the condition optimized in the previous chapter. Moreover, the effect on cell size and cell wall thickness of growing the co-culture in presence of winery wastewaters was investigated. Variation of protein expression as function of photobioreactor configuration was also assessed.

Chapter 5- The energetical recovery of the produced co-culture biomass was investigated. The pyrolysis process was carried out on microalgal biomass obtained from wastewater treatment in membrane photobioreactor. The operational condition of the process and, the distribution of reaction products and their composition ware studied.

Chapter 6- A final discussion of the process was performed based on the results obtained from the previous chapters.

Summary

1 Literature review

1.1 Biorefinery concept

The growing industrialization, the high level of pollution, and the reduction of resources lead to an urgent necessity to find an alternative way to handle the entire production chain from the raw materials to the finished products. The zero-waste strategy and the circular economy are the best solutions to remediate the dramatic conditions in which our planet pours. In this contest, the biorefinery concept is a sustainable alternative to that of a conventional refinery. The definition of biorefinery was described by the IEA Bioenergy Task 42: "Biorefinery is a sustainable processing of biomass into a spectrum of marketable products and energy"[1].

A biorefinery is an infrastructure facility where different conversion technologies, such as biochemicals or thermochemical processes, and combustion are applied on biomasses or microorganisms to produce efficiently bio-based products, in particular biofuels, bioenergy, biochemicals, and high added products [2]. A biorefinery contrary to conventional refinery requires ecological perspectives, consumption of non-renewable energy during processes, and related environmental impacts should be minimized, while the complete use of biomass should be maximized [3]. The ecological perspectives can be summarized as follow:

- analyses of the carbon cycle (respiration, photosynthesis, and organic matter decomposition), water cycle (precipitation, evaporation, infiltration, and runoff) and nitrogen cycle (N fixation, mineralization, and denitrification) [4];
- evaluation of performance on a system at plant scale [5],
- evaluation of environmental impact carried out using LCA (Life Cycle Assessment) [6].

In the past years, several guidelines for the development of biorefinery were suggested by authors [3,6]:

- a biorefinery should produce at least one high-added value component (chemical or material), as well as low-grade and high-volume product. Between them the most important in terms of market value are chemicals (fine chemicals, bulk chemicals, and biological macromolecules such as protein, lipid and carbohydrates), organic acids (lactic, succinic, etc.), polymers and resins, biomaterials, food and animal feed, and fertilizers.
- a biorefinery should produce at least one energy product (heat and electricity) or biofuels: liquid for transportation (bioethanol, biodiesel and bio-oil), solid (pellets, lignin, and charcoal), gaseous (biogas, syngas, and biomethane).

The biorefinery concept was employed to efficiently produce bioproducts from different feedstocks such as lignocellulosic biomass [7–9], food wastes [2,10], manures [11,12], and micro and macroalgal biomass [13,14]. The selection of the biorefinery feedstock is one of the main issues of its development; it must be renewable, consistent, and guarantee a regular supply. Moreover, the biofuels or bioproducts obtained from raw material in competition with food and feed industries (first generation biorefinery) give rise to ethical, environmental, and political concerns. To overcome this issue food waste, residual and non-food crop biomass gained increased interest as feedstocks for biorefinery (second generation). In the recent year, microorganisms such as microalgae-based biorefinery (third generation) are attracting more and more attention thanks to their high growth rate and productivity, their capacity to grow in marginal land, the wide range of value components contained in the cell wall and the non-seasonal biomass production [15]. Furthermore, microalgae-based biorefinery can be included as an integral part of the "Blue BioEconomy (BBE)". BBE represents economic-based activity associated with aquatic biomass focusing on creating innovations, making models, and providing a solution to environmental issues sustainably with scope to reduce global dependency on fossils fuel [16]. Moreover, the biorefinery concept applied to microalgae cultivation overcomes the related economic problems. In fact, the key bottleneck of microalgal derivates products in industrial application is weighing the cost of algal conversion against its benefits [15].

1.2 Microalgae and cyanobacteria

Microalgae and cyanobacteria are considered as one of the oldest life-forms, they are typically found in the marine system and freshwater. Compared to other photosynthetic plants, microalgae are the most productive carbon dioxide consumers. The microalgae biodiversity is enormous, it has been estimated that around 20,000–800,000 species could be found in nature in which about 40,000–50,000 species are described. They are unicellular species, that can be found individually, in chains, or in groups with size ranging from a few micrometers to a few hundreds of micrometers. From the taxonomic point of view, microalgae could be divided into different families, with common metabolism and ultrastructure but their own characteristics. Prokaryotic cells (cyanobacteria) are more similar to bacteria rather than microalgae, and lack membrane-bound organelles such as plastids, mitochondria, nuclei, Golgi bodies, and flagella. The eukaryotic cells could be categorized into classes defined by their pigmentation, namely *Chlorophyta* (green algae), *Rhodophyta* (red algae), and *Stramenopiles* (brown algae) [16].

In the recent years, the microalgae most studied and used, thanks to their multiple application in several fields, are *Chlorella vulgaris* a green microalga and *Arthrospira platensis* a green-blue cyanobacterium.

1.2.1 Chlorella vulgaris

Chlorella vulgaris is one of the first life forms appeared on Earth, it is a monocellular green microalga that grows spontaneously in mineral rich stagnant waters and is abundant in equatorial Africa (Figure 1.1.). *C. vulgaris* is an eukaryotic microscopic spherical cell with a diameter of about 2–10 μm, the cell wall is rigid to preserve the integrity of the cell and it is basically a protection against invaders and harsh environment. The cell wall thickness changes during the microalgae growth until it reaches around 17-21 nm after maturation [17]. The rigidity of the cell ensured for by a microfibrillar layer made by glucosamine. While, the cytoplasm is the gel-like substance, composed of water, soluble protein, and minerals confined within the cell membrane. The cell contains internal organelles such as a small nucleus (containing the large part of the genetic material), a single chloroplast (with a double enveloping membrane composed of phospholipids), the Golgi apparatus, mitochondria (with genetic materials, the respiratory apparatus and has a double layer membrane), and vacuoles. The whole organelles are surrounded by an outer membrane composed of an equal ratio of proteins and phospholipids [18]. The *C. vulgaris* composition, like the other microalgae, is made by lipids, carbohydrates, and proteins.

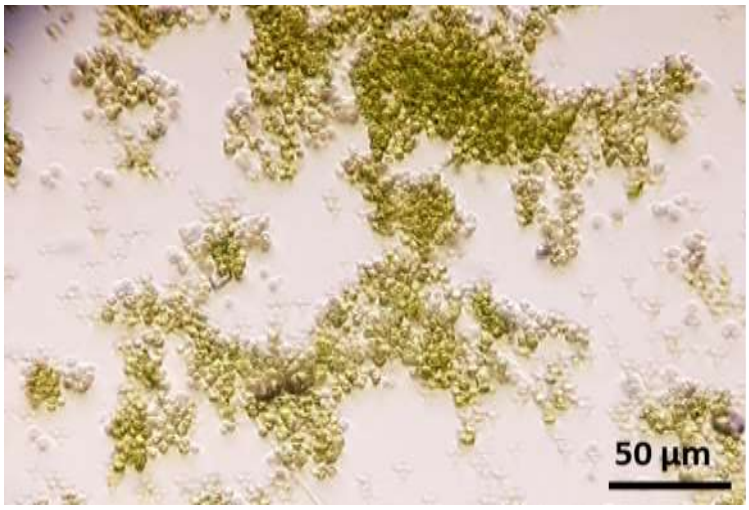

Figure 1.1. Optical observation of *Chlorella vulgaris* cells by light microscope (magnification 20x).

The lipid content can reach 40% of lipids per dry weight of biomass during light growth conditions. The lipids are synthesized by chloroplasts and are principally located in the cell wall and organelles

membrane. The main lipids are composed of glycolipids, waxes, hydrocarbons, phospholipids, and a small number of fatty acids [18], while the protein content represents around 40-58% of dried biomass and varies according to growth conditions. The most abundant amino acids are glutamic acid, alanine, and aspartic acid. Almost 20% of proteins are bound to the cell wall, around 50% are internal and 30% migrate in and out of the cell [19]. The remaining part of the cell is constituted by carbohydrates that are the energy storage of the cell. Between them, starch is the most abundant polysaccharide, which it is generally located in the chloroplast and it is composed of amylose and amylopectin. Cellulose is the structural polysaccharide, serving as the protective fibrous layer of the cell wall [20]. Chlorophyll (α and β) is the most abundant pigment and is situated into the thylakoids, which can reach 1-2% by dry weight. *C. vulgaris* has an important content of carotenoids, like β-carotene (7-12 µg/g by dry weight) [21].

1.2.2 Arthrospira platensis

Arthrospira platensis is a filamentous cyanobacterium, where the single cells are arranged into a multicellular cylindrical trichomes in an open left-hand helix along the entire length (Figure 1.2.). The helix shape of trichome may be affected by environmental factors, mainly temperature, physical and chemical conditions. The cell organization of *A. platensis* is typical of that of prokaryotic organisms, being devoid of a morphologically limited nucleus and of plastids and displaying an outer cell wall. The cell wall (about 40-60 nm) consists of four layers, namely layer of innermost polysaccharide fibrilla, a second layer of peptidoglycan, a third layer composed of proteins and outermost layer. The thylakoid membranes, located between peripheral and central cytoplasm, are filled with ribosomes and fibrils of DNA.

Figure 1.2. Optical observation of *Arthrospira platensis* cells by light microscope (magnification 20x).

A. platensis, as all the cyanobacteria, is generally poor in lipids which account for only 6-13% of dry weight of biomass; half of them are fatty acids, while the triglycerides are a minor component (1-2%). It is especially reach in proteins (60-70%) and contains 16 amino acids, eight of which are essential, such as leucine, valine, and isoleucine, while glutamic acid and aspartic acid are present in higher amount. The *A. platensis* proteins with greater economic value, especially as food pigment, are the phycobiliproteins, c-phycocyanin and allophycocyanin (20 % of total protein). The carbohydrates, mainly branched polymers of glucose, account for about of 10-20% of the dry weight, among them the most abundant sugar being glucose (7-8%). The pigment composition of *A. platensis* is typical of cyanobacteria, that is only α- chlorophyll (0.8-1.5% of dry weight), and carotenoids are β-carotene and zeaxanthin [22].

1.2.3 Microalgae metabolism

The microalgae metabolism could be both autotrophic or heterotrophic depending on carbon source and light condition in which they are submitted during growth.

Autotrophic growth takes place when microalgae utilize light as their energy source and CO_2 as the carbon source for anabolic reactions through photosynthesis and carbon fixation. Photosynthesis is a biological process utilizing ATP/NADPH to fix and convert CO_2 captured from the air to produce glucose and other sugars through a metabolic pathway known as the Calvin cycle. The carbohydrates are accumulated in the plastids as starch (reserve materials) or in the cell wall (cellulose, pectin, and sulfated polysaccharides).

Light intensity plays a critical role in autotrophic cultivation, since high microalgae concentration in the growth system does not allow light to diffuse properly, thus the cells suffer light limitation and inhibition of growth. Nevertheless, autotrophic cultivation is the most generally microalgae growth condition technically and economically viable for large-scale production of microalgae biomass [22].

The heterotrophic cultivation mode requires organic carbon as energy source. Thus, the microalgae are grown in the absence of light and convert sugar (glucose) into lipids [23]. Heterotrophic cultivation has several advantages including elimination of illumination cost, simple operation, higher growth rates and higher lipid productivity, compared to autotrophic mode. This growth mode offers the possibility of greatly increasing microalgal biomass concentration and yield on an industrial scale; however, only a few industrial heterotrophic processes have yet been tackled due to the limited number of available heterotrophic microalgal species and the inhibition of microalgae growth by organic substrates at low concentrations. Although heterotrophic mode yields high algal density and

lipid content, but the cost of alternative organic carbon source makes this microalgae cultivation economically unfeasible [24].

Moreover, microalgae and cyanobacteria could be mixotrophic, having the ability to use both organic and inorganic carbon for their metabolism. Mixotrophic cultivation allows microalgae to live under both autotrophic and heterotrophic conditions: microalgae adapt both organic compounds and CO_2 as carbon sources [25]. For these reasons mixotrophic cultivation requires low light intensity reducing light energy costs. Consequently, mixotrophic system can get benefits from both autotrophic and heterotrophic metabolism and minimize their individual limitations, even if supplementation of nutrients is still required [26].

1.2.4 Technologies for microalgae biomass production

Microalgae can be cultivated both in open and closed systems, the selection of a culture system depends on the final product: wastewater treatment and biofuel production can be done in either open or closed systems. On the other hand, to produce biomass suitable for the extraction of components that requires less contamination they should be preferably cultivated in closed systems [27].

Open ponds, which were the first developed, in the 1950s, have long been used for large scale cultivation given their simple construction and relatively easy operation. The main problems related to open systems are the requirement of high area for cultivation, mixing systems, and availability of sources such as light and nutrients. There are different types and configurations of an open system, between them the most used are inclined systems, circular ponds, and raceway ponds [28]. Raceway ponds have paddlewheels attached to the system to maintain constant mixing of the culture in order to guarantee the correct light intensity to all the cells. Open systems require low construction and operational costs, but on the other hand, they need larger land areas, which may increase the capital cost. In open ponds, until today, only a few species of microalgae have been found to grow well at a commercial scale. In fact, this type of system is generally used to cultivate *Chlorella*, *Phaeodactylum*, *Artrospira*, and *Scenedesmus* species because they can survive under a repetitive mixing environment [29].

Photobioreactors (PBR) are the closed system used to grow the microalgae; several configurations were developed such as vertical tank, helical tube, air-lift, horizontal tube, tubular, flat-plate, and vertical column. The diameter of the photobioreactor is usually small to ensure that light can penetrate through the system and reach the cells.

Closed systems allow growing microalgae under a controlled environment, in terms of pH, mixing, maximum sunlight intensity, culture density, and temperature [30]. Among the PBR, airlift photobioreactor is the most suitable for the cultivation of microalgae. The circulating air ensures that

all the microalgae have enough carbon dioxide and access to light, especially near the surfaces of the columns [28]. Generally, in a closed system, the typical surface-to-volume ratio is 80–100 m^{-1} [27]. Hybrid systems combine the properties of open pond and closed bioreactor systems. During the first cultivation stage, the desired species is cultivated in an open pond. Then, during the second stage, the open pond culture with the preferred strain is cultivated in closed system. This hybrid system can expose the algal culture to nutrient stresses and may increases the biomass productivity and lipid accumulation [29].

1.2.5 Microalgae application

1.2.5.1 Wastewater treatment

Microalgae can be used for wastewater treatments, in particular for ternary and quaternary treatments. These treatments consist of the removal of ions (such as ammonia, nitrate, and phosphate), heavy metals, and organic compounds. A wide range of wastewaters such as piggery effluent, industrial wastes, agricultural wastes, or human sewage, have also been treated by microalgae.

Microalgae use the nutrients available in the wastewater during mixotrophic and heterotrophic growth. They use principally organic carbon present in the wastewaters as a carbon source instead of carbon dioxide. The main challenge in cultivating microalgae in raw wastewater is the presence of other microbes that affect biomass productivity [31]. Although the microalgae are photosynthetic organisms, heterotrophic growth has also been made in practice to prove that algae can grow independently of light; however, this resulted in a slow growth rate compared to autotrophic mode. In comparison to conventional biological treatment, the advantage of using microalgae in raw wastewater is that they act as flocculants, which enhances sedimentation rate. They are usually used in addition to the traditional process, after secondary treatments [32].

Microalgae have the capacity to metabolize and destroy a wide range of pollutants among which recalcitrant organic molecules (such as polyphenols). Microalgal biomass generated during wastewater could be successfully converted into various bioproducts, which thus excel for life-cycle impact assessment. Some microalgal species very efficiently degrade organic contaminants, including persistent molecules such as tannins and detergents; moreover, they have also been successfully used to treat effluents generated from anaerobic digestion, biogas, olive processing, swine manure, pulp/paper mills, sand, among others [33].

1.2.5.1.1 Wastewater treatment by co-culture

Many studies have analyzed the advantages and limitations of using microalgal consortia in the wastewater purification processes, due to the difficulty of maintaining a pure microalgal monoculture

in them [34]. These consortia can naturally occur during the process or they could be artificially engineered, by the combination of microorganisms that do not necessarily co-occur, for a specific purpose [35]. The most common consortia used for wastewater treatment are microalgal consortia, which are constituted exclusively by photosynthetic microorganisms (eukaryotic and/or prokaryotic), and microalgal-bacterial consortia, which are constituted by photosynthetic microorganisms and heterotrophic bacteria [36].

Interactions between photosynthetic microorganisms can lead to an overall increase in biomass production and nutrient removal efficiency, but also to the formation of allelochemicals, secondary metabolites detrimental to co-culture growth [37] . Factors that favor the formation of allelochemicals include nutrients starvation, low light intensities, and temperature, high pH values, and concentrations of the involved microorganisms [38].

Microalgal-bacterial consortia are disadvantaged by the increase in pH and temperature caused by the photo-synthetic activity and the excretion of microalgal metabolites presenting a bactericidal effect [39]. On the other hand, microalgae can serve as a habitat for bacteria and enhance bacterial growth through the release of extracellular metabolites, bacteria release the CO_2 required photosynthetic reactions and microalgae release organic compounds that can be used by bacteria as carbon and energy source[40]. Apart from being effective in nutrients removal, these systems can further improve current wastewater treatment processes because [41]: the costs associated with the oxygenation of activated sludge tanks can be significantly reduced and the greenhouse effects associated with wastewater treatment plants can be considered negligible since the CO_2 released by bacteria is converted into organic matter by microalgae [42].

1.2.5.1.2 Winery wastewater treatment by microalgae

Winery wastewater (WWW) results from a large number of wines making process activities that include cleaning of tanks, washing of floors and equipment, rinsing of transfer lines, barrel cleaning, spent wine and product losses, bottling facilities and filtration units. Wastewater volume generated is highly variable (0.5-14 L per liter of wine produced) and principally depends on seasonal operation, working period and kind of wine. The winery wastewaters are produced in the highest quantity in the period of pre-harvesting (from August to February), considering the water used for cleaning the cellar and the harvest tools. However, these wastewaters even if are produced in large amount show a lower pollution impact. In fact, the variation in wastewater pollution parameters is mainly due to the seasonality of wine production. In general, during the wine harvest period (from February to May), the pollutants reach their higher value because of the mixing with wine. For these reasons, WWW treatment plants must be flexible to flow variations and quickly to adapt to starts-ups and close-downs

[43,44]. Chemical composition of WWW is also variable and depends on the substances contained in the grapes (berries, stalks, seeds, pulp), the residual products of the various vinification processes (such as yeasts, other micro-organisms, sugars, methyl and ethyl alcohols, butyl aldehyde, acetone, formaldehyde, formic, acetic and tartaric acids) and also all those substances that are involved in wine processing [45,46]. The qualitative characteristics of the winery wastewater, according to the literature, are presented in Table 1.1. In general, it is acidic and has high COD and BOD contents and high concentration of total solids.

Table 1.1. Characteristics of winery wastewater found by different authors [47]

Parameters	Unit	Min	Max	Average
COD [a]	mg/L	340	49105	14426
BOD_5 [b]	mg/L	181	22418	9574
pH	-	3.5	7.9	4.9
Total solid	mg/L	190	18000	4151
Electrical conductivity	S/m	1.2	7.2	4.16
Suspended solid	mg/L	1000	5137	2845

[a] Chemical Oxygen Demand, [b] Biological Oxygen Demand.

Untreated winery wastewater discharge in watercourses, rivers and lakes can cause eutrophication (nutrient enrichment) as well as lack of oxygen for aquatic animals due to high levels of chemical/biological oxygen demand (COD/BOD), and the high acidity can affect the vigor of plants, reducing the availability of plant nutrients (phosphorus and calcium) and decreasing the population of useful microorganisms. The high percentage of organic compounds, as well as salts contained in wine effluents, can cause significant inhibitory effects on plant growth, while the high electrical conductivity can cause delay of germination [48].

Moreover, different phenolic compounds could be found in WWW because they are extracted from grape skins, pulp, and seeds. Although the phenolic compounds constitute a relatively small portion of the WWW organic load, they can cause significant environmental problems if released into the environment without proper treatment. This is due to their toxicity to humans, to animals and many microorganisms, even at relatively low concentrations. The phenolic compounds are also particularly resistant to degradation reactions [49].

Conventional winery wastewater treatment is composed by four main phases: 1- Pretreatment, which consists of mechanical sorting; 2- Primary treatment, which consists of heavy solids sedimentation and removal; 3- Secondary treatment, which can be either chemical or biological and removes the suspended solids; 4- Tertiary treatments, which remove in particular substances such as nitrogen or

phosphorus and break down the bacterial load. Conventional winery wastewater treatment can be classified in: physicochemical, biological, membrane filtration and separation, advanced oxidation processes (AOPs), and combined biological with advanced chemical processes [47].

Physicochemical processes (coagulation/flocculation) have been found to be effective for winery wastewater pre-treatment, and more specifically for reducing the TSS content (Total Suspended Solids), the turbidity, and COD [50] . In particular, the physicochemical process with the highest efficiency in COD removal (up to 73%) is coagulation using chitosan [51].

Membrane filtration and separation processes are particularly appropriate when high quality of resulting treated wastewater is required. These processes can achieve high removals of constituents such as dissolved solids, organic carbon, inorganic ions, and regulated and unregulated organic compounds.

Only few studies of WWW treatment by membrane filtration and separation technologies (nanofiltration (NF) and reverse osmosis (RO)) have been reported so far. The RO process is a promising process with a COD reduction up to 95%. But, an appropriate management of the concentrate produced and the fouling of the membranes, are the two major problems that limit the use of these processes [52] .

Biological treatments use bacteria (*Pseudomonas sp.*, *Enterobacter sp.* and *Klebsiella sp.*) and fungi (*Penicillium sp*. and *Aspergillus sp.*) to purify effluents through the consumption of organic substances, carbon dioxide, and volatile acids. However, these conventional biological methods are not able to adequately remove the organic matter present in high levels in WWWs, and the residual organic load of effluents is often higher than the limits established by the Legislative Decree n. 152/2006 concerning wastewater. Wastewaters, as already mentioned, are rich in COD and colored substances, generally have acidic pH and may contain phenolic compounds that can inhibit the microorganisms responsible for biological treatment [53] .

The selection of microorganisms that could be used for biological treatments is considered in terms of their ability to grow under undesirable conditions. In fact, the concentration of nitrogen and phosphates is low in WWW, then additional nutrients are often required to increase the activity of bacteria and fungi.

Photosynthetic unicellular organisms, such as microalgae and cyanobacteria, can overcome these limits, because of their ability to grow easily under suboptimal conditions such as nitrogen and phosphate depletion, excess of sodium chloride, and the low light intensity [54]. Unfortunately, despite the numerous studies on the effectiveness of the use of algae for treating various types of wastewater, including those from dairies, slaughterhouses, piggeries, and municipal waters, literature concerning the treatment of waste deriving from the wine industry is poor.

1.2.5.2 Biofuels

Fossil fuels, accounting for 88% of the primary energy consumption, are frequently used because they are produced at lower cost compared to the other fuels; however, they are the largest contributor of greenhouse gases (GHGs) to the biosphere; in 2019, CO_2 emissions were 38 Gtonnes. It is estimated that natural processes remove only about 12 Gtonnes; therefore, compatible mitigation strategies are required to neutralize the excess CO_2. The global strategies for the CO_2 emissions mitigation include the increase of the energy efficiency, the use of clean fossil energy (fossil fuels whit CO_2 separation system) and the use of renewable energy [55].

The only possible solution to this crisis is to find a sustainable (renewable) and economically feasible source of alternative energy; the best option are biofuels, particularly those made from readily available biomass feedstock. Biofuels are liquid or gaseous fuels for the transport sector that are predominantly produced from a variety of bio-feedstocks. Bio-feedstocks or biomass refers to all the vegetable matter that can be obtained from photosynthesis. They are renewable, sustainable, biodegradable, carbon neutral for the whole life cycle and environmentally friendly. The great versatility of biomass as a feedstock is evident from the range of materials that can be converted into various solid, liquid and gaseous fuels using biological and thermochemical conversion processes. Several biofuels, including bioethanol, biomethanol, biodiesel and biohydrogen, appear to be attractive options for the future of transport sector [56]. There are different conversion technologies to produce biofuel which depend on the types and sources of biomass. The conversion technologies can be divided into three basic categories, namely thermochemical, chemical and biochemical conversion. Factors that influence the choice of the conversion process include the type and quantity of biomass feedstock, the desired forms of energy, economic consideration, desired end form of the products [57].

Thermochemical conversion consists in the thermal decomposition of organic components in biomass to yield fuel products. The thermochemical conversion processes include direct combustion, gasification, liquefaction and pyrolysis. When biomass is heated under oxygen deficient condition, it generates syngas, which consists primarily of hydrogen and carbon monoxide. The syngas can be directly burn of further processed for biofuels production. In this case thermal or chemical conversion of biomass is very similar to that of coal. Chemical conversion is a process that allows the production of biofuel from microalgae through chemical reactions. The chemical processes include the transesterification reaction to produce biodiesel and the hydrogen production. The biological processes of energy conversion of biomass into fuels includes anaerobic digestion, alcoholic fermentation, photo-biological hydrogen production, transesterification [58].

1.2.5.2.1 Pyrolysis from microalgal biomass

Pyrolysis is a thermochemical process that can efficiently recover most of the energy available in biomass, in which chemicals, in the absence of oxygen, are converted into bio-oil, biogas and bio-char [59]. The pyrolysis process is characterized by complex mechanisms, in which decarboxylation, dehydration, cracking, dehydrogenation and rearrangement reactions take place. Pyrolysis bio-oil is generally composed of a wide range of different compounds including hydrocarbons, acids, alcohols, polyaromatics, nitrogenated compounds, indole, and carbonyls, with molecular weight from 18 to 5000 g/mol. The presence of oxygenated and nitrogenated compounds in the bio-oil leads to few undesirable properties such as low heating value and high viscosity, which do not allow miscibility with fossil fuels [60].

The pyrolysis processes mentioned above could be used also with algal biomass. Some studies show that bio-oil produced from microalgae and other proteinaceous biomass have better properties than lignocellulosic biomass-based bio-oil. Generally, the bio-oils that arise from the pyrolysis of microalgae and proteinaceous biomass are more stable, have lower oxygen content and an improved higher heating value (HHV) compared to bio-oil from lignocellulosic sources. The improved properties of bio-oil obtained from the pyrolysis of a proteinaceous feedstock is associated with the conversion of its components to compounds present in the resulting oil. For example, linear hydrocarbons present in microalgae bio-oil result from the pyrolysis of lipids initially present in the algae [61]. Microalgae bio-oil is produced not only from triglyceride conversion, but also from the conversion of proteins and carbohydrates. In fact, in the pyrolysis of algae the aromatic hydrocarbons are mainly derived from the protein fraction. *Arthrospira platensis* could be a potential feedstock for pyrolysis due to its low lipid content and high protein content [62]. Moreover, it adapts easier to cultivation conditions and can be grown in wastewater with faster growth rates than high-lipid algae. High protein biomass produces bio-oil with a greater content of nitrogen and lower content of oxygen than lignocellulosic biomass, following the trend of the elemental composition of the feedstock. To improve microalgae pyrolysis performance and upgrade the quality of reaction products, it is possible to add catalysts during thermal treatment, or to follow a second biooil refining catalytic treatment [63]. The catalytic pyrolysis process can be used to reduce the content of N- and O-components and increase that of aromatic hydrocarbons [64].

1.2.5.3 Bioactive compounds

Microalgae are known to have metabolites that are suitable for pharmaceutical and food industry, such as astaxanthin, omega three fatty acids, sterols, proteins, enzymes, vitamins and pigments [65]. Pigments are essentially colored molecules that absorb light from the visible spectrum. They are used for a large variety of products including food colorants or additives, pharmaceutical and nutraceutical

products, and aquaculture. In general, these pigments are currently produced on an industrial scale by non-renewable synthetic sources (petrochemicals, inorganic chemicals, and organic acids), because the raw material and production cost are cheaper. However, on the market, there is a rising demand for naturally produced pigments due to safety and environmental concern associated with their synthetic counterparts [66]. In fact, the food industry is very keen on utilizing natural pigments as food colorants. The pigments produced by microalgae depend on the species and their corresponding color. Green microalgae contain chlorophylls, orange and red microalgae produce carotenoids and red and blue microalgae phycobilins [67] . The global market of carotenoids is about 1.53 billion US$; they have a therapeutic effect on humans and animals, protecting them from oxidative and free-radical stresses due to their strong antioxidant properties [68]. Chlorophyll, which is synthesized by all the photoautotrophic microalgal species and constitutes about 0.5–1.5% of the dry cell matter, it is a natural source of green pigments. This pigment possesses antioxidant, antigenotoxic, anticarcinogenic, and antimutagenic properties. Moreover, the consumption of chlorophyll has shown the potential to increase bile secretion and further stimulate the recovery of the liver [69]. Phycobiliproteins are hydrophilic protein complexes that capture light energy and thus assist in the photosynthesis of cyanobacteria and other red microalgae. They are mainly present in *Arthrospira sp., Porphydrium sp.,* and *Aphanizomenon flos-aquae*. Phycocyanin is a natural blue colorant belonging to the family of Phycobiliproteins, generally extracted from *Arthrospira sp*. It is used as coloring molecule in chewing gum, confectionery, wasabi, dairy products, and soft drinks. These pigments also have anti-inflammatory, anti-oxidative, anti-viral, hepatoprotective, and neuroprotective properties [70]. Pigment production from microalgae can be enhanced by nutritional and light stress conditions. The microalgae grown in wastewater can be used for the extraction of pigments, which can significantly reduce their high production cost making them more marketable.

Proteins are the building blocks of the human body, are the essential macronutrients responsible for the growth of an individual. They are made of long chains of essential and non-essential amino acids, linked by peptide bonds. The essential amino acids need to be assumed as food items because the human body is not able to synthesize them. The common sources that have a complete profile of essential amino acids are poultry meat, red meat, eggs, fish, soy, tofu, and dairy products. Contrariwise, plants do not have a complete profile of essential amino acids, which represents a problem for the population following a vegetarian and vegan diet [71]. A solution to this problem could be the assumption of microalgae that, on the other hand, are an excellent source of essential amino acids. Among them, *Chlorella sp.* and *Arthrospira sp.* are constituted by about 70% of protein. According to WHO/FAO/UNU recommendations, they contain well balanced essential amino acids content required for human consumption. Moreover, the amino acid content (such as isoleucine,

tryptophan, methionine, valine, lysine, threonine, and histidine) in some microalgae is also comparable to that of high protein content sources (eggs and soybean) [72].

The human body, apart from macronutrients (fats, proteins, and carbohydrates), requires several micronutrients to survive. These micronutrients are both active electron/proton carriers in the macronutrient breakdown process or co-enzymes. Vitamins are an important group of micronutrients that play a major role in the energy metabolism of humans. In humans, a deficit of vitamins is responsible for various diseases such as rickets, scurvy, methyl-malonic acidemia, and beriberi. Microalgal biomass is rich in vitamins [73]. In general, microalgae contain a high concentration of provitamin A, vitamin E, vitamin B1, and folic acid. Anyways, there are different species of microalgae that contain other vitamins at high concentration. *Chlorella sp.* and *Arthrospira sp.* are also rich in vitamin B8 (biotin) and B12 (cobalamin). Vegetables and fruits are poor in vitamin B12; consequently, people that follow a vegetarian/vegan diet need to assume it from different sources such as microalgae [74]. Although microalgae vitamin content is comparable to that of certain vegetables and fruit, the extraction of bioactive compounds from microalgal biomass in a biorefinery concept deems it a superior source compared to conventional sources [75].

External stress such as excessive exposure to sunlight or smoking causes the production of free radicals or reactive oxygen species by the human body. To fight the free radicals the human cells synthesize antioxidants. Moreover, the human body needs an equilibrium between the oxidant to antioxidant ratio, any disturbance of which resulting in the accumulation of free radicals. This phenomenon, called oxidative stress, plays a crucial role in cell and tissue damage and is related to several diseases such as atherosclerosis, diabetes, aging, rheumatoid arthritis, Alzheimer's disease, auto-immune disorder and motor neuron disease [76]. The human body is able to synthesize *in situ* or internally endogenous antioxidants by enzymatic or non-enzymatic pathways. Alternatively, it can consume the exogenous antioxidants (*ex-situ*) through food supplements. The external antioxidants such as carotenoids, flavonoids, fatty acids (ω-3 and ω-6 fatty acids), vitamin C and E are also known as nutrient oxidants due to their nature. Microalgae, thanks to their higher antioxidant production capacity compared to conventional plant-derived sources, are considered to be a superior source of nutritional antioxidants. Moreover, there is a huge demand for naturally sourced antioxidants, as these compounds are included in functional foods and pharmaceuticals. The bioavailability of the antioxidant that could be extracted from microalgae is higher than from synthetic sources and provide better protection [77].

1.2.5.3.1 Extraction of bioactive compounds

The conventional extraction methods require the use of a solvent (alcohol-water mixture, or non-polar solvent) which could affect the qualitative and quantitative characteristics of bioactive compounds such as biological activities and yield. These methods include Soxhlet, maceration, infusion, digestion, hydrodistillation, and percolation. The characteristics of the bioactive compound are essential for the selection of the extraction method that depends on the characteristic of the solvent (polarity, viscosity, dipole moment, surface tension, and dielectric constant), temperature, and mechanical agitation or mixing [78]. It is also desirable to select safe and ecological extraction techniques to extract them efficiently and sustainably. This allows not only to increase the quality of the end products but also to fulfill clean label requirements. For this reason, the use of a green solvent that is obtained from renewable sources has been proposed to replace hazardous solvents (petroleum derived). These solvents include principally water, sub/supercritical fluids, deep eutectic solvents, and ionic liquids [79]. The concept of green extraction not only requires the use of green solvent but also is based on the discovery and design of extraction processes which will reduce energy consumption and ensure a high-quality extract/product. Several novel technologies have been studied for the extraction of a range of bioactive compounds for food and pharmaceutical applications[80]. These methods require a disruption force that allows an easy release of the bioactive components into the solvent. The disruption methods can be classified depending on the nature of the disruption force as mechanical (homogenization and bead milling), physical (sonication, drying, microwave radiation, and pulsed electric field), and chemical/biological (enzymes and acid/base). These novel technologies are particularly appropriate for the extraction of bioactive compounds from microalgae because the intracellular components are often prevented by the intrinsic rigidity of their cell wall. To overcome this limit an initial operation unit of cell disruption is required to permit the complete release of the internal components and facilitate the extraction process. The selection of the correct cell disruption method depends on the cell wall structure, size, product location, solubility, and applied energy [81].

1.2.5.4 Food and feed application

Microalgae and cyanobacteria have been used in human nutrition for thousands of years and their supplementation helps the reduction of the stress on intense resource-demanding terrestrial crops. Microalgae are excellent sources of minerals such as potassium, iron, magnesium, calcium, and iodine and as well vitamins such as A, B1, B2, B6, B12, C, and E [82]. Currently, the microalgae for human nutrition are marketed as healthy foods and are available as capsules, tablets, powders, and liquids or they could be also mixed with snacks, pastes, breakfast cereals, candies, gums wine, and other beverages [77]. The microalgae and cyanobacteria species widely used for food include *Arthrospira platensis* (*Spirulina*), *Chlorella sp.*, *Dunaliella sp.*, and *Aflosaquae* due to their high protein content

and nutritional value. However, in recent years, *Chlorella* and *Arthrospira* species, thank their nutrient-rich profiles, are dominating the global microalgae market as they are gaining popularity in the health-food supermarkets and stores.

Chlorella sp. has a global market of around 160 million US$, it is usually marketed as 'healthy food' and is being promoted as a functional food to prevent or help common diseases or acute diseases like Alzheimer's disease, cancer, etc. In fact, it is an excellent hepatoprotective and hypocholesterolemic agent during malnutrition and ethionine intoxication, decreases blood sugar concentration and increases hemoglobin concentration. *Chlorella sp*. also contain an active immunestimulator-β-1,3-glucan, which reduces blood lipids and acts as a free radical scavenger [83].

Arthrospira sp. was labeled by the World Health Organization (WHO) as a "superfood". It has been used as food by indigenous people of Mexico and Africa since the 1950s. It is an excellent natural source of proteins, vitamins such as A, B1, B2, B8 and B12, essential fatty acids, and useful pigments (xanthophyll and carotenoids). In fact, a full spoon (around 7 g) of dried *Arthrospira sp.* biomass contains almost 4 g of protein, 1 g of fat including PUFAs (omega-3 and omega-6 fatty acids), minerals in a small amount (magnesium, manganese, and potassium) and 15%, 11% and 4% of Required Daily Allowance (RDA) of Vitamin B1, B2, and B3, respectively.

Moreover, *Arthrospira*, *Chlorella*, *Nannochloropsis*, *Scenedesmus*, *Crypthecodinium* are reported to be used as feed for terrestrial as well as aquatic animals [84]. Anyways, the high production cost is a major bottleneck for the application of microalgae in the feed industry, so the use of wastewater to generate microalgal biomass is an economical route to reduce the feed cost. Some studies have been conducted on the use of microalgae as animal feed. Guihéneuf and Stengel (2015) reported that cultured *Porphyridium purpureum* in open ponds has fatty acids that are suitable for animal nutrition [85]. Phang et al. (2000) found that the biomass composition of *A. platensis* used for the treatment of sago wastewater can be used as high-quality animal feed, especially in the aquaculture industry [86].

2 Winery Wastewater Treatment by Microalgae to Produce Low-Cost Biomass

The reduction of fossil fuels reserves and the continuously increasing demand for energy around the world has led to the necessity to find an eco-sustainable alternative to conventional fuels. In the last few years, biofuel production from different plant sources has been increasingly studied by researchers [56]. The production of third-generation biofuels from raw materials that do not compete with food crops is in fact attracting more and more attention. Third-generation biofuels can be produced from microalgal biomasses or from their intracellular components such as lipids. Moreover, their production, if compared to conventional biomasses, reduces land and water utilization along with the use of pesticides [87].

Microalgae are unicellular microorganisms able to grow under autotrophic, heterotrophic or mixotrophic conditions depending on the carbon source used in their metabolism as well as light conditions[88]. They are composed mainly of lipids, proteins and carbohydrates, whose relative proportions depend in particular on the species and growth conditions [89]. They are generally used for human or animal nutrition [90], or extraction of added-values components for chemical and pharmaceutical industries [91], but also for biofuel production [92].

The main limit of the production of biofuels from microalgae is due to the high cost of biomass cultivation, making them not cost-competitive, on a large scale, when compared with conventional biofuels produced from agricultural waste or conventional biomasses. The only possibility to make the microalgae biofuels more competitive on the global market is to apply the biorefinery concept. Thanks to their capability of also metabolizing organic carbon, microalgae can in fact be grown in wastewaters, thus reducing the use of fresh water, the cost of growth medium, the energy consumption and, at the same time, the wastewater polluting impact [47]. There are several studies in the literature focusing on the use of microalgae to treat wastewaters such as municipal wastewater [93] and textile wastewater [94], among others.

Winery wastewaters (WWWs) are released from different activities of the wine making process, namely tank washing, transfer, bottling and filtration [95]. The polluting impact of WWWs is related to their high organic load (polyphenolic compounds, sugars, organic acids and esters), low pH (3–5), content of suspended particles and large volumes (0.5–14 L per liter of wine produced) [93]. Among them, polyphenols are considered hazardous compounds because they are not

mineralized by conventional biological treatments [94]. Owing to the release of organic compounds and inorganic ions, their disposal in land without adequate treatment can change the physicochemical properties of groundwater such as color, pH and electrical conductivity, among others [95]. WWWs can be treated by biological or physicochemical processes, membrane filtration and separation, advanced oxidation or combined biological and advanced chemical processes [93]. Among these, biological processes are the most appropriate to treat WWWs because of their high organic load.

The aim of this chapter is to grow a co-culture of *Arthrospira platensis* and *Chlorella vulgaris*, the most common microalgae belonging to the prokaryotic and eukaryotic phyla, respectively, using WWWs as culture media in order to reduce, on one hand, the production cost of biomass to be used for energy production purposes and, on the other, the wastewater pollution load. Particularly, three different WWWs, namely the first and second tank washing waters and that from the filtration equipment. The reduction of the wastewater pollution load was evaluated in terms of reduction of polyphenol concentration and chemical oxygen demand.

At first, the best concentration of winery wastewaters in Bold Basal's Medium (10, 20, 50 and, 100%) was investigated. Then, the influences of light conditions on co-culture growth and winery wastewater treatment were studied by growing it under dark and light-dark cycles conditions. At least, the co-culture was grown in different photobioreactor configurations: multitubular photobioreactor, open pond, and column photobioreactor.

2.1 Determination of optimal winery wastewater concentration in microalgae growth medium

The data that will be discussed in this section has been already published:
E. Spennati, A.A. Casazza, A. Converti, Winery wastewater treatment by microalgae to produce low-cost biomass for energy production purposes, Energies. 13 (2020). doi:10.3390/en13102490.

In this section a co-culture of *Chlorella vulgaris* and *Arthrospira platensis* was used to treat three different winery wastewaters from different steps of the wine production process, in order to produce low-cost biomass intended for biofuel production. Growth of the co-culture and reduction of wastewater pollutant impact were followed by daily determinations of biomass concentration, COD and polyphenol content. The highest productivities of biomass (0.66 gDry Weight /L·day) and lipids (7.10 ± 0.22 gLipid/100 L·day) were obtained using 20% of second washing winery wastewater after 4 days of treatment. Moreover, COD and polyphenol content of the three different wastewaters were reduced by the co-culture by more than 92% and 50%, respectively. These results suggest that winery wastewaters can be used successfully for the growth of *A. platensis* and *C. vulgaris* co-culture in order to obtain inexpensive biomass for energy production purposes.

2.1.1 Materials and methods

2.1.1.1 Microalgae Strains and Culture Conditions

To produce the inoculum, the co-culture (Figure 2.1.) of *Chlorella vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK) and *Arthrospira platensis* UTEX 1926 (University of Texas Culture Collection, TX, USA) was cultivated in Erlenmeyer flasks (1000 mL) using Bold Basal Medium (BBM) with a continuous air supply at room temperature (25 °C). The inoculum was prepared mixing two solutions at same volume and concentration of pure *C. vulgaris* and *A. platensis*. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The three second fermentation winery wastewaters (WWWs), namely those from the first (W1) and second (W2) tank washings as well as that from the filtration equipment (W3), were provided by a winery cellar located in the Piemonte region, Italy.

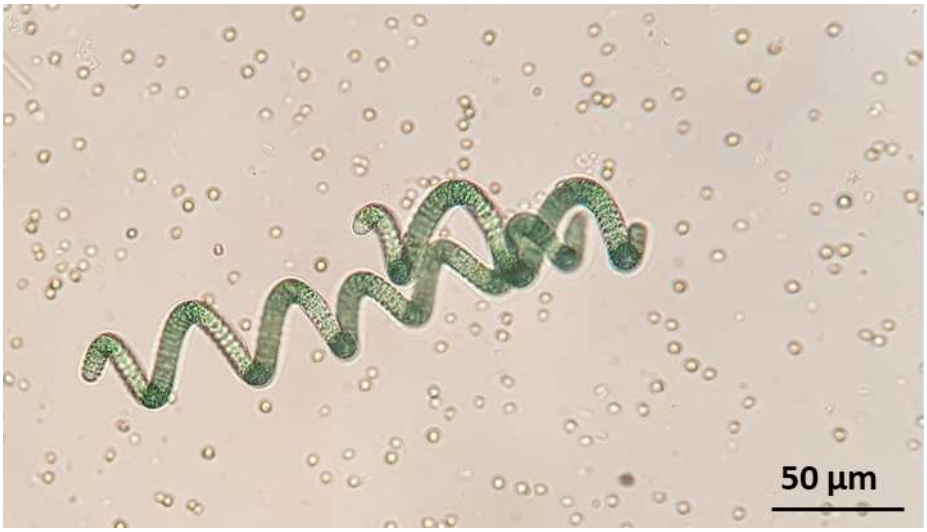

Figure 2.1. Image of *Chlorella vulgaris* and *Arthrospira platensis* co-culture obtained by optical microscope (magnification 20x).

2.1.1.2 Experimental design

After inoculation (0.5 g_{DW}/L), the co-culture was grown in 200−mL bubblers using the three WWWs diluted with BBM up to 10%, 20%, 50% and 100% (v/v) under continuous air supply and illumination (82 ± 5 µmol photons/m^2 s) for 15 days (Figure 2.2.). WWWs were used without preliminary treatments. Two different sets of control runs were performed, where the co-culture was grown under the same conditions using only BBM (C). Moreover, the three wastewaters were exposed to air bubbling and light without microalgal inoculum, in order to check the possible effect of autochthonous microorganisms on pollutant removal (C1W, C2W and C3W for 1W, 2W and 3W, respectively) (Figure 2.2.).

Total microalgae concentration was determined daily by dry weight, taking into account the total suspended solid (TSS) content of WWWs expressed in g/L. Aliquots of the medium were collected and filtered daily to evaluate the reduction in the concentrations of the pollutants. At the end of the growth period, the microalgal biomass was centrifuged at 6036 ×*g* for 15 min (MF20-R, Alliance Bio Expertise, Guipry, France) and freeze dried (Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). Supernatant, microalgal biomass and filtrate were collected and frozen at −20°C for subsequent analyses.

Runs were carried out in duplicate, while biomass concentration and WWW analyses were done in triplicate. Results were expressed as mean values ± standard deviations.

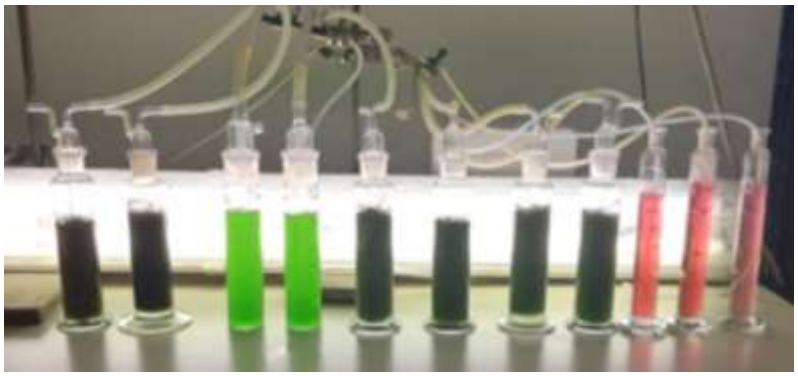

Figure 2.2. 200 mL-bubblers used for co-culture growth and winery wastewater treatment. Form the left part of image: 1W, C, 2W, 3W, C1W, C2W and, C3W.

2.1.1.3 Biomass and winery wastewaters characterization

WWWs were characterized in terms of contents of total solids (TS), total suspended solids (TSS) and volatile solids (VS) according to the standard methods of APHA (APHA, 1999).

Chemical oxygen demand (COD) was determined by colorimetric analysis. Briefly, 1.5 mL of sample and 2.5 mL of $HgSO_4$-H_2SO_4:$K_2Cr_2O_7$ (1:4 v/v) solution were added to 10− mL glass tubes. The tubes were then heated at 150 °C for 2 h, and the absorbance was measured at 620 nm (ABS_{620}) with a UV-Vis spectrophotometer (Photometer PF-12^{plus}, Macherey-Nagel, Düren, Germany). A calibration curve was made with standard solutions of potassium hydrogen phthalate, and the unknown COD (X_1) estimated by the equation (R^2 = 0.9999) and expressed in grams of oxygen per liter (g_{O_2}/L).

$$ABS_{620} = 0.0004\ X_1 - 0.0011 \quad (2.1)$$

Polyphenolic compounds (PC) were quantified by the Folin–Ciocalteu assay [96]. The absorbance was read by UV spectrophotometer (Genova, Jenway, Stone, UK) at 725 nm and expressed as milligrams of gallic acid equivalents per milliliter of solvent (mg_{GA}/mL). A calibration curve was prepared with standard solutions of gallic acid, and the unknown total polyphenol concentration (X_2) estimated by the equation (R^2 = 0.9988):

$$ABS_{725} = 0.0018\ X_2 \quad (2.2)$$

both COD and total polyphenol content of the WWWs were quantified daily, in order to evaluate the degradation efficiency of the microalgal co-culture.

The lipid fraction was extracted with a 2:1 (v/v) chloroform/methanol solution as solvent, following a modified version of the Folch method [97].

The co-culture elemental composition, in terms of carbon, hydrogen, nitrogen and sulfur contents, was determined with a CHNS-O elemental analyzer (FLASH EA1112, ThermoQuest, Cleveland,

OH, USA) following the methodology described by Ortiz Montoya et al. [97] and expressed as percentages.

2.1.1.4 Kinetic Parameters of Microalgae Growth

The specific grow rate (μ), expressed in day^{-1}, was calculated by the equation:

$$\mu = \frac{1}{t} \ln\left(\frac{X_f}{X_0}\right) \quad (2.3)$$

where t is the overall cultivation time (days), while X_0 and X_f are the starting and final biomass concentrations (g_{DW}/L), respectively.

The value of μ at maximum biomass concentration (μ_{max}), expressed in day^{-1}, was calculated by the equation:

$$\mu_{max} = \frac{1}{t_{max}} \ln\left(\frac{X_{max}}{X_0}\right) \quad (2.4)$$

where X_{max} is the maximum biomass concentration (g_{DW}/L) and t_{max} the time needed to reach it.

Biomass productivity at the end of cultivation (v) and its value at X_{max} (v_{max}), both expressed in $g_{DW}/L \cdot day$, were calculated as follows:

$$v = \frac{X_f}{t} \quad (2.5)$$

$$v_{max} = \frac{X_{max}}{t} \quad (2.6)$$

Defining the lipid content of biomass (C_L, $g_L/100\ g_{DW}$) as the fraction of lipid mass referred to 100 g of dry biomass, the lipid productivity (v_L), expressed in $g_L/100 \cdot L \cdot day$, was calculated as:

$$v_L = \frac{C_L (X_f - X_0)}{t} \quad (2.7)$$

2.1.1.5 Statistical analysis

Statistical analysis was carried out by Statistica v 10 software (StatSoft, Tulsa, OK, USA). The statistically significant differences between the results were evaluated by the analysis of variance (ANOVA) with Tuckey's HSD, post-hoc multiple comparison test.

2.1.2 Results and discussion

2.1.2.1 Winery wastewater characterization

Three different winery wastewaters (WWWs), from three different steps of the wine making process, namely first (W1) and second (W2) tank washings, and filtration (W3), were used as media to grow the *Arthrospira platensis* and *Chlorella vulgaris* co-culture. Wastewaters were characterized in terms of contents of total solids (TS), total suspended solids (TSS) and polyphenol

compounds (PC) as well as pH and chemical oxygen demand (COD), whose results are listed in Table 2.1.

Table 2.1. Winery wastewaters characterization.

	TS [a] (g/L)	TSS [b] (g/L)	pH	COD [c] (g_{O_2}/L)	PC [d] (mg $_{GAE}$/L)
W1 [e]	13.15 ± 0.48	1.26 ± 0.02	3.42	116.30 ± 8.13	143.33 ± 0.13
W2 [f]	11.51 ± 0.24	0.39 ± 0.04	3.31	119.30 ± 1.06	139.72 ± 0.03
W3 [g]	4.69 ± 0.24	0.60 ± 0.05	3.82	36.90 ± 0.88	98.52 ± 0.23

[a] total solids, [b] total suspended solids, [c] chemical oxygen demand, [d] polyphenol content, [e] first tank washing wastewater, [f] second tank washing wastewater, [g] wastewater from the filtration equipment.

The COD content was especially high in W1 (116.30 ± 8.13 g_{O_2}/L) and W2 (119.30 ± 1.06 g_{O_2}/L) mainly due to the presence of organic compounds such as sugar and ethanol, but it was remarkably lower in W3 (36.90 ± 0.88 g_{O_2}/L) probably due to the lower content of total dissolved compounds (11.89, 11.12 and 4.09 g/L in W1, W2 and W3, respectively). The lower content of dissolved compounds in W3 could be related to the increased water volume used in the filtration step, which leads to dilution of the dissolved solids present in W1 and W2. On the other hand, the high polyphenol concentration in the three WWWs was the likely result of the presence of soluble acidic phenolic compounds in grapes such as gallic, vanillic, syringic and protocatechuic acids [98]. In general, the qualitative characteristics of the different WWWs were comparable with the literature's data [47].

2.1.2.2 Microalgal Biomass Growth Using Different Type and Concentration of Winery Wastewaters

To reduce the polluting impact of WWWs and to increase the final biomass concentration, the co-culture was grown on each of them after previous dilution in Bold's Basal Medium up to 10%, 20%, 50% and 100% (v/v). Figure 2.3. illustrates the co-culture growth curves considering the TSS content of WWWs. No significant changes of dry weight in the controls (C1W, C2W and C3W) were observed during the 15 days, suggesting that there was no proliferation of other microorganisms.

Table 2.2. Growth and lipid production parameters of co-cultures used in the mixotrophic treatment of winery wastewaters from the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3) at different concentrations in Bold's Basal Medium.

Concentration	10% (v/v)			20% (v/v)			50% (v/v)			100% (v/v)			
Wastewater	W1	W2	W3	W1	W2	W3	W1	W2	W3	W1	W2	W3	Control
X_f [a] (g_{DW}/L)	1.03± 0.37	1.85± 0.24	1.85± 0.15	0.67± 0.10	1.04± 0.41	2.07± 0.20	0.18± 0.10	0.84± 0.00	0.34± 0.00	0.40± 0.10	1.07± 0.24	0.03± 0.01	2.14± 0.06
X_{max} [b] (g_{DW}/L)	1.03± 0.37	1.85± 0.024	1.85± 0.10	1.87± 0.32	2.63± 0.00	2.07± 0.20	1.66± 0.05	2.12± 0.01	0.72± 0.00	1.64± 0.01	2.07± 0.00	0.91± 0.10	2.04± 0.29
μ [c] (day^{-1})	0.07± 0.02	0.12± 0.00	0.10± 0.00	0.01± 0.00	0.04± 0.03	0.08± 0.00	0.00± 0.00	0.08± 0.00	0.00± 0.00	0.00± 0.00	0.05± 0.01	0.00± 0.00	0.09± 0.01
μ_{max} [d] (day^{-1})	0.07± 0.02	0.12± 0.00	0.10± 0.00	0.29± 0.01	0.39± 0.00	0.09± 0.00	0.48± 0.01	0.04± 0.02	0.03± 0.00	0.60± 0.00	0.17± 0.00	0.07± 0.01	0.10± 0.00
ν [e] (g_{DW}/L day)	0.07± 0.02	0.12± 0.016	0.11± 0.01	0.04± 0.00	0.49± 0.00	0.08± 0.01	0.00± 0.00	0.06± 0.00	0.01± 0.00	0.03± 0.00	0.07± 0.01	0.00± 0.00	0.14± 0.02
ν_{max} [f] (g_{DW}/L day)	0.07± 0.02	0.12± 0.016	0.11± 0.01	0.47± 0.08	0.66± 0.03	0.15± 0.01	0.33± 0.01	0.42± 0.00	0.05± 0.00	0.82± 0.00	0.25± 0.01	0.11± 0.00	0.14± 0.01

[a] final biomass concentration, [b] maximum biomass concentration, [c] specific growth rate, [d] specific growth rate at X_{max}, [e] biomass productivity, [f] biomass productivity at X_{max}.

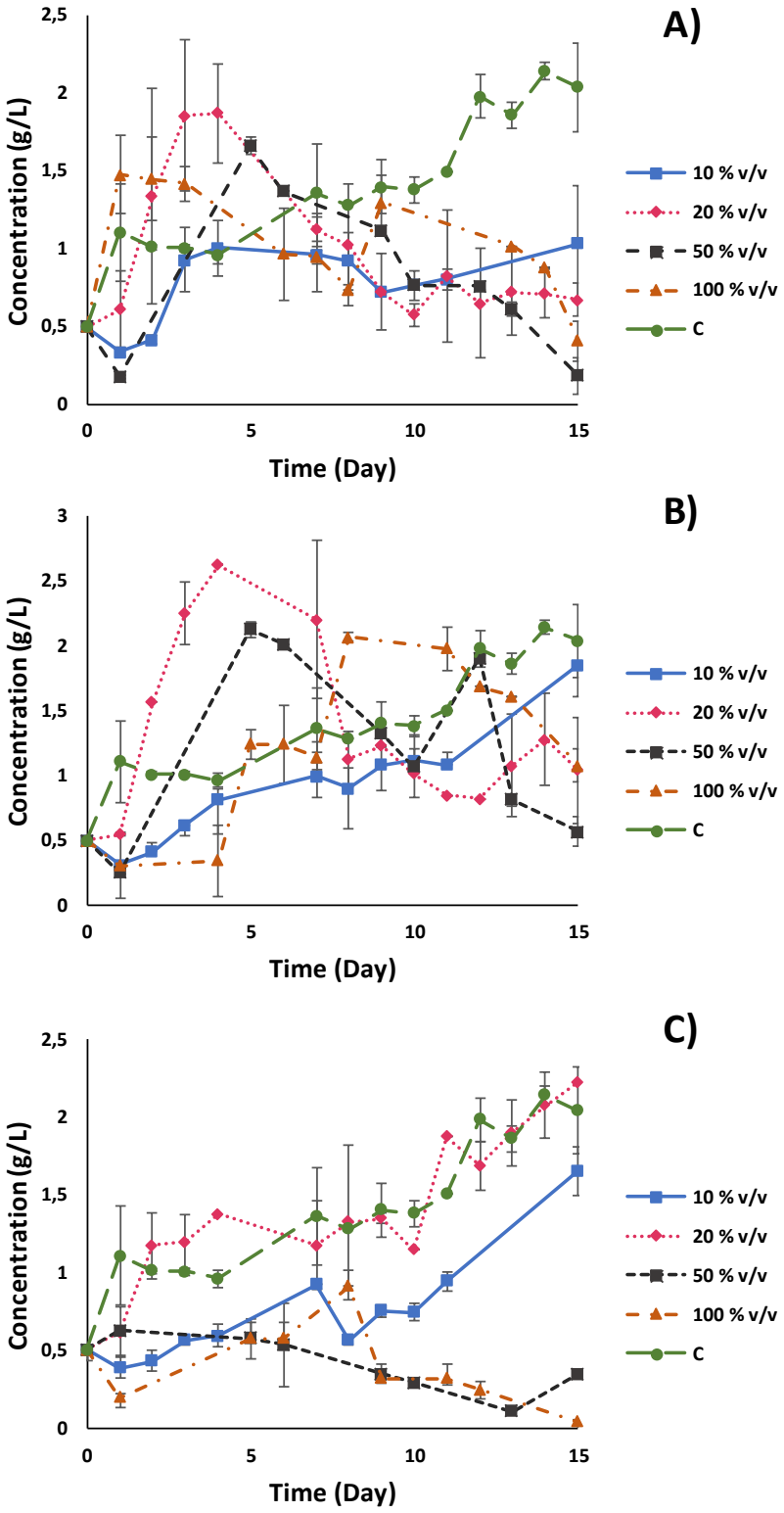

Figure 2.3. Co-culture growth curves in (A) the first tank washing wastewater (W1), (B) second tank washing wastewater (W2) and (C) wastewater from the filtration equipment (W3), after their dilution with Bold's Basal Medium up to 10%, 20%, 50% and 100% (v/v). Biomass concentration expressed as g dry weight per liter.

The co-cultures carried out in W1 quickly reached exponential growth without a clear lag phase, achieving biomass concentrations (X) much higher than those obtained in the control run (Figure 2.3A), while a progressive X decrease took place after 5 days. The only exception was the 10% (v/v) W1 co-culture during which growth was always poorer than in the control run especially after 10 days. A maximum biomass concentration (X_{max}) of 1.87 ± 0.32 g_{DW}/L was reached in 20% (v/v) W1 after 4 days. A qualitatively similar behavior was observed in W2 (Figure 2.3B), with achievement of X_{max} = 2.63 ± 0.00 g_{DW}/L and 2.12 ± 0.00 g_{DW}/L in 20% and 50% (v/v), respectively, and subsequent decay, also in this case the co-culture behavior in 10% (v/v) was as poor as in the control run. These results taken together suggest that the co-culture growth was inhibited by the wastewater as such, i.e., with no dilution, and limited by excess dilution (10% v/v). Finally, in 10% and 20% (v/v) W3 (Figure 2.1C), the co-culture behaved similarly to the control run, reaching X_{max} = 1.85 ± 0.010 g_{DW}/L in the latter after as long as 14 days, while the growth was strongly inhibited at either poor (50% v/v) or no dilution. It is likely that filtration removed mainly readily metabolizable rather than recalcitrant carbon sources, hence especially affecting the runs carried out in more concentrated W3.

The specific grow rate (μ) of the co-culture grown only in BBM (C) calculated at the end of the growth period was higher if compared with those in the presence of W1, W2 and W3 at the different concentrations (Table 2). Instead, the specific growth rates at maximum biomass concentration (μ_{max}) in W1 and W2 without any dilution were higher than in the control, with the highest value of this parameter (0.60 ± 0.00 day^{-1}) being obtained in the former wastewater. The same trend was observed for biomass productivity (v) along with its value at X_{max} (v_{max}) (Table 2). Consistently with these findings, several authors reported higher biomass concentrations for microalgae cultivated under mixotrophic rather than autotrophic conditions. To provide only a few examples limited to the microorganisms used in this study, *C. vulgaris* concentration in the presence of 4 g/L of glucose was (1.40 ± 0.10 g/L) more than 3 times higher compared with its autotrophic growth [19] and that of *A. platensis* in the presence of 0.75 g/L (2.52 g/L) 42.4% higher [20]. The same trend of microalgae concentration obtained in 100% (v/v) WWWs was reported by Ganeshkumar et al., who observed a reduction of *Chlorella* sp. concentration from 3×10^6 to 1.2×10^6 cells/mL after 10 days of growth in concentrated winery wastewater [99].

2.1.2.3 Lipid accumulation and elemental composition in co-culture biomass

The lipid content of microalgae ranges between 10% to 50% of dry weight, depending on the species and growth conditions. Their high lipid accumulation makes them a suitable source for biodiesel production by transesterification of fatty acids [100]. Moreover, the lipid content can be further increased if microalgae are grown under abiotic stress conditions in terms of light intensity, pH and temperature, or changing the medium composition. Particularly, microalgae cultivation in wastewater streams may increase the lipid content and help the process to become more environmentally friendly and economically advantageous[101].

In general, the co-culture cultivated in WWW had its lipid content (C_L) increased with respect to that in the control. For instance, biomass grown on 50% (v/v) WWWs had a C_L value that was about twice the one of control biomass (Table 2.3), 19.66 ± 0.00 and 21.95 ± 0.00 $g_L/100g_{DW}$ for W2 and W3, respectively. Moreover, the lipid productivity (ν_{Lmax}) at X_{max} obtained in the presence of WWW was higher than in the control for almost all the tests. In particular, with 20% and 50% W2 the lipid productivity reached values of 7.10 ± 0.22 and 6.37 ± 0.18 $g_L/100$ L·day, respectively. Similar results were reported by several authors. Santana et al. [24] obtained a 50% increase in fatty acid productivity when cultured *Micractium sp.* in vinasse was diluted in 50% BBM. Kwak et al. [102] observed an increase in the lipid content of different microalgae strains grown under different stress conditions using microfluidic systems. They observed that the lipid production was highly improved in the presence of a combination of more than three stress conditions.

Table 2.3. Lipid production parameters of co-cultures used in winery wastewaters treatment from the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3) at different concentrations in BBM.

Concentration	Wastewater	C_L [a] (g_L/100 g_{DW})	ν_L [b] (g_L/100 L·day)	ν_{Lmax} [c] (g_L/100 L·day)
10%	W1	8.36 ± 1.70	0.29 ± 0.01	0.29 ± 0.01
	W2	12.61 ± 0.00	1.13 ± 0.01	1.13 ± 0.01
	W3	12.31 ± 0.00	1.07 ± 0.02	1.07 ± 0.02
20%	W1	10.03 ± 0.68	0.29 ± 0.00	3.43 ± 0.10
	W2	13.34 ± 3.00	0.11 ± 0.01	7.10 ± 0.22
	W3	10.93 ± 0.02	1.14 ± 0.01	1.14 ± 0.01
50%	W1	9.98 ± 0.04	0.00 ± 0.00	2.31 ± 0.09
	W2	19.66 ± 0.00	0.00 ± 0.00	6.37 ± 0.18
	W3	21.95 ± 0.00	0.00 ± 0.00	2.41 ± 0.12
100%	W1	7.37 ± 2.27	0.00 ± 0.00	2.80 ± 0.14
	W2	7.30 ± 0.00	0.00 ± 0.00	1.43 ± 0.13
	W3	8.39 ± 2.02	0.00 ± 0.00	0.43 ± 0.07
	Control	10.54 ± 0.00	1.08 ± 0.09	1.08 ± 0.09

[a] lipid content of biomass, [b] lipid productivity, [c] lipid productivity at $X_{max.}$

The elementary composition of the co-culture biomass at the end of the growth was determined by a CHNS-O elemental analyzer (Table 2.4). Comparing nitrogen, sulfur and carbon contents of the positive control (C) with the biomass obtained after WWWs treatments, no significant difference could be observed. The hydrogen content was strongly increased from 2.93 ± 0.43 to 6.40 ± 0.39 using W3 at 10% (v/v) and reduced to 0.09 ± 0.13 with 100% (v/v) of W3.

Table 2.4. Co-culture elemental composition after the mixotrophic treatment of winery wastewaters from the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3) at different concentrations in Bold's Basal Medium.

Concentration	Wastewater	H	C	N	S
	Control	2.93 ± 0.43 ^{b,c,d,e}	38.43 ± 1.08 ^{a,b}	6.30 ± 0.37 ^{a,b,c}	0.00 ± 0.37 ^{a}
10% (v/v)	W1	4.78 ± 0.43 ^{d,e,f}	40.60 ± 1.08 ^{a,b}	6.40 ± 0.37 ^{a,b,c}	0.49 ± 0.49 ^{a}
	W2	5.48 ± 0.56 ^{e,f}	43.20 ± 1.12 ^{a,b}	6.84 ± 0.17 ^{b,c}	0.00 ± 0.00^{a}
	W3	6.40 ± 0.39 ^{f}	42.50 ± 3.64 ^{a,b}	7.29 ± 0.22 ^{a,b,c}	0.08 ± 0.09 ^{a}
20% (v/v)	W1	4.21 ± 0.53 ^{d,e,f}	39.65 ± 3.74 ^{a,b}	6.20 ± 0.3 ^{a,b,c}	0.04 ± 0.05 ^{a}
	W2	4.14 ± 0.25 ^{c,d,e,f}	40.40 ± 1.42 ^{a,b}	6.60 ± 0.33 ^{a,b,c}	0.00 ± 0.00 ^{a}
	W3	3.32 ± 0.40 ^{b,c,d,e}	41.95 ± 1.02 ^{a,b}	6.91 ± 0.68 ^{b,c}	0.00 ± 0.00 ^{a}
50% (v/v)	W1	1.14 ± 0.07 ^{a,b}	42.33 ± 1.12 ^{a,b}	6.49 ± 0.14 ^{a,b,c}	0.00 ± 0.00 ^{a}
	W2	1.56 ± 0.24 ^{a,b,c}	42.74 ± 1.12 ^{a,b}	6.82 ± 0.25 ^{b,c}	0.04 ± 0.00 ^{a}
	W3	2.84 ± 0.12 ^{b,c,d,e}	36.58 ± 1.02 ^{a}	5.38 ± 0.40 ^{a}	0.00 ± 0.00 ^{a}
100% (v/v)	W1	1.04 ± 0.60 ^{a,b}	45.05 ± 0.94 ^{b}	5.79 ± 0.28 ^{a,b}	0.00 ± 0.00 ^{a}
	W2	2.62 ± 2.03 ^{a,b,c,d}	39.67 ± 1.63 ^{a,b}	6.24 ± 0.20 ^{a,b,c}	0.00 ± 0.00 ^{a}
	W3	0.09 ± 0.13 ^{a}	43.32 ± 3.25 ^{a,b}	6.58 ± 0.68 ^{a,b,c}	0.00 ± 0.00 ^{a}

Different letters (a-f) in the same column indicate statistically significant differences among mean values.

2.1.2.3.1 COD removal from winery wastewater

Chemical oxygen demand (COD) has been accepted as a national standard for the evaluation of organic pollution in wastewater, besides being the most frequently used parameter to assess the efficiency of biological wastewater treatments. Since microalgae cultivated under mixotrophic condition have the ability to consume organic pollutants as a carbon source and inorganic nutrients for their growth, COD removal by the co-culture from the three different WWWs was daily determined to compare reduction trends.

The COD reduction curves followed the same trend in the different WWWs either as such or differently diluted. One can see in Figure 2.4. that the organic matter was quickly removed from the non-diluted WWWs by no less than 85% during the first 5 days of mixotrophic treatment, while after the exponential phase its removal proceeded slowly up to the end.

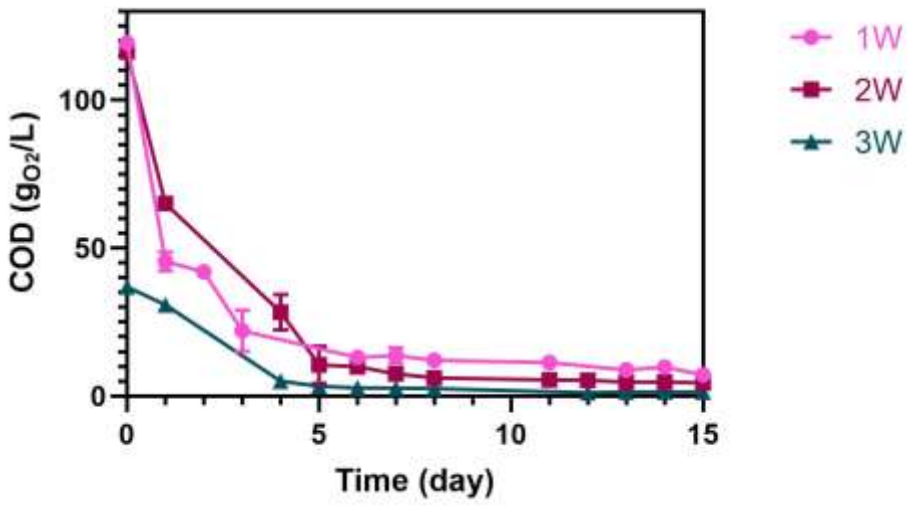

Figure 2.4. Time behavior of chemical oxygen demand in non-diluted winery wastewaters from the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3) during treatment with the co-culture.

The residual COD assessed at the end of each treatment was used to calculate its percentage removal with respect to the initial COD, for which the results are depicted Figure 2.5A. One can see that at all the concentrations, the removal was higher than 90% in the three WWWs, with the highest value (around 99%) being detected in 50% (v/v) W2. In addition, Figure 2.5B, which illustrates the percent increases in COD removal at the end of every treatment compared with the respective negative controls (without inoculum), clearly shows that the co-culture, as expected,

was much more effective than the autochthonous species (mainly yeast) present in the WWWs in reducing their COD contents.

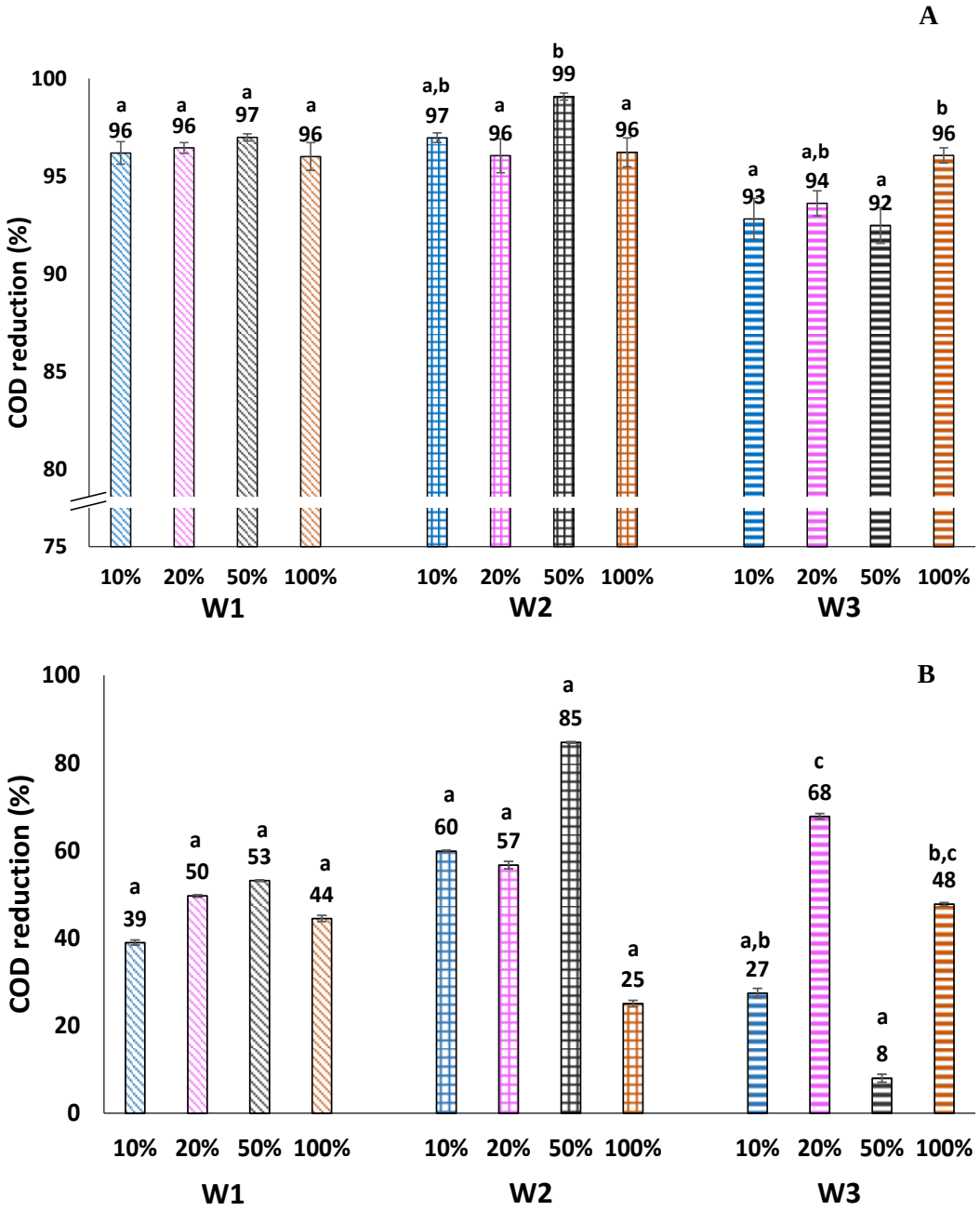

Figure 2.5. Chemical oxygen demand removals by the co-culture in winery wastewaters from the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3) at different concentrations in Bold's Basal Medium. (A) Final COD removal compared to the start of each treatment. (B) Increase in final COD removal compared to the negative control of each treatment. Different letters (a-c) in the same group indicate statistically significant differences among mean values.

Other microrganisms such as bacteria and fungi have been used to purify WWWs. Malandra et al. [103] reported the ability of a new yeast isolate (MEA5) to reduce by 95% the COD of a synthetic wastewater in a rotating biological contactor under aerated conditions, demonstrating the potential of a dynamic microbial population to treat WWW. Zhang et al. [103] observed a 90% reduction of WWW COD by microfungi such as *Trichoderma viride*, *Aspergillus niger* and *Aspergillus oryzae*.

Most of the attempts reported in the literature to reduce wastewater COD by microalgae have shown comparable or even better performance than conventional treatments. The most common winery wastewater treatment systems are conventional activated sludge plants or different anaerobic systems. However, despite their simplicity, they require long retention times to degrade the organic matter and can only be applied when cells have a small size and large surfaces are available. For instance, Torrijios and Moletta [104] reported a COD reduction in winery wastewater as high as 97.5% in a sequencing batch reactor.

2.1.2.4 Polyphenols removal

It is well-known that microalgae have the ability to degrade polyphenols through two different mechanisms, namely mineralization to carbon dioxide [105] or biotransformation to other compounds, as suggested by Cerniglia et al. [106], who observed the biotransformation of naphthalene into other similar metabolites, such as 1-naphthol. So, in order to check the capability of the selected co-culture to reduce the polyphenol content (PC) of WWWs, the total concentration of these substances was determined by the Folin–Ciocalteu assay before and after treatments.

Polyphenol degradation by the co-culture at the end of treatments was higher than 50% under all the tested conditions (Figure 2.6A). In general, the higher the WWW concentration and the PC content, the lower the polyphenol degradation. For instance, PC removal from W2 progressively decreased from 100% to 53% when its concentration in BBM was increased from 10 to 100 (v/v). Consistently, PC removal was the highest (100%) in 10 and 20 (v/v) W3 and decreased to 77% and 60% in 50 and 100 (v/v), respectively. When comparing the three different WWWs, as expected W1 was the most recalcitrant to PC degradation, followed by W2 and W3, even though the PC were completely degraded in all three WWWs at the lowest concentration (10 v/v). In the same ways as COD, the percent increases in PC removal at the end of every treatment compared with the respective negative controls (without inoculum) were always quite high, ranging from 63% to 96% (Figure 2.6B). The most diluted WWWs (10 v/v) were the only exceptions, being all

completely decontaminated from PC by either the microalgal co-culture or the autochthonous microflora.

Similar results were obtained by other authors. To provide only a few examples, Pinto et al. [107], who investigated the removal of different phenolic compounds by *Scenedesmus obliquus*, reported removals of tyrosol and hydroxytyrosol higher than 50% and 68%, respectively. Moreover, the same inverse correlation between polyphenol concentration and removal was observed by Papazi et al. [108] for olive mill wastewater treatment by the same microalga, in that tyrosol removal decreased from 75% to 15% when its concentration was increased from 0.05 to 0.3 mM.

Polyphenols and other high molecular weight pollutants of winery wastewater are not mineralized by WWW conventional biological treatments [94]. To achieve reductions in the content of polyphenolic compounds comparable to that obtained in the present study, expensive and complex technologies must be applied such as reverse osmosis, nanofiltration and ultrafiltration [109].

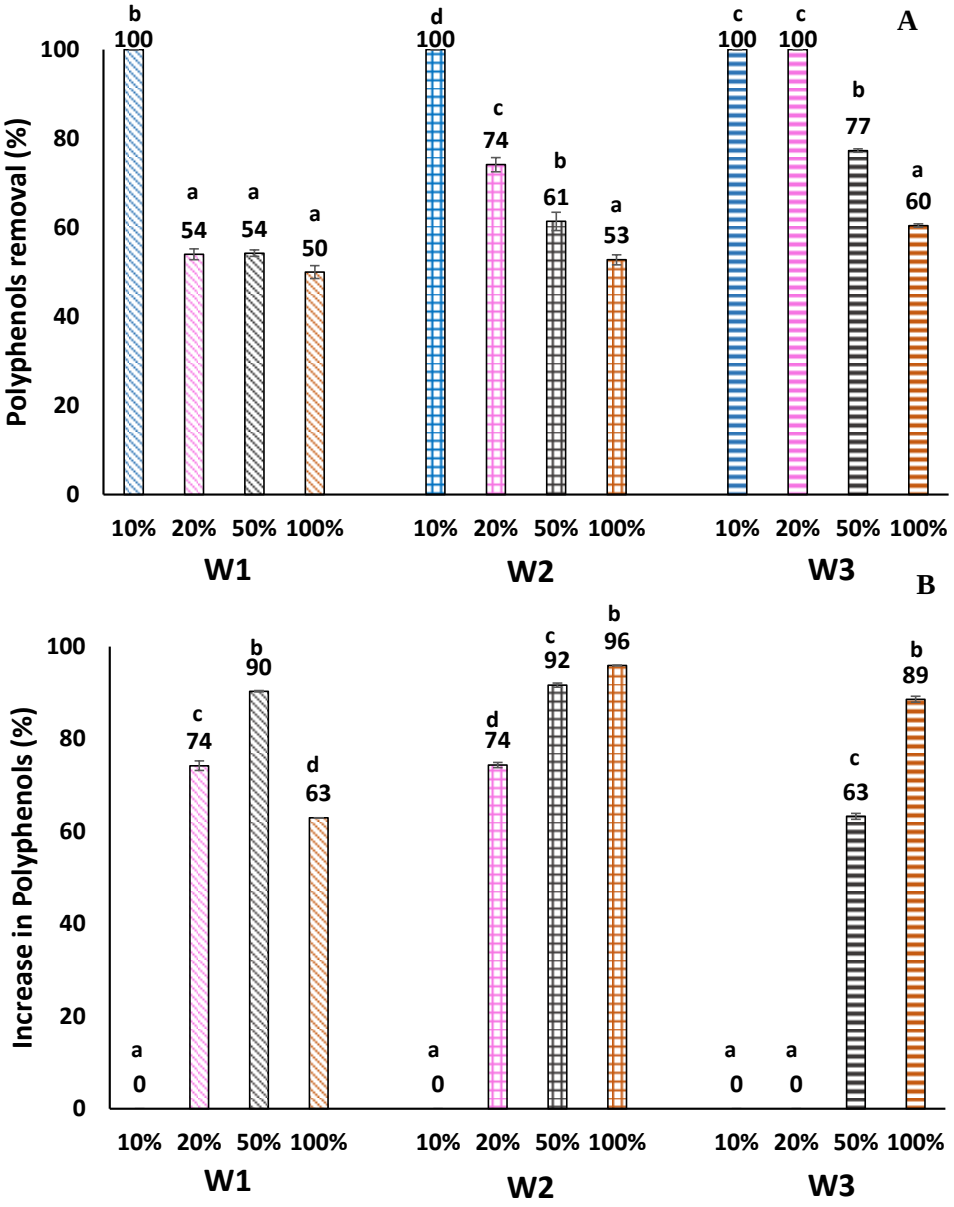

Figure 2.6. Removals of polyphenols (PC) by the co-culture in winery wastewaters from the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3) at different concentrations in Bold's Basal Medium. A) Final PC removal compared to the start of each treatment. B) Increase in final PC removal compared to the negative control of each treatment. Different letters (a-c) in the same group indicate statistically significant differences among mean values.

2.1.3 Conclusions

A *Chlorella vulgaris* and *Arthrospira platensis* co-culture was grown in three different winery wastewaters (WWWs), namely those from the first (W1) and second (W2) tank washings as well as that from the filtration equipment (W3).

The highest biomass concentration (2.63 ± 0.00 g_{DW}/L) was obtained in 20% (v/v) W2 after only 4 days of treatment, corresponding to a biomass productivity of 0.66 ± 0.03 g_{DW}/L·day.

The co-culture was able to reduce the chemical oxygen demand and polyphenol content of the three WWWs by more than 92% and 50%, respectively. The lipid productivity increased considerably after the wastewater treatment. The results suggest it is possible to stop the co-culture cultivation after the achievement of maximum biomass concentration in order to increase biomass and lipid productivities without losing the benefits in terms of reduction of the pollution load.

This study demonstrates the feasibility of using WWWs as a culture medium for the growth of microalgae in order to reduce their production costs and exploit the resulting biomass as a source of biofuels.

2.2 Investigation of light conditions on co-culture growth

The research work regarding the co-culture growth under dark conditions, that will be discussed in this section has been already published:

E. Spennati, A.A. Casazza, P. Perego, C. Solisio, Microalgae Growth in Winery Wastewater under Dark Conditions, Chemical Engeneering Transactions, 74 (2019). 1471–1476. doi:10.3303/CET1974246.

Microalgae often used to treat civil and different industrial wastewaters, are unicellular organisms that can be grown either in autotrophic or heterotrophic mode using various organic and inorganic carbon sources. The purposes of this work were to reduce WWW environmental impact and to find a cheap growth medium able to reduce the microalgae production costs. In this study, three different wastewaters were used, namely WWW from first (1W) and second (2W) washing tanks, and WWW from filtration apparatus (3W). They were 20% (v/v) diluted with Bold Basal medium and treated batchwise with a co-culture of *Arthrospira platensis* and *Chlorella vulgaris* under dark conditions and, light-dark cycle (12 h, 12 h). The aim of this research was to investigate the effect of co-culture metabolism on microalgal biomass production and removal of winery wastewater pollutants.

2.2.1 Material and methods

2.2.1.1 Microalgae strains and culture condition

The co-culture of *Arthrospira platensis* UTEX 1926 (University of Texas Culture Collection, TX, USA) and *Chlorella vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK) was cultivated in Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) with continuous air supply at room temperature (25 °C). All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The three WWWs were provided from a winery cellar located in Piemonte region, Italy.

2.2.1.2 Experimental design

The co-culture (inoculum of 0.5 g_{DW}/L) was grown in 500-mL Erlenmeyer flask, under dark conditions (DC) in the presence of 20% (v/v) WWWs/BBM for 15 days (Figure 2.7.A). WWWs were used without any preliminary treatment. The microalgae concentration was daily determined

by dry weight (DW) measurements taking into account the suspended solid content of WWWs ($g/100g_{DW}$). All the measurements were carried out in triplicate, and cell concentration (X) was expressed in grams of dried biomass per liter of medium (g_{DW}/L).

Microalgae growth (C) in absence of WWW, using BBM as culture media, was performed at the same conditions to compare it with those in presence of WWWs.

WWWs under the same above-described conditions, but without any inoculum, were used as growth controls (C1W, C2W, and C3W for 1W, 2W, and 3W, respectively). After growing, biomass was collected and processed as reported in section 2.1.1.2.

The kind of wastewater that produced the best results in terms of biomass productivity and removal of pollutant impact under dark conditions was selected for the investigation in the next experiment. Thus, the co-culture was grown in 1000-mL flask at 20% of 2W under dark-light cycles (12h light/12h dark) for 15 days (DLC) (Figure 2.7.B). The experiment was conducted following the same methodologies and procedures explained above (DC).

Figure 2.7. Erlenmeyer flask for co-culture growth and winery wastewater treatment: A-under dark conditions, B-under light-dark cycle.

2.2.1.3 Biomass and winery wastewater characterization

The lipid content (C_L) was evaluated as explained in section 2.1.1.3 and the kinetic growth parameters were evaluated as explained in section 2.1.1.4 Co-culture biomass was observed by optical microscope (Leica DMLS, Wetzlar, Germany) before and after winery wastewater treatment under dark conditions.

Chemical oxygen demand (COD) and total polyphenol content (by the Folin-Ciocalteu method) of WWWs were quantified daily, in order to evaluate the degradation efficiency of microalgae following the methodologies reported in section 2.1.1.3.

2.2.1.4 Statistical analysis

Statistical analysis was carried out by Statistica v 10 software (StatSoft, Tulsa, OK, USA) as explained in section 2.1.1.5.

2.2.2 Results and discussion

2.2.2.1 Effect of the type of WWW on the co-culture growth in dark condition

The co-culture growth curves obtained by growing the co-culture under dark conditions were shown in Figures 2.8. The growth curves obtained exhibited almost the same trend for WWWs from the first washing tank (1W), the filtration apparatus (3W), and the control (C), while for that from the second washing tank (2W) the concentration determined with the former were always higher.

During the whole experiment, no significant changes in weight were observed for the three controls (C1W, C2W, and C3W), suggesting no proliferation of autochthonous microorganisms. The co-culture growth in 1W and 2W quickly entered the exponential phase of growth (within only 2 days) and reached, at the end of the runs, a biomass concentration about 2 times higher than the control. Instead, 3W presented the same trend as the control, probably because of the low content of organic carbon source.

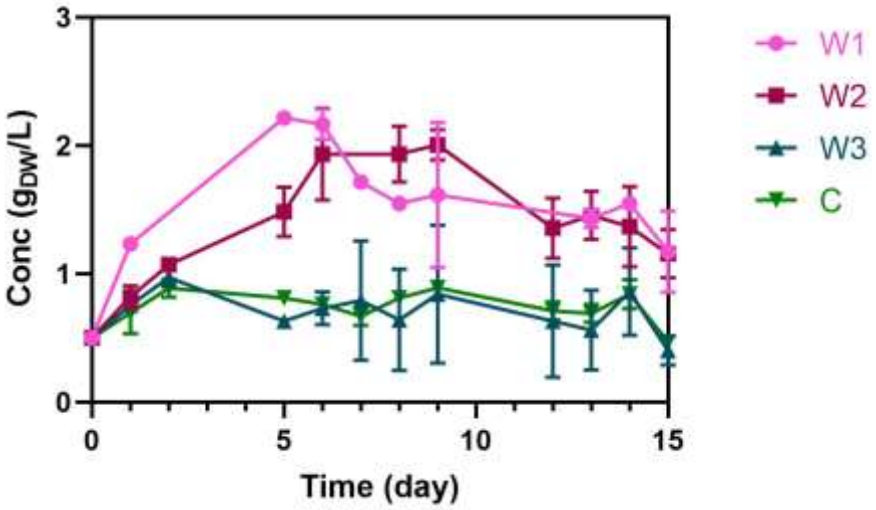

Figure 2.8. Microalgae growth curves obtained under dark condition (DC) in the first (W1) and second (W2) washing tanks and from the filtration apparatus (W3).

A maximum biomass concentration of 2.22±0.01 g_{DW}/L was reached after 5 days on 1W, while a comparable value (X_{max} = 2.01±0.12 g_{DW}/L) required no less than 9 days on 2W. On the other hand, it achieved only twice the starting biomass concentration either on 3W or C. Since the specific grow rate at the end of cultures (μ) was quite low on all WWWs (0.24±0.10 and 0.22±0.03 d^{-1} on 1W and 2W, respectively) because of the decrease in concentration occurred after the stationary phase, μ_{max} values were more than twice those of μ, as shown in Table 2.5. A different trend could be observed for biomass productivity, in that it was on 1W (0.41±0.05 g_{DW}/Ld) about 41% higher than on 2W, while the lipid content of biomass increased in presence of WWWs from 7.55±0.00 to 11.71±0.59, 10.15±0.00 and 15.38±5.89 $g_{DW}/100g_{Dw}$ on 1W, 2W and 3W, respectively.

Table 2.5. Growth parameters and lipid and biomass productivities, determined by dry weight, in the presence of wine wastewaters from the first (1W) and second (2W) washing tanks and from the filtration apparatus (3W).

	X_f[a] (g_{DW}/L)	X_{max}[b] (g_{DW}/L)	μ[c] (day^{-1})	μ_{max}[d] (day^{-1})	v[e] (g_{DW}/Ld)	v_{max}[f] (g_{DW}/Ld)	C_L[g] ($g_L/100g_{DW}$)	v_L[h] ($g_L/100g_{DW}d$)
1W	1.51±0.80	2.22±0.01	0.07±0.04	0.24±0.10	0.10±0.05	0.41±0.05	11.71±0.59	0.01±0.00
2W	1.16±0.19	2.01±0.12	0.06±0.01	0.22±0.03	0.08±0.01	0.29±0.11	10.15±0.00	0.00±0.00
3W	0.40±0.11	0.97±0.07	0.00±0.00	0.08±0.17	0.03±0.01	0.02±0.23	15.38±5.89	0.00±0.00
C	0.50±0.08	0.89±0.07	0.00±0.01	0.06±0.01	0.03±0.01	0.10±0.00	7.55±0.00	0.00±0.00

[a] final biomass concentration, [b] maximum biomass concentration, [c] specific growth rate, [d] specific growth rate at X_{max}, [e] mean biomass productivity, [f] biomass productivity at X_{max}, [g] lipid content of biomass, [h] lipid productivity.

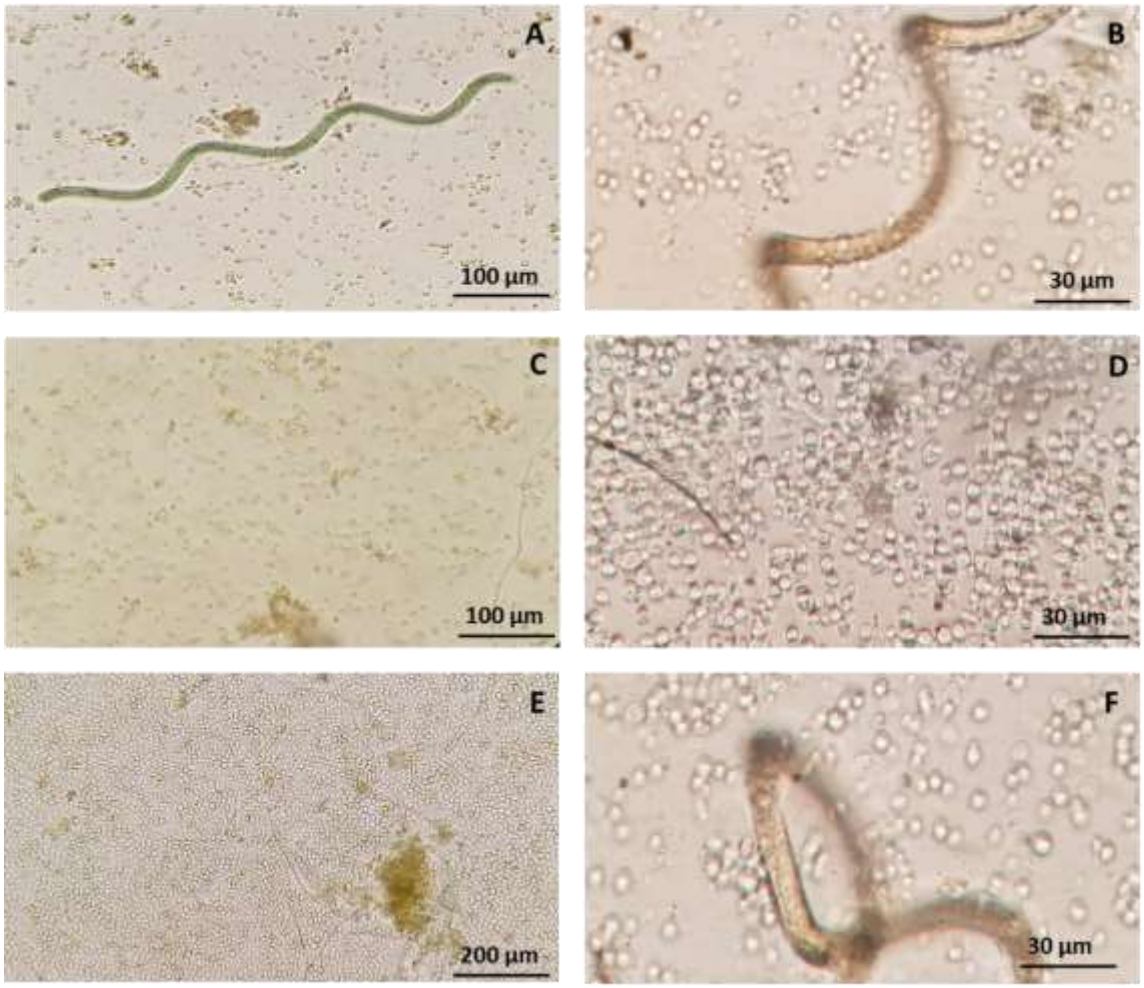

Figure 2.9. Optical observation of the co-culture before (A, C, and E) and after (B, D and F) winery wastewater treatment under dark condition. A, B- with 1W; C, D- with 2W; E, F- with 3W (A, C, and E magnification 20x while B, D, and F magnification 40x).

The co-culture was observed before and after winery wastewater treatment under dark conditions by optical microscope, the recorded images were shown in Figure 2.9. The co-culture after the treatment changed drastically the color, losing the typical green color of the chlorophyll pigment. This result suggested, as expected, that the co-culture growth under dark conditions and in presence of the WWW organic carbon growth prevalently uses heterotrophic metabolism instead that autotrophic.

2.2.2.2 Co-culture growth curve with 20% of 2W under light-dark cycle conditions

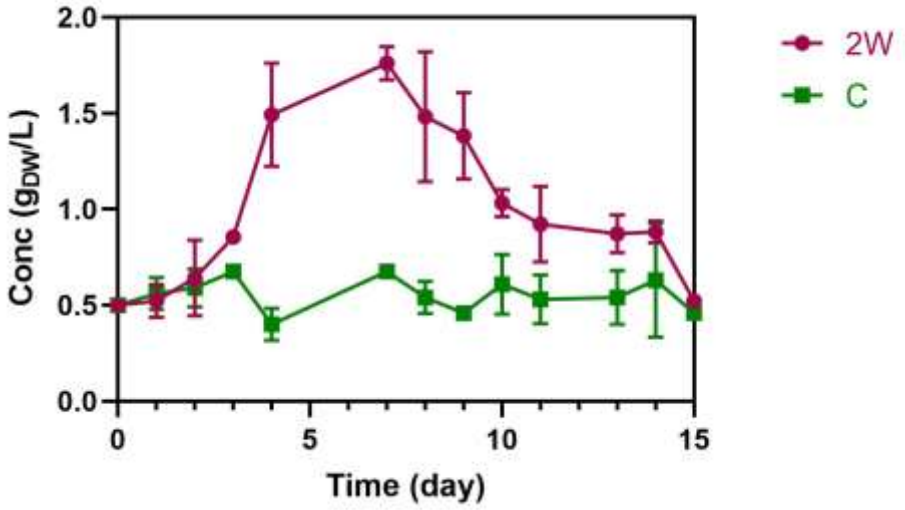

Figure 2.10. Microalgae growth curves obtained under light-dark cycle condition (LDC).

The growth curves obtained by growing the co-culture under light-dark cycles condition (LDC), 12 hours of light and 12 hours of dark, in presence of 20% (v/v) of 2W in BBM and only BBM (C) were shown in Figure 2.10. The curves showed different behavior during all the co-culture growth. The co-culture growth in 2W quickly reached the maximum biomass concentration of 1.53± 0.12 g_{DW}/L on the 7th day of growth. Instead, the biomass concentration of co-culture growth in BMM remained almost constant during all the growth periods. This result suggested that probably the light exposure was not enough to guarantee a correct co-culture growth only using autotrophic metabolism.

Table 2.6.. Growth parameters and lipid and biomass productivities, determined by dry weight, in the presence of second (2W) washing tanks winery wastewater.

	X_f[a] (g_{DW}/L)	X_{max}[b] (g_{DW}/L)	μ[c] (day^{-1})	μ_{max}[d] (day^{-1})	v[e] (g_{DW}/Ld)	C_L[g] (g_L/100g_{DW})
2W	0.39±0.19	1,53±0.12	0.00	0.09	0.03	32.95
C	0.28±0.08	0,68±0.07	0.00	0.06	0.02	9.70

[a] final biomass concentration, [b] maximum biomass concentration, [c] specific growth rate, [d] specific growth rate at X_{max}, [e] mean biomass productivity, [f] biomass productivity at X_{max}, [g] lipid content of biomass, [h] lipid productivity.

In Table 2.6. were shown the kinetic growth parameters and the lipid content (C_L) obtained from the LDC experiment. The biomass productivity and the specific growth rate were very low. These results were conducible to the results previously discussed. The lipid content obtained after 2W treatment was much higher (39.95 $g_L/100g_{DW}$), compared with the control run (9.70 $g_L/100g_{DW}$).

2.2.2.3 COD reduction by co-culture in CD and LDC experiments

The reduction of polluting power of WWWs by co-culture was evaluated in terms of Chemical Oxygen Demand (COD) decrease versus time for both experiments (dark conditions and light-dark cycle condition). The COD curves, Figure 2.11., showed the same trend in the three different WWWs and experimental conditions, in that the COD decreased quickly during the first 6 days of cultivation and then more slowly up to the achievement of a minimum threshold value. A final COD value of 0.90, 0.53, and 0.41 g_{O2}/L was obtained after co-culture treatment under dark conditions for 1W, 2W and, 3W, respectively.

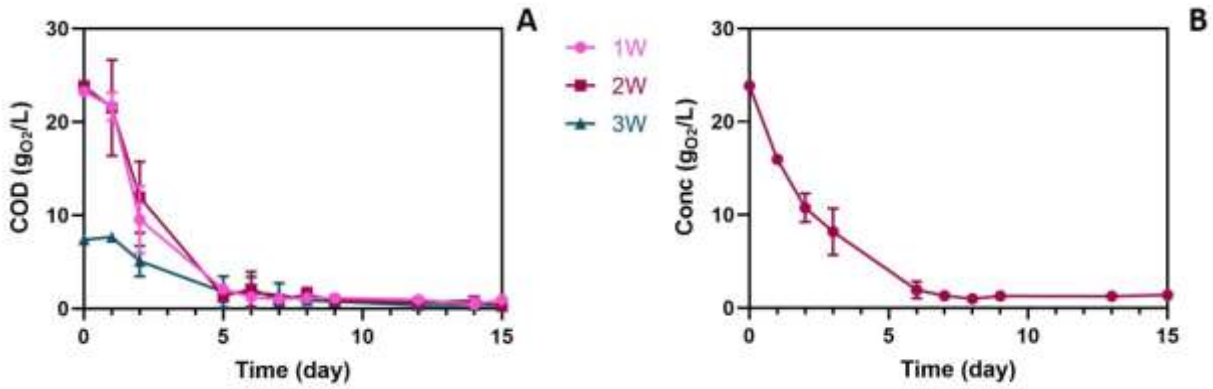

Figure 2.11. Efficiency of WWW Chemical Oxygen Demand removal by a co-culture of Arthrospira platensis and Chlorella vulgaris. A-under dark condition and, B- under light-dark cycle condition.

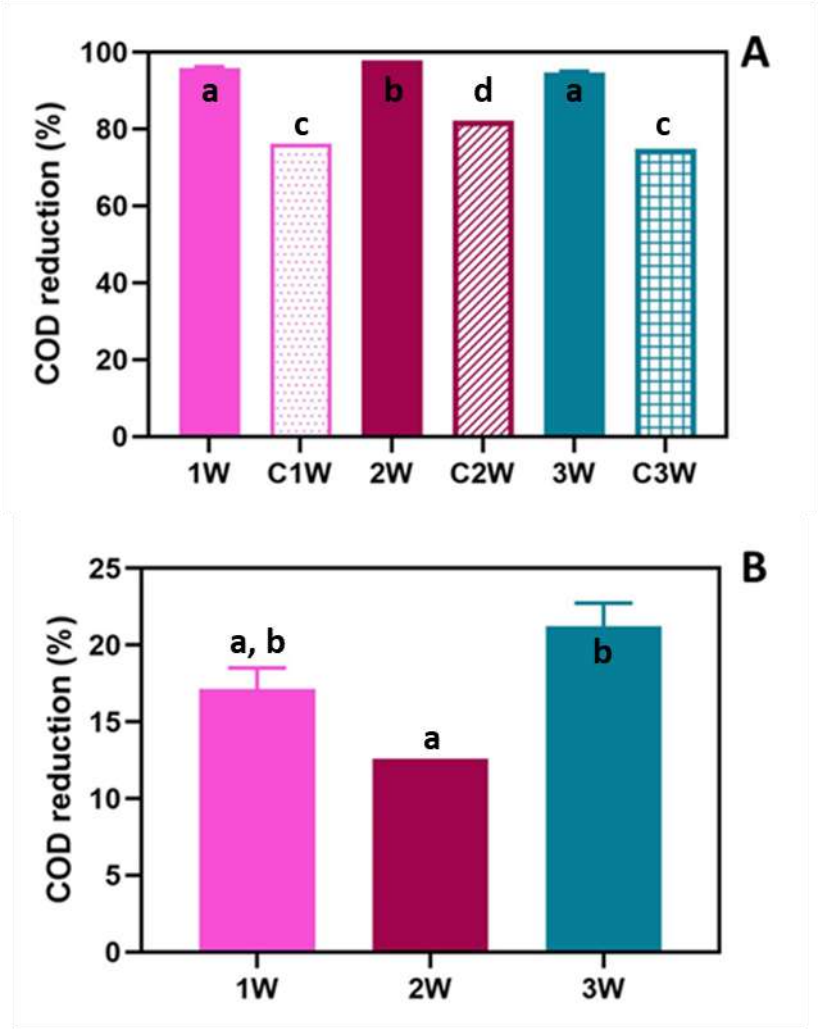

Figure 2.12. Chemical oxygen demand removals by the co-culture in winery wastewaters from W1, W2 and, W3. A- Final COD removal compared to the start of each treatment. B- Increase in final COD removal compared to the negative control of each treatment. Different letters (a-d) in the same group indicate statistically significant differences among mean values.

The COD reduction of WWWs by co-culture treatment under dark condition and from the three different negative controls (C1W, C2W, and C3W) was evaluated, as showed in Figure2.12A. The COD reduction by co-culture was higher than the negative control run in all the WWWs. In fact, an increase of COD reduction of 17.16, 12.59, and 21.21% by co-culture was obtained for W1, W2, and W3, respectively. This confirms that microalgae can use the organic load of WWW as a carbon source for their metabolism [110].

Table 2.7. COD values of 2W and C2W after light-dark cycle treatment

	COD (g_{O2}/L)		COD reduction (%)	COD reduction respect C2W (%)
	Start	End		
2W	23.26±0.01	1.37±0.10	94.22	54.10%
C2W	23.20±0.01	2.53±0.01	84.42	-

The same behavior observed under dark conditions, in terms of COD reduction, could be obtained under light-dark cycle conditions. In fact, the co-culture reduced the COD content up to 94.22%, corresponding to an increase in COD reduction of 54% in comparison with the negative control (Table 2.7.).

The COD removal efficiency, under dark and light-dark cycle conditions, is comparable or even better than those of conventional WWW treatment. To give only a few examples, Ioannou et al. (2017) reported values of WWW COD removal by Advanced Oxidation Processes (AOPs) from 50 to 95%, while the combination of biological processes and AOPs lead to removals in the range 30 to 96%.

2.2.2.4 Polyphenol removal in WWWs by co-culture in DC and LDC experiments

Polyphenol content of WWWs was determined either before or after treatment with the microalgae co-culture by the Folin-Ciocalteu method.

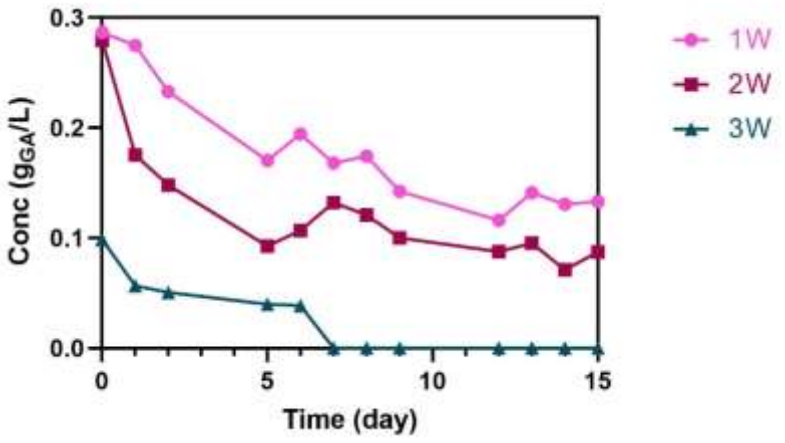

Figure 2.13. Time behavior of polyphenol removal efficiency during WWW treatment with a co-culture of *Arthrospira platensis* and *Chlorella vulgaris*

As illustrated in Figure 2.13, polyphenols were removed following a similar trend in all the three WWWs under dark conditions, in that their content decreased quickly during the first 5 days of growth and then slowly achieved an almost constant minimum value.

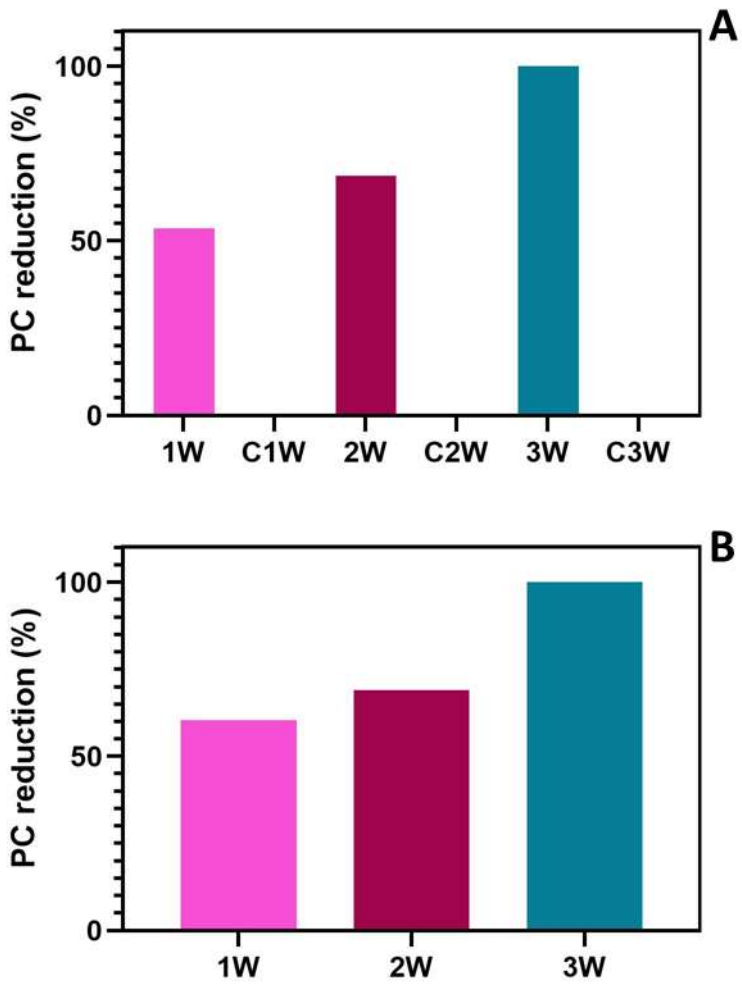

Figure 2.14. Removals of polyphenols (PC) by the co-culture in winery wastewaters W1, W2, W3. A) Final PC removal compared to the start of each treatment. B) Increase in final PC removal compared to the negative control of each treatment.

At the end of treatments, polyphenol content, expressed as gallic acid equivalents (GA) per liter, was reduced from 0.29±0.02 to 0.13±0.01 g_{GAE}/L in 1W and to 0.08±0.00 g_{GAE}/L in 2W, while total removal occurred in 3W (Figure 2.14). Corresponding to a PC reduction of 53.5, 68.7 and 100.0%, for W1, W2 and W3, respectively.

Table 2.8. PC values of 2W and C2W after light-dark cycle treatment

	PC (g_{GAE}/L)		PC reduction (%)	PC reduction respect C2W (%)
	Start	End		
2W	0.28±0.01	0.25±0.02	10.71	-19.00%
C2W	0.28±0.01	0.21±0.01	25.00	-

In Table 2.8 were shown the PC value obtained from the co-culture growth under light-dark cycle conditions. A COD reduction of 10.71% was obtained by co-culture, and 25.00% in the negative control.

2.2.3 Conclusions

The results obtained in this study demonstrated that winery wastewater (WWW) may be used to cultivate microalgae under dark and light-dark cycles conditions. The co-culture of *Arthrospira platensis* and *Chlorella vulgaris* was able to effectively grow in WWWs from the first and second washing tanks as culture media under dark condition, achieving maximum biomass concentrations after 5 and 9 days, respectively, and a COD reduction as high as 95% and quite low polyphenol content. Therefore, the growth could be stopped when the maximum concentration is reached. This could give a profit in terms of specific growth rate, lipid accumulation and productivity.

The use of winery wastewater as a culture medium for microalgae growth in the absence of light could significantly reduce microalgae production costs. The resulting microalgae biomass may then be used for biodiesel production, for extraction of value-added compounds and/or as protein-rich animal feed.

2.3 Microalgae growth in winery wastewaters using different photobioreactor configurations.

In this section, winery wastewater was used as a growth medium for a co-culture of *Chlorella vulgaris* and *Arthospira platensis*. Three different microalgae growth systems (multitubular photobioreactor, open pond, column photobioreactor) were used to evaluate the differences in terms of biomass productivity and removal of winery wastewater. A high concentration of biomass (more than 2.19 $g_{DryWeight}/L$) and reduction of pollutant impact was obtained after winery wastewater treatment in all the growth system configuration. The obtained results suggested that microalgae co-culture was effectively able to grow and purify winery wastewater.

2.3.1 Materials and Methods

2.3.1.1 Microalgae strains and culture condition

The co-culture of *Arthrospira platensis* UTEX 1926 (University of Texas Culture Collection, TX, USA) and *Chlorella vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK) was cultivated in Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) with continuous air supply at room temperature (25 °C). All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The three WWWs were provided from a winery cellar located in Piemonte region, Italy.

2.3.1.2 Experimental design

Considering the results obtained growing the co-culture (Section 2.1) in different kinds of winery wastewater and at different concentrations, we selected second washing of tanks wastewaters(2W) at 20% (v/v) in BBM as the best medium for microalgae growth.

The co-culture was grown in three different growth systems: 1- Multitubular photobioreactor (MTP), 2- Open pond (OP) and, 3- Column photobioreactor (CP). The inoculum concentration was 0.5 g_{DW}/L, and the culture medium was made by a solution 20% (v/v) 2W/BBM for 15 days. The growths in presence of 2W were compared with a control run (C) consisting of only BBM as growth medium. Moreover, the results in terms of COD and polyphenols reduction were compared with the ones obtained from the degradation of 2W.

The biomass concentration was daily determined by dry weight (DW) measurements considering the content of suspended solid in 2W ($g/100g_{DW}$). The co-culture growth was carried out in

duplicate, while all the measurements in triplicate. After growing, the biomass was collected and processed as reported in section 2.1.1.2.

2.3.1.3 Multitubular photobioreactor

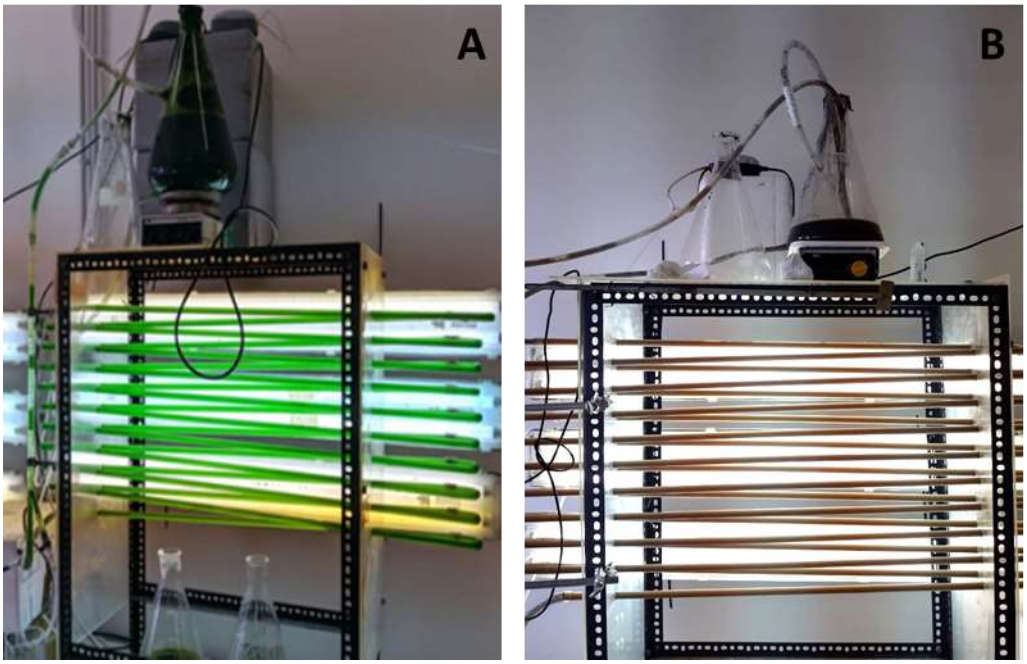

Figure 2.15. Multitubular photobioreactor A-with 2W (20% v/v) on the left, B- control run.

The multitubular reactor (MTP) was composed of a set of 21 glass tubes, each having inner and outer diameters of 12 and 14 mm and a length of 1 m (Figure 2.15). The tubes were located into two parallel planes with a slight inclination and arranged horizontally. This configuration allowed the same light exposure of the tubes. The continuous mixing of the culture was guaranteed by injecting compressed air at the bottom of the system into a vertical pipe used as a riser by an air pump. A 5.0 L Erlenmeyer flask was placed at the top of the system, used as storage and degasser. The total volume of the photobioreactor was 5.0 L. The system was illuminated by three 40-W-fluorescent with a light intensity of 60 µmol photons $m^{-2}s^{-1}$ from one side only. the light intensity was measured by a light meter model LX-107 (Lutron, Taipei, Taiwan). The surface/volume ratio evaluated of the system was 1.94 cm^{-1}.

2.3.1.4 Open pond

Figure 2.16. Open ponds.

The open pond (OP) has a total volume of 5 L, a liquid depth of 5 cm and, a surface area of 0.13 m^2 (Figure 2.16). The system was continuously illuminated by two fluorescent lamps (40 W) located above the surface in order to allow a light intensity of 60 µmol photons $m^{-2}s^{-1}$. Culture mixing and was ensured with paddled wheels at 30 rpm. The surface/volume ratio evaluated of the system was 0.25 cm^{-1}.

2.3.1.5 Column photobioreactor

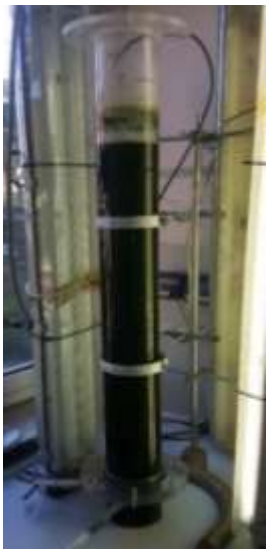

Figure 2.17. Column photobioreactor.

The column photobioreactor (CP) was made by a plastic tube with a total volume of 7L (Figure 2.17).

The continuous mixing of the culture was guaranteed by injecting compressed air at the bottom of the system. The system was continuously illuminated by two fluorescent lamps (40 W) located around the column. The light intensity was 60 µmol photons $m^{-2}s^{-1}$.

2.3.1.6 Biomass characterization

The lipid fraction (C_L) was extracted with a 2:1 (v/v) chloroform/methanol solution as solvent, following a modified version of the Folch method [111], as previously describes in details (section 2.1.1.3)

2.3.1.7 Winery wastewater characterization

Chemical oxygen demand (COD) and total polyphenol content (by the Folin-Ciocalteu method) [112] of 2W were quantified daily, in order to evaluate the degradation efficiency of microalgae following the methodologies reported in the previous sections (section 2.1.1.3).

2.3.1.8 Kinetic parameters of cultures

Kinetic parameters were evaluated for each growth in the different photobioreactors. The kinetics parameters: specific grow rate (μ), μ at maximum biomass concentration (μ_{max}), biomass productivity at the end of cultivation (v) and its value at X_{max} (v_{max}) and, lipid productivity (v_L) were calculated as described in the previous sections (section 2.1.1.4).

2.3.1.9 Statistical analysis

Statistical analysis was carried out by Statistica v 10 software (StatSoft, Tulsa, OK, USA) as explained in section 2.1.1.5.

2.3.2 Results and Discussion

2.3.2.1 Microalgal Biomass Growth Using Different Type of photobioreactors

The *A. platensis* and *C. vulgaris* co-culture was grown in a medium made by 20% of 2W in BBM in different photobioreactor configuration multitubolar photobioreactor, open pond and, column photobioreactor. The co-culture growth curves obtained growing the co-culture in different growth systems and in presence of 2W were shown in Figure 2.18. The growth curves were compared with the respective control run (C) obtained by growing the co-culture in BBM. While the kinetic growth parameters were listed in Table 2.9.

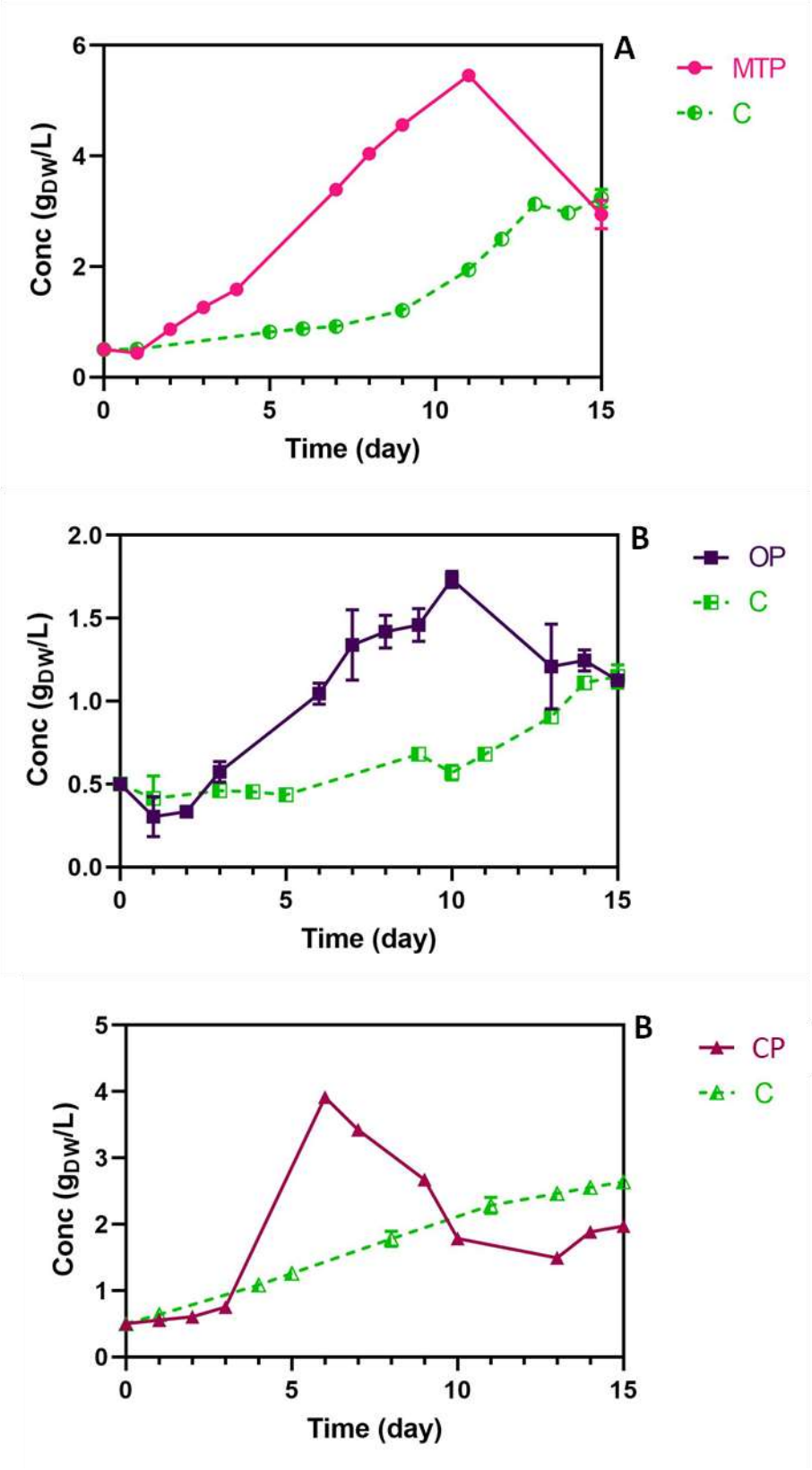

Figure 2.18. Co-culture growth curves in A- Multitubular photobioreactor (MTP), B- Open pond (OP) and, C- Column photobioreactor (CP). Biomass concentration expressed as g dry weight per liter.

In presence of 2W, the maximum biomass concentration obtained was higher than control using all the growth configurations. Moreover, the X_{max} was reached in less time compared with the control. In fact, maximum biomass concentration of 5.54, 3.90, and 2.19 g_{DW}/L was obtained after 11, 6 and, 10 days for MTP, CP, and OP, respectively. At the end of the growth period biomass productivity of 0.10, 0.13, and 0.09 g_{DW}/L day were obtained for MTP, CP, and OP in presence of 2W, respectively. These values can be highly increased if the co-culture growth is stopped when the maximum concentration was reached. In fact, maximum biomass productivity of 0.50, 0.65, and 0.18 g_{DW}/L day could be obtained for MTP, CP, and OP in presence of 2W, after 11, 6, and 10 days of growth, respectively. The same trend obtained for biomass productivity could be observed for the specific growth rate.

Table 2.9. Growth and lipid production parameters of co-cultures used in the mixotrophic treatment of winery wastewaters (W) and BBM (C) in different photobioreactors: Multi-tubular photobioreactor (MTP), Column photobioreactor (CP) and, Open pond (OP).

	MTP		CP		OP	
	W	C	W	C	W	C
X_f [a] (g_{DW}/L)	1.56	3.70	1.96	2.68	0.93	1.12
X_{max} [b] (g_{DW}/L)	5.54	3.70	3.90	2.68	2.19	1.12
μ [c] (day^{-1})	0.07	0.12	0.09	0.11	0.10	0.09
μ_{max} [d] (day^{-1})	0.22	0.12	0.34	0.11	0.18	0.09
ν [e] (g_{DW}/L day)	0.10	0.22	0.13	0.18	0.09	0.09
ν_{max} [f] (g_{DW}/L day)	0.50	0.22	0.65	0.18	0.18	0.09

[a] final biomass concentration, [b] maximum biomass concentration, [c] specific growth rate, [d] specific growth rate at X_{max}, [e] biomass productivity, f biomass productivity at X_{max}.

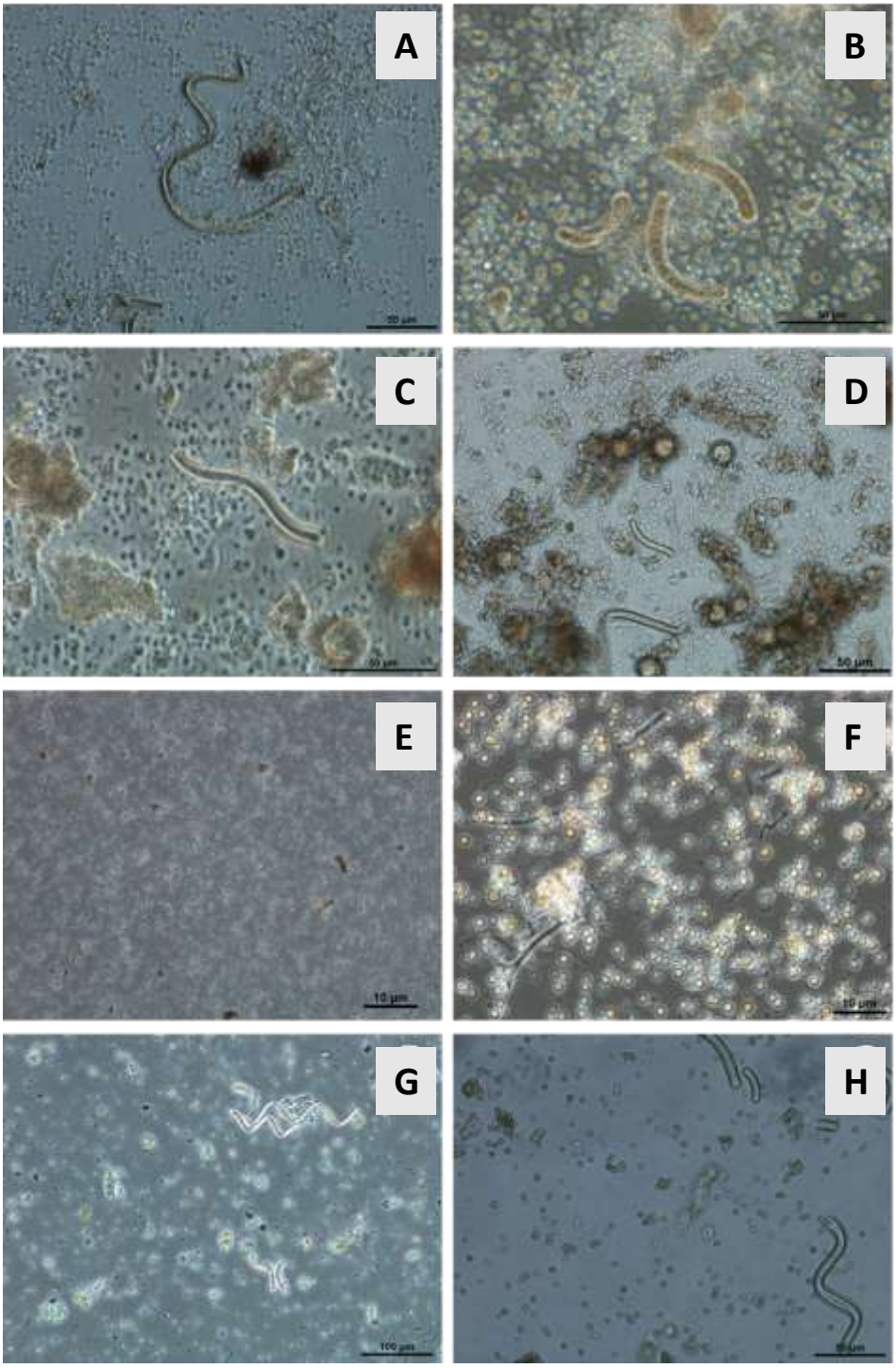

Figure 2.19. Optical observation of co-culture growth in 2W in the different growth system configurations by optical microscope at different magnification. A, B- multitubolar photobiorerator, C, D- open pond, E, F- column photobioreactor, G, H- Control run. A, E, and G magnification 10x; C, D and H magnification 20x; F and B magnification 40x.

The co-culture was observed by optical microscope after winery wastewater treatment in the different photobioreactor configurations, the recorded images were shown in Figure 2.19. The co-culture after the treatment changed its typical green color of the chlorophyll pigment to a more brownish one. This result suggested the co-culture growth in presence of the WWW organic carbon growth prevalently using heterotrophic metabolism instead that autotrophic, as already observed in the experiment carried out under dark conditions. In fact, the co-culture growth in BBM presented a more greenish color.

2.3.2.2 COD Removal from Winery Wastewater by co-culture

In microalgae cultivation, microalgal cells can consume nutrients, such as inorganic anions, as well organic matter for their cell generation to produce biomass. Chemical oxygen demand (COD) is the most used measure for organic pollution, it estimates the portion of oxygen required to oxidize the organic matter contained in the wastewater. In this research work, the evaluation of COD was used to estimate the consumption of organic matter by the co-culture needed for its metabolism.

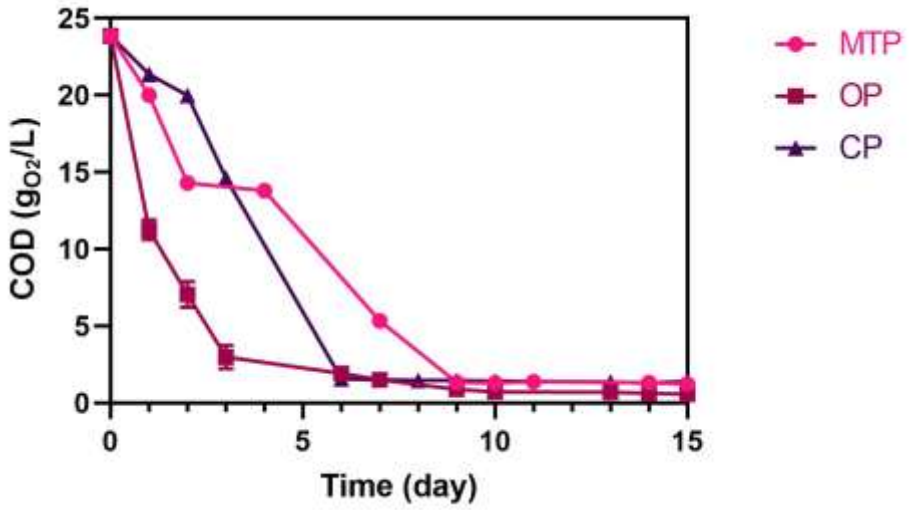

Figure 2.20. Cod reduction by microalgae co-culture. Multitubular photobioreactor (MTP), Column photobioreactor (CP) and, Open pond (OP).

Chemical oxygen demand and polyphenols content was determined daily to evaluate and, compare the reduction efficiency by co-culture and plot the reduction curves. At the end of WWW treatment, the COD content was reduced by more than 95% using all the different growth systems.

A COD content of 1.16, 0.55, and 1.41 g_{O2}/L, was reached after WWW treatment in MPT, OP, and CP, respectively. As shown in Figure 2.20, the COD reduction trends obtained from different reactors configuration was quite different in the first days of co-culture grown. A rapid COD consumption was obtained by growing the co-culture in OP reaching a reduction of about 80% on the third day of growth. Instead, to obtain comparable results in terms of COD reduction, 9 and 6 days were needed for MTP and CP, respectively.

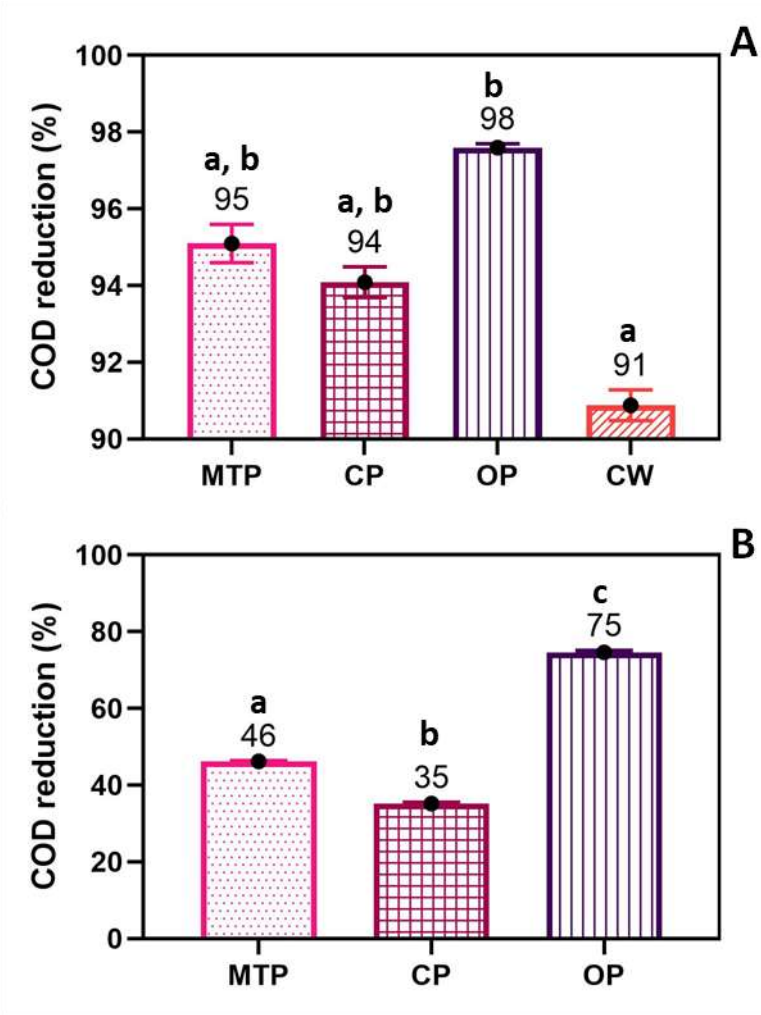

Figure 2.21. Removals of Chemical Oxygen Demand (COD) by the co-culture in winery wastewaters in multitubolar photobioreactor, open pond, and column photobioreactor. A- Final COD removal compared to the start of each treatment. B- Increase in final COD removal compared to the negative control of each treatment. Different letters (a-c) in the same group indicate statistically significant differences among mean values.

The COD reduction of 2W by co-culture treatment in MTP, OP and CP and from negative control (CW) was evaluated, as showed in Figure 2.21. The COD reduction by co-culture was higher that the negative control run for all the growth system configurations. In fact, an increase of COD reduction of 46, 35 and 75% by co-culture was obtained for MTP, CP and OP, respectively.

2.3.2.3 PC Removal from Winery Wastewater by co-culture

Considering the high polyphenols content in wine, and consequently into winery wastewater, it was calculated the polyphenols concentration during all the co-culture growth to evaluate the co-culture ability to assimilate and consume these compounds.

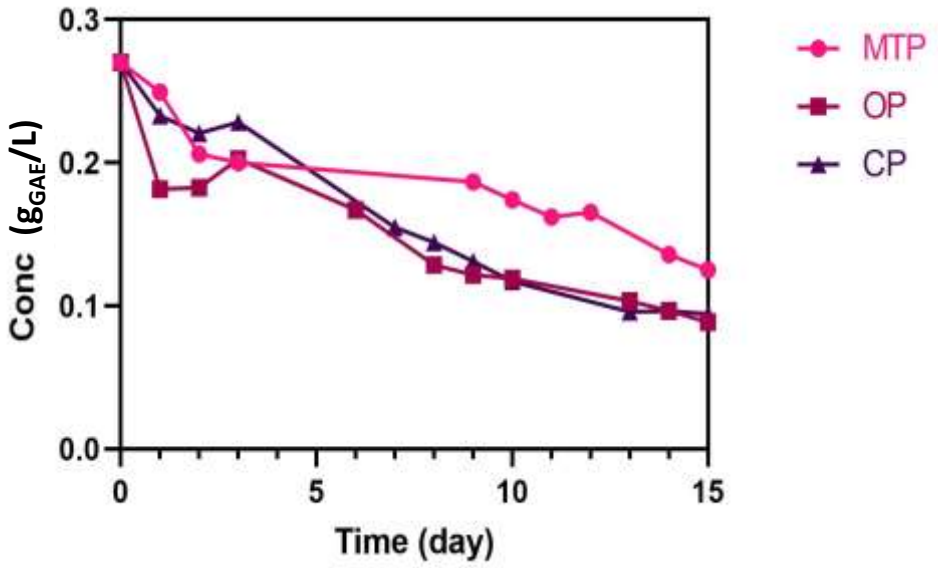

Figure 2.22. PC reduction by microalgae co-culture. Multitubular photobioreactor (MTP), Column photobioreactor (CP) and, Open pond (OP).

Polyphenol degradation by the co-culture at the end of treatments was higher than 50% in all the tested growth system configurations. A final PC concentration of 120, 90, and 90 mg_{GA}/mL was obtained at the end of the co-culture growth in MTP, OP, and CP, respectively. The curves of PC removal curve obtained in OP and CP follows the same trend, as shown in Figure 2.22. Instead, a slow PC reduction was obtained growing the co-culture in MTP. Microalgae can consume polyphenols components through two different mechanisms biotransformation to other structurally similar compounds or mineralizing to carbon dioxide [106]. Moreover, polyphenols biotransformation by microalgae can occur in both presences of the absence of light. For example, Pinto et al. (2003) observed biotransformation of 95 and 100% of tyrosol by S. *quadricauda* and

A. braunii under dark conditions, and a reduction of about 58 and 95% under light conditions, respectively [113].

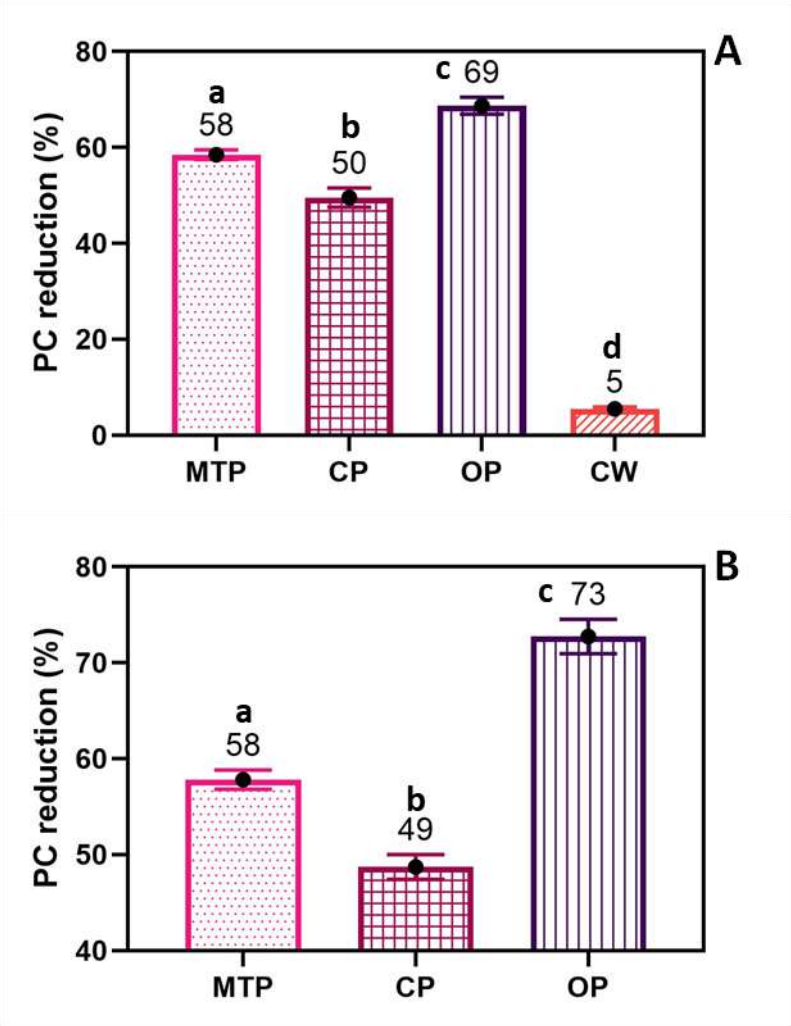

Figure 2.23. Removals of polyphenols content (PC) by the co-culture in winery wastewaters in multitubolar photobioreactor, open pond, and column photobioreactor. A- Final PC removal compared to the start of each treatment. B- Increase in final PC removal compared to the negative control of each treatment. Different letters (a-d) in the same group indicate statistically significant differences among mean values.

As observed previously for the COD removal, the PC reduction of 2W by co-culture treatment in MTP, OP, and CP and from negative control (CW) was evaluated, as showed in Figure 2.23. The PC reduction by co-culture was higher than the negative control run for all the growth system configurations. In fact, an increase of PC reduction of 58, 49, and 73% by co-culture was obtained for MTP, CP, and OP, respectively.

2.3.2.4 Anions Removal from Winery Wastewater by co-culture

The quantification of the characteristic anions contaminant of wastewater (fluoride (F^-), chloride (Cl^-), nitrite (N-NO_2), nitrate (N-NO_3), phosphate (PO_4^{3-}), sulfate (SO_4^{2-}) and sulfite (SO_3^-)), were estimated before and after winery wastewater treatment to better understanding the overall co-culture metabolism.

Table 2.10. Winery wastewaters parameters before and after co-culture treatment in different photobioreactors: Multitubular photobioreactor (MTP), Column photobioreactor (CP) and, Open pond (OP).

		MTP	OP	CP
Fluoride, F^- (mg/L)	Initial	13.00±0.14	13.00±0.14	13.00±0.14
	Final	0.71±0.03	0.40±0.03	n.d.
Chloride, Cl^- (mg/L)	Initial	73.20±1.13	73.20±1.13	73.20±1.13
	Final	79.80±0.14	75.80±2.55	80.45±0.32
Nitrite, N-NO_2 (mg/L)	Initial	0.20±0.00	0.20±0.00	0.20±0.00
	Final	0.30±0.00	1.53±0.29	0.61±0.01
Nitrate, N-NO_3 (mg/L)	Initial	191.35±0.04	191.35±0.04	191.35±0.04
	Final	0.20±0.00	30.95±6.40	0.21±0.01
Sufite, SO_3^- (mg/L)	Initial	9.25±0.04	9.25±0.04	9.25±0.04
	Final	0.35±0.01	4.40±0.47	0.24±0.01
Phosphate, PO_4^{3-} (mg/L)	Initial	126.00±0.99	126.00±0.99	126.00±0.99
	Final	130.37±10.14	113.37±2.73	115.38±8.73
Sulfate, SO_4^{2-} (mg/L)	Initial	97.30±0.07	97.30±0.07	97.30±0.07
	Final	39.27±3.30	60.78±2.65	76.69±5.50

Microalgae can utilize both organic (urea) and inorganic nitrogen (nitrate, nitrite, and ammonia) for their growth. Generally, into wastewaters inorganic nitrogen is present in the form of NO_3^-, NO_2^- and $NH4^+$. The conversion of inorganic carbon into organic form is carried out by microalgae via assimilation across the plasma membrane. Briefly, the mechanism is a sequence of nitrogenates reduction, NO_3^- to NO_2^- and at least NH_4^+ is directly integrated into amino acids [114]. Almost a complete reduction of NO_3^- occurred at the end of the co-culture treatment in all the growth system configurations. Instead, an increase of NO_2^- content from 0.20 mg/L to 0.30, 1.53 and 0.61 mg/L was observed (Table 2.10.) in MTP, OP, and CP, respectively. Inorganic phosphorus plays an important role in microalgae growth and energy metabolism. It is generally in form of HPO_4^{2-} and

$H_2PO_4^-$ and is integrated into the microalgae membrane by phosphorylation mechanism, in which is produced ADP and subsequently ATP [114]. A reduction of PO_4^{3-} form 116 mg/L to 113 and 115 mg/L was observed in OP and CP, while PO_4^{3-} concentration remained constant in MTP. Almost a complete reduction of F^-, and SO_3^- was observed after co-culture treatment in all the growth systems. A reduction of about 59, 38 and, 21% of SO_4^{2-} was observed in MTP, OP, and CP, respectively. Moreover, no reduction of anion chloride took place after wastewater treatment.

2.3.3 Conclusions

In this research work, winery wastewater was used as a growth medium for a co-culture of *Chlorella vulgaris* and *Arthrospira platensis*. Three different microalgae growth systems (multitubular photobioreactor, open pond and, column photobioreactor) were studied to evaluate the differences in terms of biomass productivity and pollutant impact removal. A maximum biomass concentration of 5.54, 3.90 and, 2.19 g/L was obtained in MTP, CP and OP, respectively. A COD reduction higher than 94% was obtained by co-culture in all the growth system configurations. While the polyphenols content was reduced around 50-60% by co-culture in the function of the growth systems. In conclusion, the co-culture was effectively able to grow and to reduce the pollutant impact of second washing of wine tanks wastewater in the three different growth system configurations.

3 Continuous winery wastewater treatment by microalgae co-culture in membrane photobioreactor

Microalgae-based wastewater treatment systems represent a sustainable, cost-effective, and environmentally friendly technology not only for the good management of wastewaters and nutrients' removal, but also for the recovery of biocomponents such as lipids, proteins, and carbohydrates. The algal biomass grown in wastewater can in fact be collected and utilized as a raw material in the production of protein-rich animal feed, biogas, biofuel, and biofertilizer [115,116].

While it is well known that high density of cells can be achieved when organic substrates (sugars and organic acids) are used as carbon sources for algal cultivation under mixotrophic or heterotrophic conditions, few recent studies have demonstrated that growing microalgae in WWW is possible. These studies, performed in batch mode, showed that it was possible to reduce the levels of most organic compounds via assimilation by microalgal cells. However, it is worth noting that the cultivation time was quite long (about 15 days) [117–119]; therefore, a large reactor volume or high biomass concentration would be required to improve WWW treatment performance using microalgae. Furthermore, microalgae are subjected to many variables during cultivation, in particular the concentrations of nutrients, which makes it difficult to attribute a variation in biomass composition to a given cause [120]. As a result of these limitations, continuous operation under steady-state conditions is more attractive on an industrial scale, owing to higher reproducibility and controllability compared to batch mode, and provide a better combination of operating conditions and biomass composition [121].

Among the types of photobioreactors for simultaneous microalgae cultivation and wastewater treatment, membrane photobioreactors (MPBRs), whose applicability has already been demonstrated in both biological and chemical processes, could be an ideal solution for WWW continuous treatment [122,123]. Membrane-inspired photobioreactors have numerous benefits for microalgae cultivation, namely, higher cell productivity, higher removal efficiency, process flexibility at high loading rates, and moderate capital cost [124,125].

The main advantage of MPBR is that the biomass retention time is independently controlled independently of the hydraulic retention time (HRT). By virtue of this characteristic, both the biomass concentration and nutrient removal efficiency can be maintained at high levels, which

reduces the costs of both harvesting and downstream dewatering operations. So far, very few studies have been carried out to assess the performance of MPBR in the continuous cultivation of microalgae using waste effluent as a culture medium [126,127].

To the best of our knowledge, no studies are available in the literature on the continuous treatment of WWW using microalgae. Hence, for the first time the present study investigated the use of MPBR to co-cultivate *Chlorella vulgaris* and *Arthrospira platensis* in order to treat winery effluent. Since the co-culture may increase the stability of the process, we evaluated the influence of HRT on microalgae growth and, as well as the removal of organic components (polyphenols' concentration and chemical oxygen demand) under continuous conditions.

3.1 Materials and methods

3.1.1 Microalgae strain and culture conditions

Freshwater microalga *Chlorella vulgaris and Arthrospira platensis* were grown as a co-culture in the present study. To produce the inoculum, both strains were pre-cultured in 1000-mL Erlemeyer flasks containing 400 mL of Bold Basal Medium (BBM) at room temperature (25 °C) with a continuous air supply and fluorescence illumination of 80 μmol photons $m^{-2}s^{-1}$ for 7 days. Subsequently, the co-culture was scaled up to a bench-scale tubular membrane photobioreactor (MPBR) made up of polymethacrylate at an initial cell dry weight of about 0.5 g L^{-1}. The co-culture was grown in a mixture of winery wastewater (WWW) coming from different steps of wine making process, namely tank washings and filtration equipment, which was provided by a winery cellar located in the Piemonte region, Italy. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.1.2 Experimental design

The lab-scale tubular photobioreactor, with an internal diameter of 30 cm and working volume of 20 L, was implemented with a membrane module with nanometer scale pore size submerged in the middle of the photobioreactor. Figure 3.1. shows the basic schematic of the lab-scale MPBR configuration. Microalgae were cultivated initially in batch mode until reaching the late logarithmic phase of growth in BBM. After 10 days of batch cultivation, the feed was switched to semi-continuous mode. 20% (v/v) of WWW in BBM without any preliminary treatment was supplied to the photobioreactor as a cultivation medium at three different hydraulic retention times (HRTs), i.e. 4.6 days (HRT1), 2.0 days (HRT2), and 1.4 days (HRT3), which were maintained for

21, 17, and 14 days, respectively. The WWW concentration in microalgae medium was selected taking into account the results shown in the previous chapter, while it has been used of a mixture of WWW instead of a single kind to simulate a more real industrial situation. The membrane was physically cleaned at the beginning of each phase at different HRT, and the selected inlet and outlet streams were pumped continuously using a peristaltic pump. The photobioreactor was illuminated by two LED lamps placed near the reactor to ensure a continuous light intensity of 60 μmol photons m^{-2} s^{-1}, while the temperature of the MFBR system was not controlled and ranged from 23 °C to 25 °C. At the bottom of the photobioreactor there was a gas distributor, through which air was feed at a constant rate to provide agitation, CO_2 supply, and membrane fouling control. During cultivation, the culture medium within the MPBR and the outlet stream were sampled daily to assess the nutrient removal and biomass production.

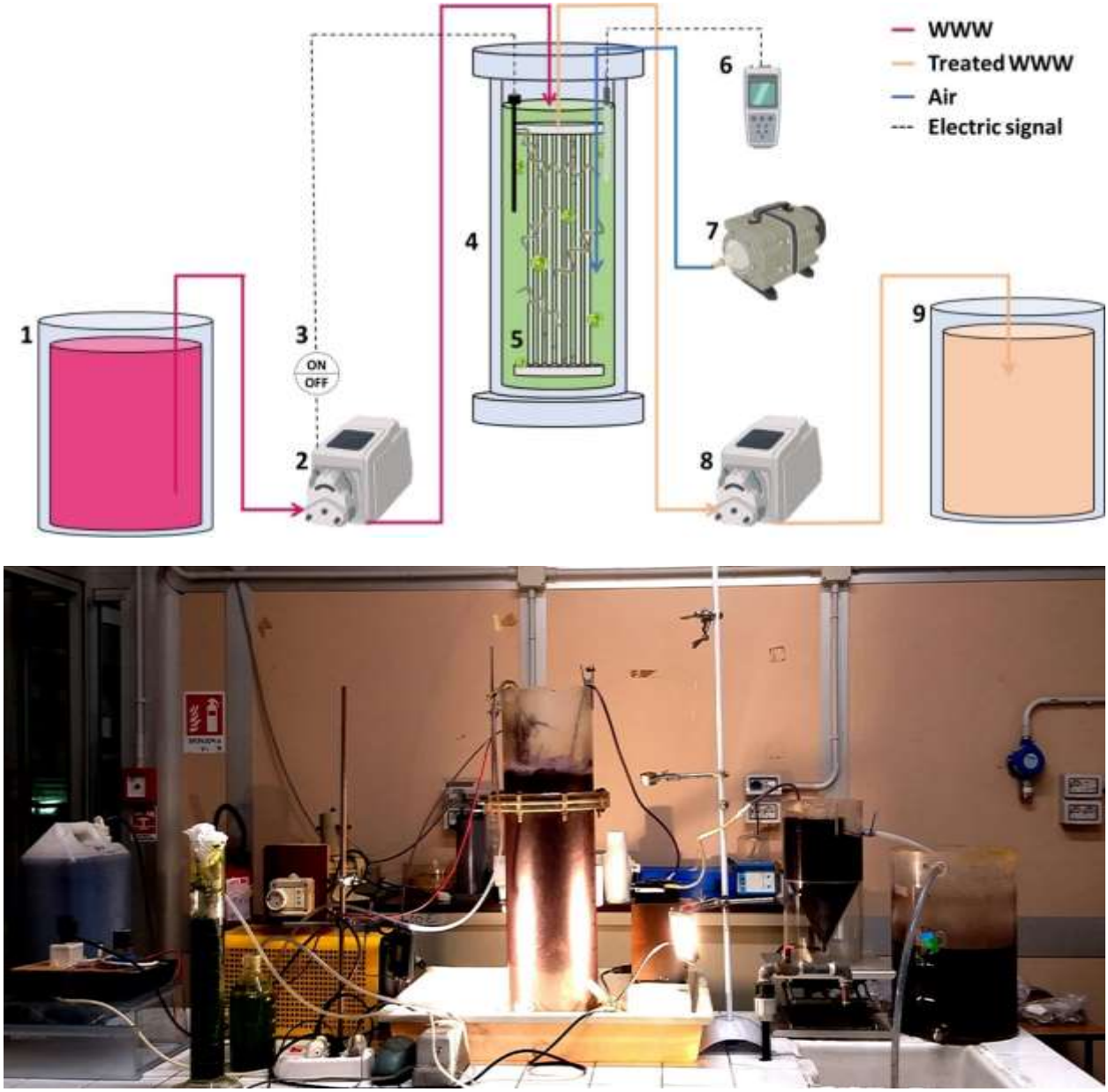

Figure 3.1 The schematic of the lab-scale MPBR configuration and WWW treatment process. 1,9- collection tank; 2,8- peristaltic pump; 3-volume controller; 4- tubular photobioreactor; 5- membrane; 6- pH controller; 7- air pump.

3.1.3 Winery wastewater characterization

WWW was characterized in terms of contents of total suspended solids (TSS) and total solids (TS) according to the standard methods of APHA (APHA, AWWA, WEF, 1999)..

Chemical Oxygen Demand (COD) was quantified by colorimetric analysis using the potassium dichromate method according to the standard methods and expressed in grams of oxygen per liter (g_{O_2}/L (APHA, AWWA, WEF, 1999).

Polyphenols concentration (PC) was measured by the Folin–Ciocalteu assay and expressed as expressed as milligrams of gallic acid equivalents per milliliter of solvent (mg_{GAE}/mL) [119].
To determine the degradation efficiency of the microalgal co-culture, the WWW COD content was measured daily.

3.1.4 Biomass characterization

Total microalgae concentration was determined daily by dry weight, taking into account the total suspended solid (TSS) content of WWW expressed in g_{DW}/L. Optical observation of the co-culture was carried out daily by light microscope (Leica DMLS, Wetzlar, Germany).
The total lipids were extracted from the microalgal co-culture using a 2.0:2.0:1.8 (v/v) methanol/chloroform/water mixture according to the modified Bligh and Dyer method (Kates et al., 1966).
Fatty acids in the lipid fractions were transesterified as described by Zunin et al. (2006) [128] and analyzed with a gas chromatograph (DANI, Milan, Italy) equipped with a ZB Vax column and a FID detector (Thermoscientific, Milan, Italy).The injector and the FID were set at 250 °C. To allow the separation of the different methyl ethers, the column temperature was changed during the analysis according to the following separation protocol: 150 °C for 2 minutes, from 150 °C to 175 °C at a rate of 3 °C/min, 175 °C for 1 minute, from 175 °C to 210 °C at a rate of 3 °C/min, 210 °C for 7 minutes. The duration analysis was around 30 minutes. The resulting chromatograms allowed identifying the different methyl ethers through the retention time and quantifying them through the peak areas.
The calorific values of the biomass grown in the stages of cultivation at different HRTs were determined by means of a calorimetric bomb (C200, IKA, Staufen, Germany).
The elemental composition of the co-culture, on the basis of carbon, hydrogen, nitrogen and sulfur contents, was determined with a CHNS-O elemental analyzer (FLASH EA1112, ThermoQuest, Cleveland, OH, USA) according to the methodology described by Casazza et al. (2015).

3.1.5 Kinetic parameters of co-culture

Kinetic parameters were evaluated for each growth with different HRT. The kinetics parameters: specific grow rate (μ) and biomass productivity at the end of cultivation (v) were calculated as described in the previous sections (section 2.1.1.4).

3.1.6 Statistical analysis

Statistical analysis was carried out by Statistica v 10 software (StatSoft, Tulsa, OK, USA). The statistically significant differences between the results were evaluated by the analysis of variance (ANOVA) with Tuckey's HSD, post-hoc multiple comparison test.

3.2 Results and discussion

3.2.1 Characterization of winery wastewater

The winery wastewater (WWW) used in this work as a medium to grow the *Arthrospira platensis* and *Chlorella vulgaris* co-culture was composed by a mixture of effluents deriving from different steps of wine making process, namely waters from tank washing and filtration equipment. Before use, WWW was characterized in terms of contents of total solids (TS), total suspended solids (TSS) and polyphenol compounds (PC) as well as pH and chemical oxygen demand (COD), whose values are listed in Table 3.1.

Table 3.1. Winery wastewaters characterization

Parameter	WWW
TS [a] (g/L)	9.78 ± 0.70
TSS [b] (g/L)	0.75± 0.15
pH	3.51±0.20
PC [c] (mg $_{GAE}$/L)	127.1± 5.00
COD [d] (g_{O_2}/L)	119.3 ± 4.70

[a] total solid, [b] total suspended solids, [c] polyphenol content, [d] chemical oxygen demand

The COD of WWW (119.3 g_{O_2}/L) was especially high mainly due to the presence of organic compounds such as sugar and ethanol, while the high polyphenol content in WWW (127.1 mg_{GAE}/L) was due to the presence of soluble acidic phenolic compounds in grapes (syringic, gallic, vanillic and protocatechuic acids and other organic acids). In general, the quantitative characteristics of WWW were comparable with those found in the literature [130].

3.2.2 Microalgal Biomass Growth

The co-culture of *C. vulgaris* and *A. platensis* was grown semi-continuously in the column photobioreactor in the presence of 20% (v/v) WWW in Bold's Basal Medium (BBM). Such a

WWW proportion in the culture medium was selected based on previous results of a study where the WWW concentration was optimized for the same co-culture in 200-mL bubblers with continuous air and light supply for 15 days [119]. medium. The wastewater was fed to the photobioreactor at three different flow rates, i.e. 3, 7 and, 10 mL/min corresponding to mg_{GAE}/L Hydraulic Retention Times (HRTs) of 4.6 days (HRT1), 2 days (HRT2) and 1.4 days (HRT3), respectively. The performance of WWW treatment was compared with that of a control run (CTR), in which the co-culture was grown batchwise, in the same photobioreactor, for 21 day and using BBM as growth medium.

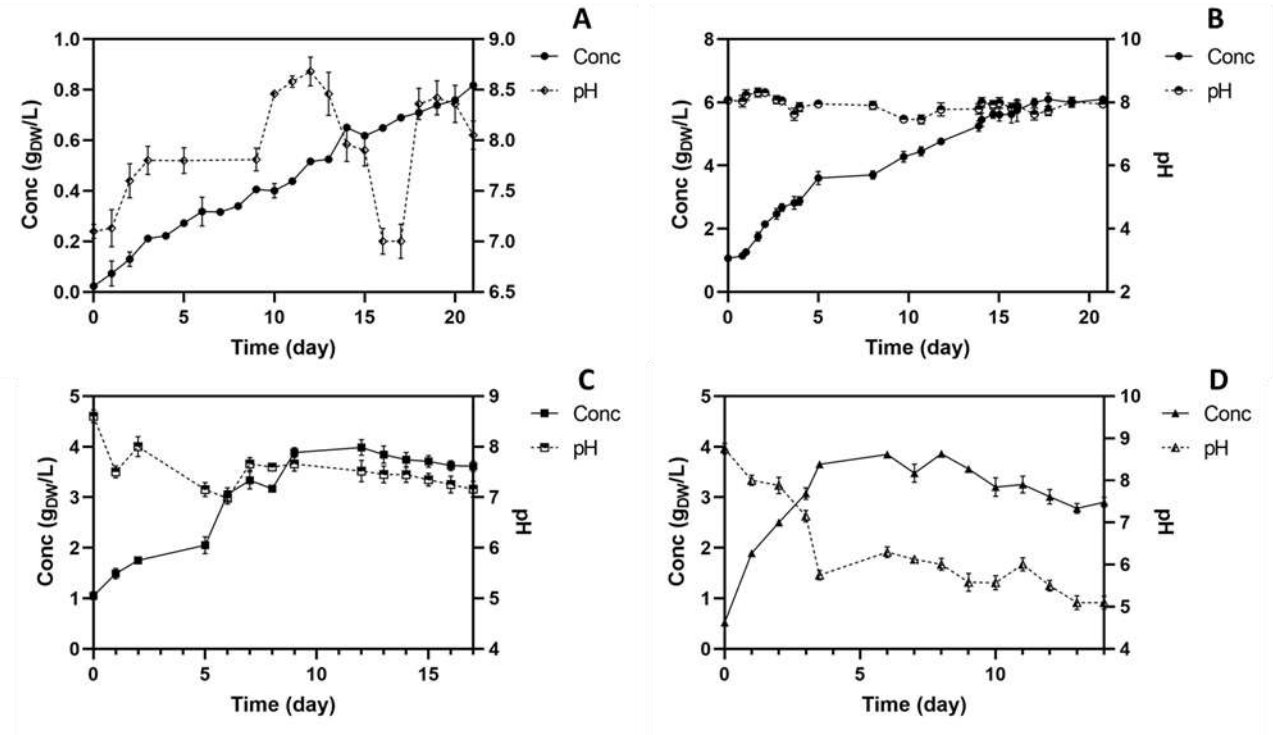

Figure 3.2. Co-culture growth curves and pH variation in (**A**) Bold's Basal Medium (CTR), (**B**) in winery wastewater with an HTR (B) 4.6 day, (C) 2 day and (D) 1.4 day. Biomass concentration expressed as g dry weight per liter.

Figure 3.2 shows the growth curves and the pH variation during the different co-cultures performed in WWW at HTR1, HRT2 and HRT3 compared to the control run in BBM. Biomass concentration in the control run after the whole growth period (0.82 ±0.02 g_{DW}/L) was much lower than the ones obtained in runs carried out in WWW (6.10±0.05 g_{DW}/L, 3.61±0.04 g_{DW}/L, and 2.90±0.02 at HTR1, HRT2 and HRT3, respectively) (Table 3.2), where exponential growth was quickly achieved without a clear lag phase. This result is in agreement with those of several authors, who

observed, culturing microalgae or cyanobacteria under mixotrophic or heterotrophic conditions, a significant increase in biomass concentration compared to autotrophic cultures [131,132]

The same improvement was observed for biomass productivity, which increased from only 38.9 ±2 mg_{DW}/Ld in the control run under autotrophic conditions up to 240 mg_{DW}/Ld in WWW at HRT of 4.6 days (Table 3.2). However, when the HRT was reduced to 1.4 days, biomass productivity decreased by 43.3%, confirming the trend of biomass concentration.

The above reductions in biomass concentration and productivity induced by an increase in WWW flowrate was probably due not only to the achievement of washout conditions but also to higher nutrient stress conditions to which the co-culture was submitted. However, it should be remembered that the appropriate HRT should be selected to ensure good biomass production and, at the same time, a satisfactory reduction of the polluting impact of wastewater. Qualitatively results were reported by Andreotti et al. (2020) , who observed, culturing *Tetraselmis suecica* in aquaculture wastewater in semi-continuous mode, that a decrease in HRT from 10 and 7 days reduced biomass concentration and productivity from 900 to 500 mg of total solids/L and from 67 to 49 mg/Ld, respectively [133].

Table 3.2. Growth parameters of *Arthrospira platensis* and *Chlorella vulgaris* co-culture grown in Bold's Basal Medium (CTR) and winery wastewater at different hydraulic retention times: HRT1 = 4.6 days; HRT2 = 2.0 days; HRT3 = 1.4 days

Run	Medium pH [1]	X_f (g_{DW}/L) [2]	μ (1/d) [3]	ν (mg_{DW}/Ld) [4]
CTR	7.5±0.5[a]	0.82±0.02[a]	0.170±0.003[a]	39 ±2 [a]
HRT1	7.9±0.2[a]	6.10±0.05[b]	0.083±0.004[b]	240 ±11[b]
HRT2	7.5±0.4[a]	3.61±0.04[c]	0.003±0.001[c]	154±13[c]
HRT3	6.3±1.1[a]	2.90±0.02[d]	0.014±0.002[c]	136 ±19[c]

[1] Medium pH, [2] final biomass concentration, [3] specific growth rate, [4] biomass productivity. Different letters (a-c) refer to statistically significant differences among results within columns ($p<0.05$, ANOVA with Tuckey's HSD, post-hoc multiple comparison test).

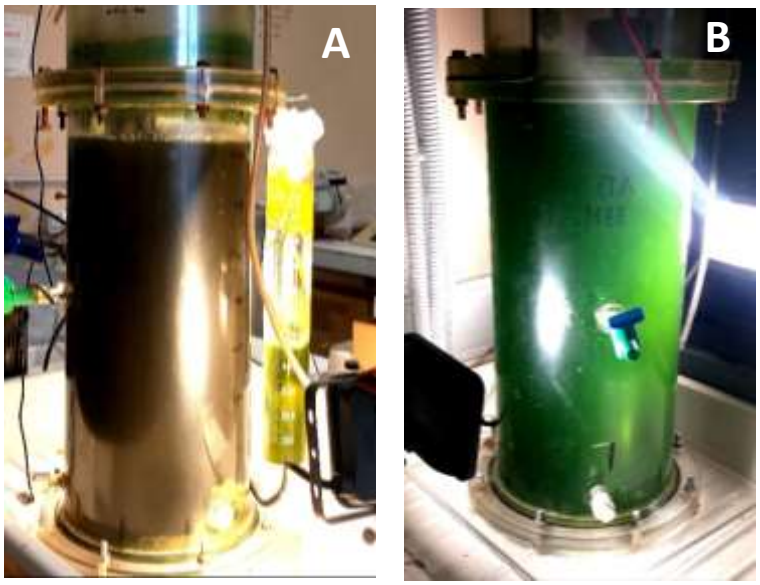

Figure 3.3. Column photobioreactor at the beginning (A) at the end (B) of the WWW treatment by the *Arthrospira platensis* and *Chlorella vulgaris* co-culture at a hydraulic retention time of 2.0 days.

Figure 3.3. shows two pictures of the photobioreactor at the beginning and the end of the WWW treatment by the co-culture at HRT2. It is clearly visible that at the end of the growth the culture color was more greenish than at the beginning because of the increase in biomass concentration from 1 to 3.61 g_{DW}/L. This result was qualitatively confirmed by optical microscopy examination of the co-culture at the end of the same run (Figure 3.4). In particular, it is possible to note the abundant presence of *C. vulgaris*, the absence of the typical trichomes of *A. platensis* and the development, in its place, of another filamentous cyanobacterium, probably autochthonous.

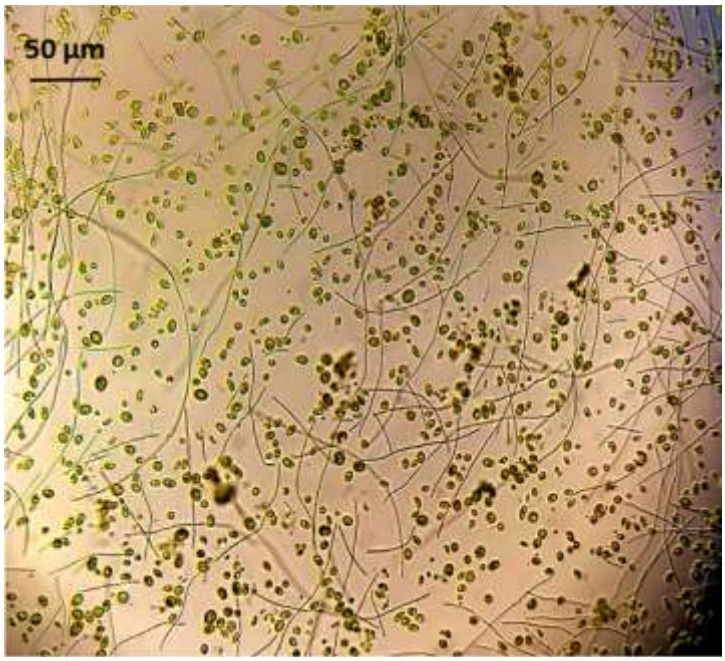

Figure 3.4. Optical microscopy examination of the co-culture after the WWW treatment at a hydraulic retention time of 2.0 days (magnification 20x).

3.2.3 Lipid Accumulation and Elemental Composition in Co-Culture Biomass

In general, the lipid content of microalgae and cyanobacteria ranges between 10 to 50% by dry weight, depending on the growth conditions and species. Thanks to their high lipid accumulation, biomass of the microorganisms is a suitable source for biofuels production by transesterification of fatty acids[133]. Moreover, the lipid content can be increased by growing the microalgae under stressful conditions in terms of medium composition, light intensity, temperature, and pH. In this sense, the use of wastewater as a microalgae growth medium not only increases the lipid content of the produced biomass but also makes the process more environmentally friendly and economically feasible [23].

Table 3.3. Lipid production parameters of co-cultures in Bold's Basal Medium (control run) and in winery wastewater at different hydraulic retention times: HRT1 = 4.6 days; HRT2 = 2.0 days; HRT3 = 1.4 days.

Run	L [1] ($g_L/100g_{DW}$)	ν_L [2] ($mg_L/L\ d$)
Control	11.0 ± 1.1^a	20.7 ± 2.4^a
HRT1	19.0 ± 3.3^b	47.8 ± 3.2^b
HRT2	27.8 ± 1.4^c	105.5 ± 10.0^c
HRT3	22.8 ± 1.8^b	67.4 ± 9.2^d

[1] lipid content of biomass, [2] lipid productivity.
Different letters (a-d) refer to statistically significant differences among results within columns ($p<0.05$, ANOVA with Tuckey's HSD, post-hoc multiple comparison test).

Values of the lipid content in biomass and lipid productivity are listed in Table 3.3 for the different operating conditions tested. In general, the lipid content in biomass grown in presence of WWW was remarkably higher (by 72-153%) than that grown autotrophically in the control run (11.0 $g_L/100g_{DW}$), which suggests that semi-continuous feeding instead of batch mode of operation and/or the presence of an organic rather than inorganic carbon source acted somewhat as environmental and nutritional stressors, respectively. The same trend in lipid accumulation by microalgae due to the wastewater treatment was observed by several other authors. Tan et al. (2018) reported an increase in the lipid content of *Chlorella pyrenoidosa* using digested starch and alcohol processing wastewaters in the growth medium, and Moon et al. (2014) observed the same increase in *Ettlia sp.* biomass when sugar factory wastewater was added to the culture medium. Assuming, as mentioned above, that the accumulation of lipids in the present study was the result of stressful conditions to which the co-culture was subjected during the treatment, we can deduce that the longer HRT (HRT1) was not able to ensure an environmental stress enough to maximize lipids accumulation and that, on the contrary, at the shorter HRT (HRT3) the excess of available organic carbon source somehow reduced the effectiveness of the nutritional stress conditions.

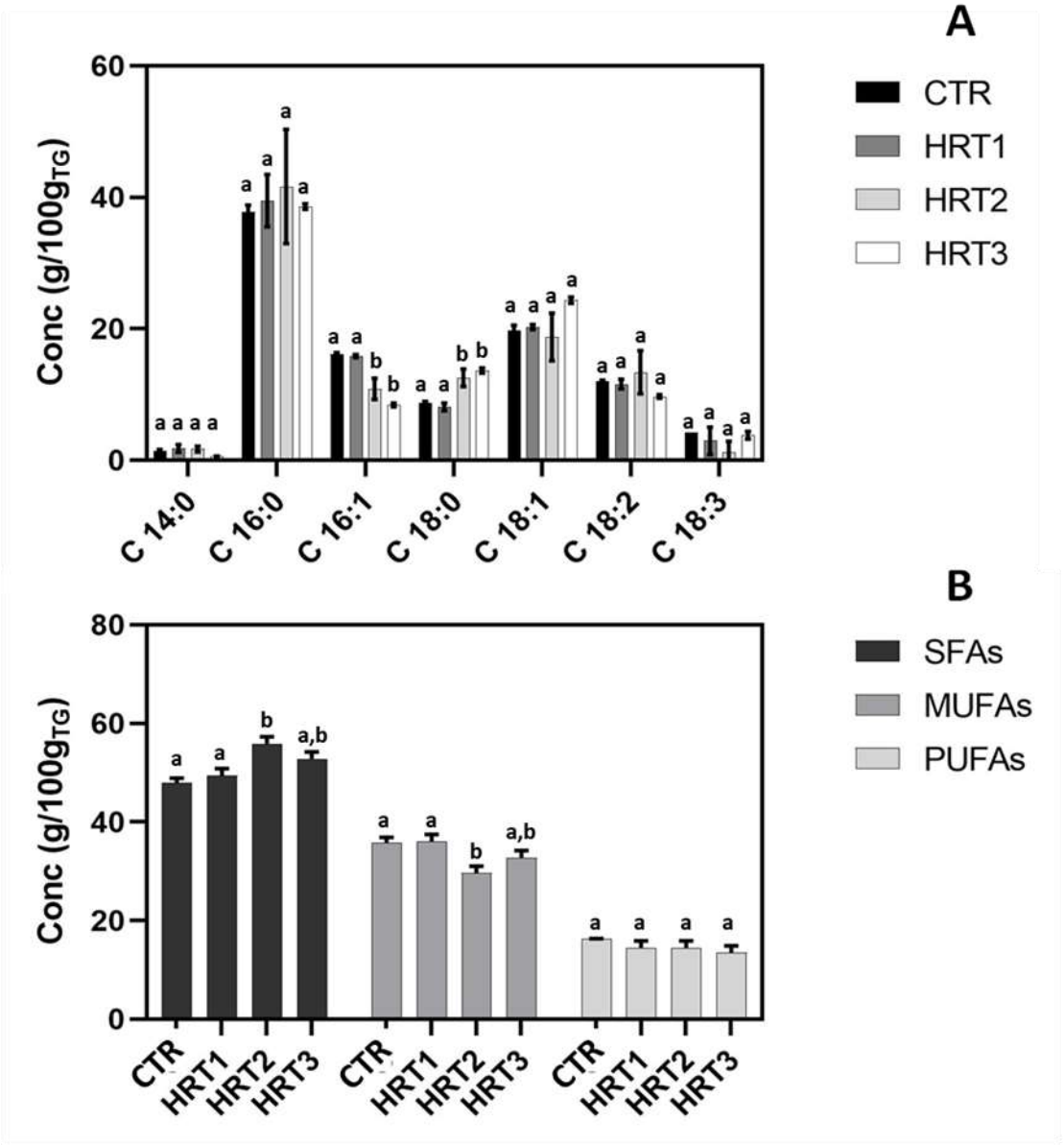

Figure 3.5. Fatty acid composition of co-culture biomass grown semi-continuously at different retention times: HRT1 = 4.6 days; HRT2 = 2.0 days; HRT3 = 1.4 days. (A) Concentrations of the most significant lipids, (B) Total contents of different classes of lipids: TG: Triglycerides, SFAs saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids. CTR = control. Different letters (a-b) refer to statistically significant differences among results inside the same group ($p<0.05$, ANOVA with Tuckey's HSD, post-hoc multiple comparison test).

The fatty acid composition (expressed as g/100g of triglycerides) of biomass was determined by gas chromatographic analysis after transesterification to their methyl esters, whose results are illustrated in Figure 3.5. The fatty acid composition was influenced by the presence of WWW in culture medium and HRT, with palmitic (C 16:0) and oleic (C 18:1) acids being always the predominant fatty acids. The palmitic acid content increased from 37.8 ± 1.00 g/100g$_{TG}$ in the

control to 39.5 ± 4.00, 41.6 ± 8.70 and, 38.6 ± 0.40 g/100g_{TG} in biomass produced at HRT1, HRT2, and HRT3, corresponding to 5.6% average increase. On the other hand, no significant difference in the oleic acid concentration (around 20 g/100g_{TG}) was observed by adding WWW to the culture medium or varying the flow rate. Instead, a reduction in palmitoleic acid (C 16:1) concentration occurred by reducing HRT, while that of stearic acid (C18:0) increased. In general, the co-culture growth in WWW led to an increase in saturated fatty acids (SFAs) content of biomass and a reduction in the monounsaturated (MUFAs) and polyunsaturated (PUFAs) ones. In particular, in biomass semi-continuously grown at HRT2, which had the highest lipid content (about 28 g_L/100g_{DW}), a 16.7% increase in SFAs content (55.9 g/100g_{TG}) and a 20.9% reduction in MUFAs content (29.6 g/100g_{TG}) were observed compared to the control. Several studies have reported a significant increase in SFAs, particularly palmitic and stearic acids, when microalgae were grown mixotrophically in wastewaters [134–136]. As known, the ratio and the amount of saturated and unsaturated fatty acids is the key factor that determines the suitability of microalgae lipids for biodiesel production. Biodiesel that contains a high presence of mono and polyunsaturated fatty acid oxidize faster than conventional diesel; this is correlated to settling of the insoluble part that interferes with engine performance. Conversely, a high amount of SFAs gives better properties to biodiesel [130]. For this reason, the produced biomass could be exploited by lipid extraction and subsequent transesterification for biodiesel production.

Table 3.4. Elemental composition and calorific value of biomass collected after the mixotrophic treatment of winery wastewaters at different hydraulic retention times: HRT1 = 4.6 days; HRT2 = 2.0 days; HRT3 = 1.4 days.

Run	N (%)	C (%)	H (%)	S (%)	O (%)	Calorific value (kJ/kg)
CTR	6.30 ± 0.30^{a}	38.43 ± 1.08^{a}	2.93 ± 0.43^{a}	n.d.	46.34 ± 0.20^{a}	20000
HRT1	5.04 ± 0.05^{a}	42.59 ± 0.19^{a}	5.96 ± 0.02^{b}	n.d.	42.41 ± 0.12^{b}	19533
HRT2	4.52 ± 0.14^{b,c}	39.18 ± 0.04^{a}	5.47 ± 0.06^{b}	n.d.	46.82 ± 0.25^{b}	17777
HRT3	3.64 ± 0.25^{c}	39.14 ± 0.69^{a}	5.74 ± 0.18^{b}	n.d.	47.48 ± 0.62^{b}	17989

Different letters (a-c) refer to statistically significant differences among results within columns ($p<0.05$, ANOVA with Tuckey's HSD, post-hoc multiple comparison test).

The elementary composition and the calorific value of biomass collected at the end of each run are listed in Table 3.4. Comparing the carbon and oxygen contents of biomass grown in the control and after WWW treatment at different HRTs, no significant difference could be observed being 39.83% and 45.76% on average, while the hydrogen content remarkably increased (from 2.93 ±

0.43% to 5.63% on average) and the nitrogen one decreased (from 6.39 ± 0.30 to 4.40% on average). Even though the average calorific value of biomass produced in WWW treatment (18433 kJ7kg) was about 7.8% lower than that grown in the control, such a difference is too low to be generalizable.

3.2.4 COD Reduction from Winery Wastewater

Chemical oxygen demand (COD) is the most used parameter to evaluate the efficiency of biological wastewater treatments, in fact has been selected as national standard for the evaluation of organic pollution in wastewater. The COD removal by co-culture during the winery wastewater treatment at the three different hydraulic retention time was evaluated daily to compare the reduction trends.

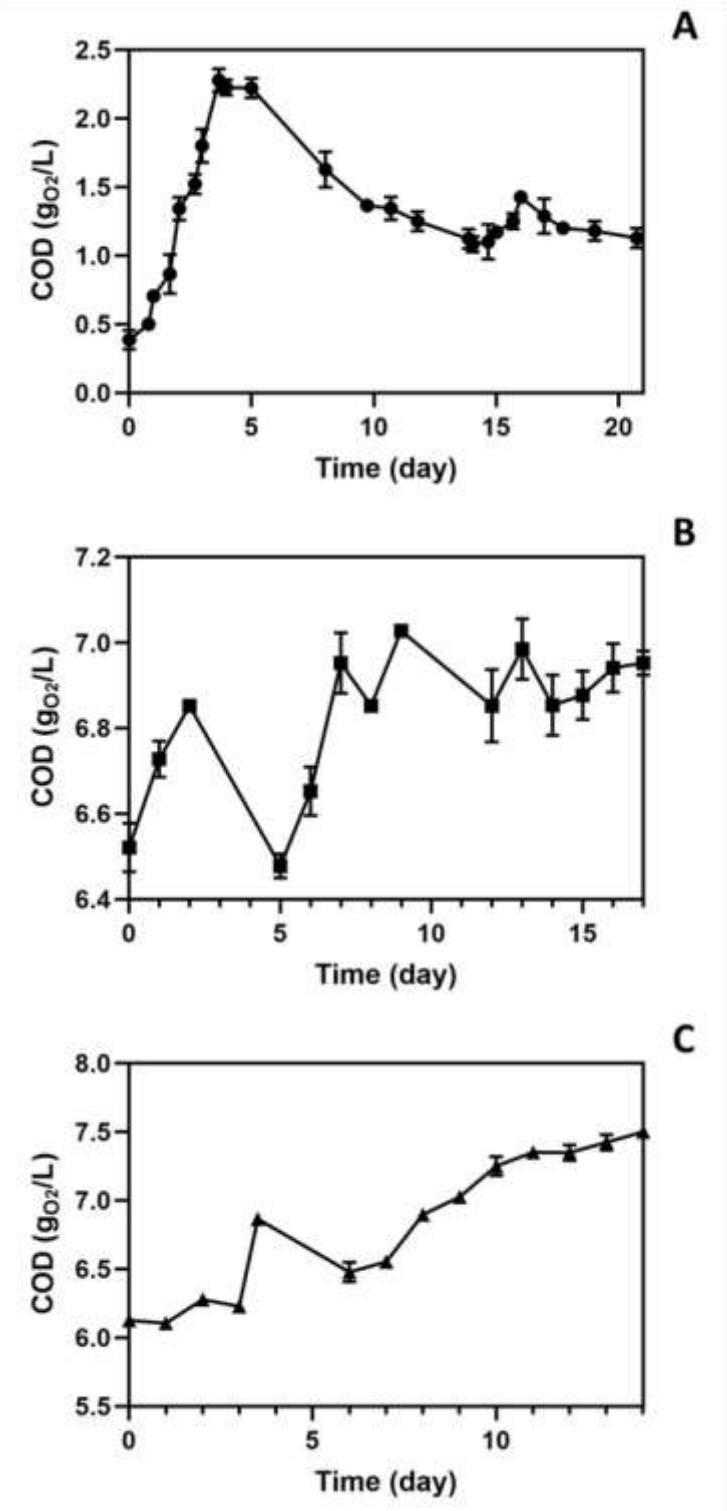

Figure 3.6. Chemical Oxygen Demand reduction during winery wastewater treatment at different hydraulic retention times (days): (A) HRT1 = 4.6; (B) HRT2 = 2.0; (C) HRT3 = 1.4.

Figure 3.6A shows that at HRT1 the COD increased during the first four days of treatment, probably due to the acclimation of the co-culture to the WWW-containing medium, and then

significantly decreased until a final value of 1.3 g_{O2}/L. Contrariwise, at HRT2 and HRT3 the COD increased during all the runs until it reached a constant value of about 7.0-7.5 g_{O2}/L, likely because the HRT was too short to allow the co-culture to effectively consume the organic pollutants present in the medium. The corresponding COD removal yield was excellent operating at HRT1 (> 90%) and satisfactory at HRT2 and HRT3 (about 75%). Comparable results were reported by Anbalagan et al. (2016), who co-cultured *Scenedesmus* sp. and *Chlorella* sp. in municipal wastewater at three different HRTs, obtaining a COD reduction yield of about 79 ± 7% at 2 days HRT.

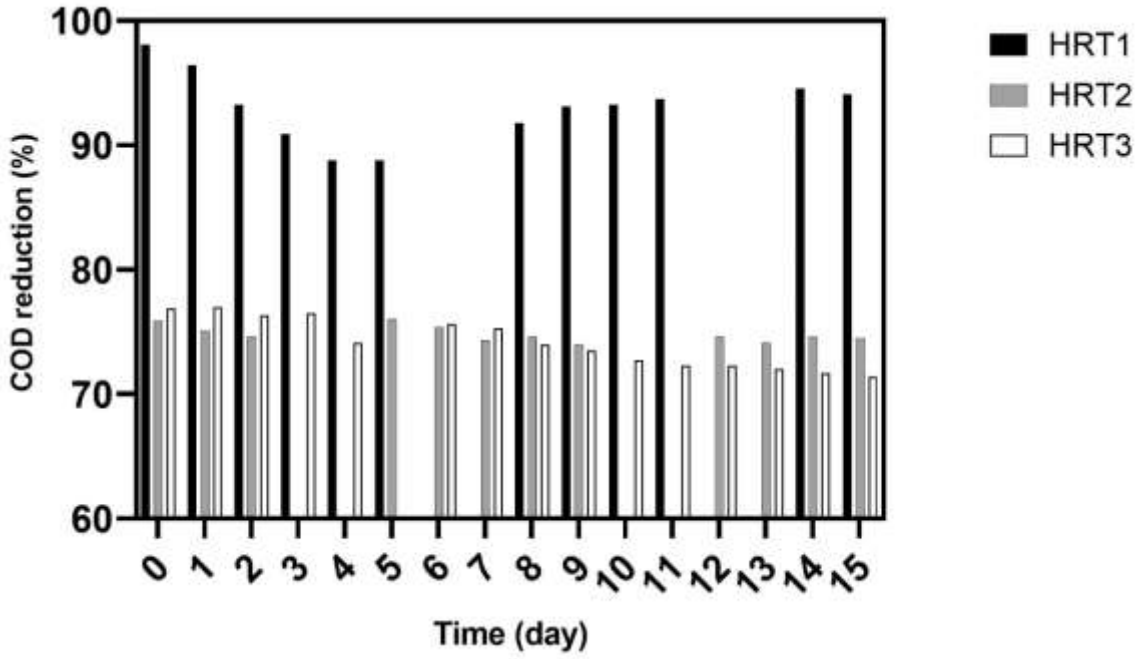

Figure 3.7. Chemical Oxygen Demand reduction during winery wastewater treatment at different at different hydraulic retention times (days): (A) HRT1 = 4.6; (B) HRT2 = 2.0; (C) HRT3 = 1.4.

Generally, winery wastewater is treated with a conventional activated sludge process or different anaerobic systems. These conventional treatments are easy to manage but require a long retention time to degrade the organic pollutants and a large surface area, therefore they can be used only for small size cellars. In this respect, the data available in the literature relating to wastewater COD reduction by microalgae have shown comparable or even better performance compared to conventional treatments. For instance, Li et al. (2011) reported a COD reduction in concentrated municipal wastewater higher than 90% using *Chlorella sp*.

3.3 Conclusions

A *Chlorella vulgaris* and *Arthrospira platensis* co-culture was grown in a semi-continuously fed membrane photobioreactor to treat winery wastewater at different hydraulic retention times. Biomass concentration reached values higher than 4 g_{DW}/L, with a lipid content higher than 20 $g_L/100g_{DW.}$ Lipids were mainly composed of saturated fatty acids. COD reduction ranged from about 75% to more than 90%. In conclusion, this study showed that winery wastewater could be used as a medium for microalgae growth significantly reducing their production cost and water consumption. The obtained biomass is suitable for lipid extraction and transesterification of fatty acids to produce biodiesel.

4 Ultrasound-assisted extraction of proteins from microalgal biomass

One of the main drawbacks regarding microalgae production is related to their high production cost, especially if the microalgae biomass will be used for the extraction of high-added value components suitable for food, cosmetical and pharmaceutical industry and the production of biofuels [138]. For this purpose, several are strategies studied to reduce microalgae production costs by the researcher to make more feasible and competitive microalgae applications. In this contest, the biorefinery concept is one of the best possibilities.

The identification of a microalgal intracellular component with a high market value, in general, useful in the pharmaceutical and cosmetics field, would make economically feasible the biofuel production from microalgal biomass. For this purpose, a good candidate may be the c-phycocyanin (c-PC) is a phycobiliprotein found in the blue-green microalgae, it is generally used as a natural blue colorant in the food industry for candy and even in cosmetic production. c-PC also showed a variety of pharmacological properties, such as antioxidant, hepatoprotective and, anti-inflammatory. Arthrospira platensis can contain up to 70 % of protein and was observed by several authors that the c-phycocyanin is its major protein [139,140].

In this chapter, the protein extraction from *A. platensis* by ultrasound-assisted extraction (UAE) using water as solvent was optimized by Box-Behnken Design for response surface methodology considering the effect of total extraction time (t), solvent volume (V), and mass of *A. platensis*. Subsequently, the obtained optimized conditions were used to set the operative condition for c-PC extraction from *A. platensis* by UAE with 1.5% (w/v) of $CaCl_2$ as solvent and the furthermore protein extraction from wet c-PC extraction residue. Moreover, the protein extraction was carried out on the co-culture biomass obtained after WWW in the three different growth systems (multitubular photobioreactor, open pond, column photobioreactor) studied in Chapter 2, Section 2.3. The differences in terms of protein content, protein expression, cell structure were investigated. Furthermore, the differences of cell resistance to UAE were investigated in terms of cell rupture and particle size distribution.

4.1 Optimization of ultrasound-assisted extraction of protein form *Arthrospira platensis* biomass

Arthrospira platensis is a protein-rich cyanobacterium, it can contain up to 70 % of proteins, in which c-phycocyanin (c-PC) is the more abundant. c-PC is a natural blue coloring that is used in the food industry for chewing gum, candies, and even cosmetic production. It also showed antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective effects [141].
The literature is rich in works on the optimization of lipid extraction from microalgae mainly for energetic purposes, while to the best of our knowledge only a few studies are available on the optimization of protein extraction, even though the great interest in food and pharmaceutical sectors, and for smart packaging production. The intrinsic rigidity of the microalgae cell wall limits the extraction of the high added-value component. Several techniques can be employed to break the cell wall, among them the ultrasound-assisted extraction is the most advantageous, because it is a low-cost technology and can work with green solvents [139]. The research work in this section aimed to optimize by Box-Behnken Design for response surface methodology the ultrasound-assisted extraction of proteins from *A. platensis*, evaluating the effect of the total extraction time (20, 30, and 40 min), solvent volume (50, 75, and 100 mL) and *A. platensis* mass (0.66, 1 and 1.33 g).

4.1.1 Materials and methods

4.1.1.1 Chemicals and microalgal strain

The optimization of protein extraction was performed on *Arthrospira platensis* powder provided by a commercial seller (Italy), in order to guarantee the same properties of the starting biomass during all the experimental tests. All the chemicals were purchased by Sigma-Aldrich (St. Louis, MO, USA).

4.1.1.2 Protein ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) of proteins from *Artrospira platensis* biomass was carried out by an ultrasonic probe (Sonicator Vibra cell 75115, 500 Watt, Bioblock Scientific Co.) using milliQ water as solvent. The ultrasonic settings were frequency 20 kHz, 60% amplitude, and on/off pulsed ratio 5/15 s/s. The influence of solvent volume, *A. platensis* mass, and total extraction time was investigated using Box-Behnken Design for response surface methodology. The temperature was controlled by an ice bath to guarantee no denaturation of the extracted molecules. After the

extraction, the solvent containing the water-soluble components (S1) was separated from the residual *A. platensis* biomass by centrifugation at 10000 xg for 10 minutes.

4.1.1.3 Design of experiment

Box-Behnken Design for response surface methodology was employed to plan the set of experiments, in which the effects of total extraction time (t), solvent volume (V) and, mass of *A. platensis* (M) were investigated. The input variables were reported in Table 4.1., which are coded into three levels (−1, 0, +1). The central point (0,0,0) was replicated 3 times, thus obtaining a set of 15 experiments. The whole experimental plan was repeated twice.

Table 4.1. The input variables for Box-Behnken Design for response surface methodology

Variable	+1	0	-1
t (min)[a]	20	30	40
M (g)[b]	0.66	1.00	1.33
V (mL)[c]	50	75	100

[a] Extraction time, [b] *A. platensis* mass, [c] Solvent volume

Protein content in S1 was investigated as response variables. Response variables were mathematically represented as functions of the input ones by using the quadratic model reported in Equation 4.1.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j<1}^{k} \beta_{ij} X_i X_j \quad \text{(Eq 4.1.)}$$

where Y represents the response variable, X_i and X_j represent the independent variables, β_0 is a constant, while β_i, β_{ii} and β_{jj} are the coefficient of linear, quadratic, and interactive terms of the equation, respectively.

The software Design Expert (Stat-Ease, Inc., Minneapolis, United States) was employed as a tool for the experimental design and to perform the analysis of variance (ANOVA) on experimental results, assessing their statistical significance. A numerical optimization technique was used to identify the optimal values of process parameters. The desirability method available in Design Expert Software was used for the multiple response optimization, in which the overall objective function (desirability) to be maximized ranged between 0 and 1. In the objective function, the same value of importance was assigned to all the response variables.

4.1.1.4 Protein recovery

Proteins were separated and recovered from others water-soluble components (S2) by a precipitation step obtained by acidification of the solution (pH 4) with HCl. Protein pellets (P) were separated from liquid solution by centrifugation at 10000 rpm for 10 minutes and then resuspended into tris-HCl buffer (pH 8.8). A schematic diagram of the water-soluble extraction process was shown in Figure 4.1.

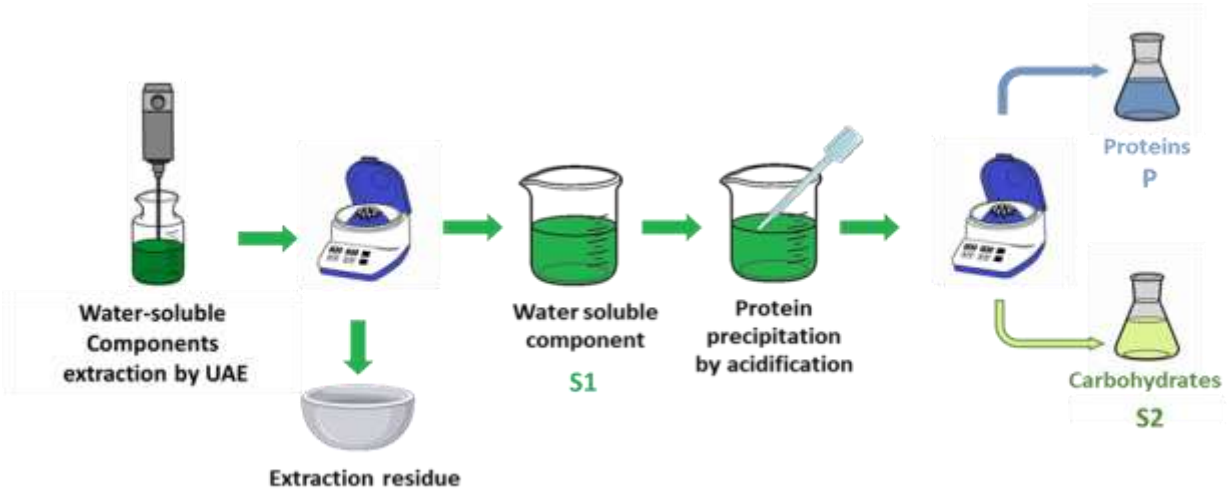

Figure 4.1. Diagram of protein extraction and recovery process

4.1.1.5 Analytical methods

Protein concentration in S1, P and S2 was evaluated by Bradford assay. Briefly, 100 µL of sample were added to 1000 µL of Bradford reagent into 2 mL cuvette [142]. The cuvettes were left 10 minutes in dark condition and the absorbance was read at 595 nm UV-spectrophotometer (Genova, Jenway, Stone, UK). The standard curve (R^2 = 0.9983) shown in equation 4.2. was produced using BSA as standard and the unknow protein concertation (PR) was expressed as g/L.

$ABS_{595} = 3.394\ PR - 0.044$ (eq 4.2.)

The Total Solids (TS) of protein fraction P and S2 obtained after UAE extraction and protein recovery process were evaluated gravimetrically by drying 10 mL of sample in an oven at 110 °C until constant weigh was reached.

The recovery of protein (η_P) fraction was calculated as ratio from the protein concentration in P fraction and S1, as reported in equation 4.3.

η_P = Protein concentration in P / Protein concentration in S1 (eq. 4.3.)

while the protein purity (v_P) was evaluate as shown in equation 4.4.

v_P = Protein concentration in P / TS of P (eq. 4.4.)

4.1.2 Results and discussion

4.1.2.1 Response surface modelling of ultrasound-assisted extraction

Box-Behnken Design was used to investigate the effect of total extraction time, *Arthrospira platensis* mass loaded and solvent volume on UAE of proteins from *A. platensis*. In Table 4.2. the values of input variables considered in the design space point and, the response variable were reported. The experiment regarding the central point (30 min, 1 g and 75 mL) was carried out in triplicate, and the total number of experiments was 15.

Table 4.2. Results and conditions of ultrasound-assisted extraction tests performed on *A. platensis* biomass according to 3^3-full factorial design.

Run	Independent variable			Response
	t (min)	M (g)	V (mL)	PR in S1 ($g/100g_{DW}$)
1	40	0.66	75	39.21
2	20	1.33	75	26.65
3	40	1.00	50	30.55
4	20	1.00	100	41.95
5	40	1.33	75	22.00
6	30	1.00	75	48.00
7	30	1.00	75	45.00
8	30	1.33	50	25.43
9	30	1.00	75	48.83
10	20	1.00	50	35.75
11	30	0.66	50	30.80
12	30	0.66	100	28.83
13	40	1.00	100	33.74
14	30	1.33	100	28.58
15	20	0.66	75	32.18

The protein concentration in fraction S1 obtained by UAE, ranged between 22 and 48.83 $g/100_{DW}$ as shown in Table 4.3. The ANOVA results for the protein concentration in S1 showed that the fitted Responded Surface Methodology model, reported in Equation 4.5. was statistically significant ($F=5.90$, $p<0.1000$) with coefficient of determination ($R^2=0.9139$). Not significant lack of fit was reported by the analysis.

$$PR = 47.28 + 3.54\,M + 1.32\,V - 12.18\,M^2 - 6.69\,V^2 \quad \text{(Eq 4.5.)}$$

In Equation 4.5. M (g) was the *A. platensis* mass loaded and, V (mL) the solvent volume. In the fitting equation the terms that resulted more relevant in the model were the quadratic term of M and V with p-value of 0.0023 and 0.0259, respectively. While the dependence of total extraction time resulted less significant as showed in Table 4.3.

Table 4.3. Analysis of variance table (Partial sum of squares- Type III) for Response Surface Quadratic Model of the output variable of protein concentration (PR) in S1.

	Coefficient estimate	Sum of Squares	Mean Squares	F-value	p-value Prob > F
Model		895.3058	99.47842	5.89964	0.0325
β_1	-1.37898	15.21279	15.21279	0.902206	0.3858
β_2	-3.54457	100.5115	100.5115	5.960911	0.0585
β_3	1.32105	13.96138	13.96138	0.82799	0.4046
β_0	-2.92098	34.12839	34.12839	2.024009	0.2141
β_{12}	-0.7529	2.267434	2.267434	0.134472	0.7288
β_{13}	1.28	6.5536	6.5536	0.388666	0.5603
β_1^2	-5.0889	95.61917	95.61917	5.670765	0.0631
β_2^2	-12.1768	547.4745	547.4745	32.46838	0.0023
β_3^2	-6.68987	165.2469	165.2469	9.800089	0.0259
Intercept	47.28				
Residual	84.30889	5	16.86178		
Lack of fit	76.18962	3	25.39654	6.25587	
Pure error	8.119267	2	4.059633		
Cor total	979.6146	14			

To better visualize the combined effect of the three independent variables on PR, the three-dimensional graphs obtained by Eq. 4.5. were reported in Figure 4.2. As confirmed by significant quadratic a linear terms of model equation, *A. platensis* mass loaded (M) and solvent volume (V) strongly influenced the protein content extracted by UAE.

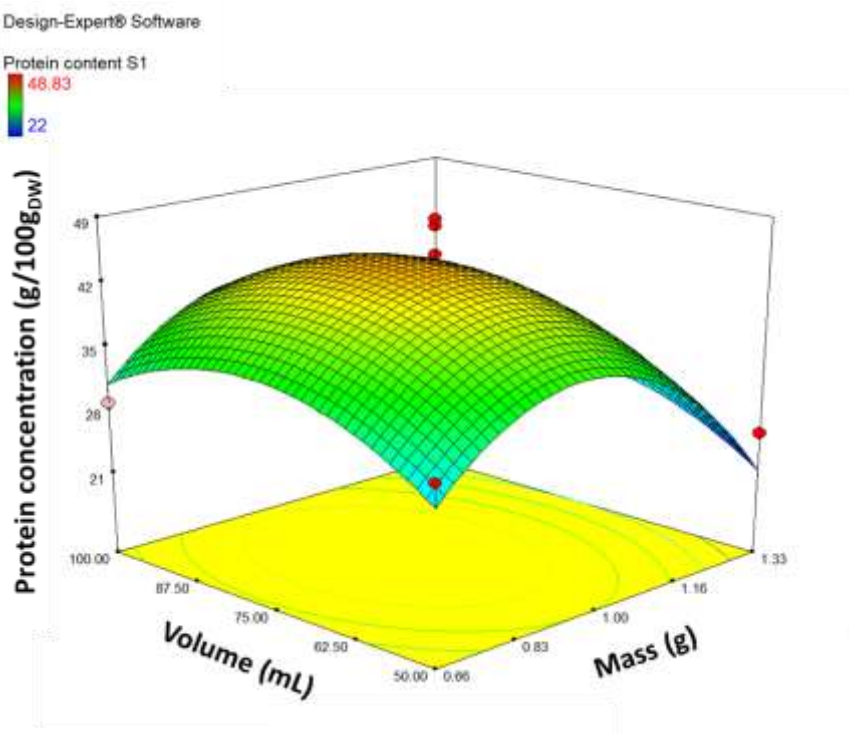

Figure 4.2. Three-dimensional surface plot of quadratic regression equation of protein concentration (PR) in S1 obtained by experimental design and response surface methodology.

4.1.2.2 Protein recovery and purity

The protein fraction was separated and recovered from the other water-soluble component (polyphenols, carbohydrate) by an acidification step. The pH of the supernatant (S1) was adjusted to a value of 4 with HCl in order to precipitate proteins.

Table 4.4. Characterization of fraction P after protein precipitation.

RUN	t (min)	M (g)	V (mL)	PR in P[a] ($g/100g_{DW}$)	TS in P[b] ($g/100g_{DW}$)	η_P[c] (%)	ν_P[d] (%)
1	40	0.66	75	31.21	54.52	79.60	57.24
2	20	1.33	75	21.52	51.23	80.75	42.01
3	40	1.00	50	28.66	48.31	93.81	59.33
4	20	1.00	100	32.19	43.52	76.72	73.96
5	40	1.33	75	20.11	30.63	91.43	65.67
6	30	1.00	75	47.00	40.28	97.92	100.00
7	30	1.00	75	42.50	40.73	94.44	100.00
8	30	1.33	50	20.11	43.63	79.10	46.10
9	30	1.00	75	47.90	40.30	98.10	100.00
10	20	1.00	50	33.00	51.32	92.31	64.30
11	30	0.66	50	20.86	58.18	67.72	35.85
12	30	0.66	100	27.02	51.58	93.73	52.39
13	40	1.00	100	32.12	46.88	95.19	68.51
14	30	1.33	100	25.15	51.29	87.99	49.03
15	20	0.66	75	30.00	58.09	93.23	51.64

[a] Protein concentration in P, [b] Total Solid in P, [c] Protein recovery, [d] Protein purity

The protein recovery (ηP) and purity (νP) were evaluated for all the testes carried out for the protein extraction optimization, as shown in Table 4.4.

The protein recovery ranges between 67.20 to 98.10%, this indicates that almost all the protein extracted by UAE was precipitated and recovered into the fraction P. While the purity of the samples was evaluated considering the total solid of the samples measured gravimetrically. As suggested by the low values the protein purity (from 35.85 to 100%), the samples contain other components that have been precipitated by HCl. The presence of the undesirable compounds could be correlated to carbohydrates and proteins extracted by water that have an isoelectric point lower than 4 that have been precipitated by HCl. Thus, the protein fraction intended for specific uses, such as food and pharmaceutical industry, may require a purification step before the use.

4.1.3 Conclusions

In this study, the ultrasound-assisted extraction of proteins from *A. platensis* was optimized varying the total extraction time, solvent volume and *A. platensis* mass.

The highest protein concentration (48.83 g/100 g) in the Supernatant 1 (S1) was obtained under the following extraction conditions: 75 mL (solvent volume), 1 g (*A. platensis* mass), 30 min (extraction time). After the protein precipitation step of supernatant S1 by acidification, it was

possible to obtain a protein recovery from 67.20 to 99 %. The fitting equation has been evaluated by ANOVA and the obtained response surfaces showed good accuracy (R2 = 0. 9139).
The statistical analysis led to the optimization of the ultrasound-assisted extraction parameters, maximizing the protein content of S1.

4.2 c-Phycocyanin extraction from Arthrospira platensis biomass

c-Phycocyanin (c-PC) is a phycobiliprotein found in the blue-green microalgae, it is generally used as a natural blue colorant in the food industry for candy and even in cosmetic production. c-PC also showed a variety of pharmacological properties, such as antioxidant, hepatoprotective and, anti-inflammatory. *Arthrospira platensis* is a protein-rich cyanobacterium, it can contain up to 70 % of proteins. Different authors observed that the c-PC is the major protein in *A. platensis*. In this study, the extraction of c-PC from *A. platensis* by UAE was performed using 1.5% of $CaCl_2$ as solvent and the purification with ammonium sulfate, and the additional protein recovery from wet c-PC extraction residue was carried out. A concentration of 9.63 ± 0.82 $mg_{c\text{-}PC}/100mg_{AP}$ was obtained. Moreover, after the purification with $(NH_4)_2SO_4$, the purity of the c-phycocyanin was compared to the crude extract. A protein-rich extract with a concentration of 12.80 ± 0.80 mgPR /100mgAp was obtained by solid/liquid extraction for 17 h.

4.2.1 Materials and methods

4.2.1.1 Chemicals and microalgae strain

Arthrospira platensis powder was provided by a commercial seller (Italy), all the chemicals were purchased by Sigma-Aldrich (St. Louis, MO, USA).

4.2.1.2 Ultrasound-assisted extraction of c-pyocyanin

The c-PC was extracted from *Arthrospira platensis* by ultrasound-assisted extraction using a of 1.5% (w/v) $CaCl_2$ solution in water as solvent. $CaCl_2$ was added to the solvent to increase the selectivity and the yield of c-PC extraction instead of the other proteins contained in *A. platensis.* The operating conditions were set considering the results obtained in the previous sections (Section 4.1) regarding the optimization of protein extraction from *A. platensis*. Briefly, the ultrasonic probe (Sonicator Vibra cell 75115, 500 Watt, Bioblock Scientific Co.) was set with an ultrasonic frequency of 20 kHz, 60% amplitude, and on/off pulsed ratio 5/15 s/s. The temperature was controlled by ice bath to guarantee the stability of the extracted c-PC. The solvent volume was 250 mL, the *A. platensis* mass 3.12 g and, total extraction time 30 min. The mass/solvent ratio was maintained constant at the best condition of protein optimization. After the extraction, the solvent containing the c-PC was separated from the extraction residue by centrifugation at 10000 rpm for 10 minutes.

4.2.1.3 Improvement of c-phycocyanin commercial grade

Ammonium sulfate was gradually added, with continuous stirring, to the c-PC crude extract to obtain a 50% (w/v) saturated solution. The resulting solution was kept for 2 hours under continuous stirring [143]. After purification, the solution was centrifuged at 1000 xg for 10 minutes. The obtained blue precipitate was dissolved into lysis buffer (7 M urea, 2 M thiourea, 1% C7BzO, 40 mMTris pH 8.7)

4.2.1.4 Protein extraction from wet c-phycocyanin extraction residue

The c-PC extraction residue was furthermore extracted using water as solvent for the recovery of proteins. The protein extraction was carried out by solid-liquid extraction and UAE on wet c-PC extraction residue in both using water as solvent.

Solid-liquid protein extraction was carried out using 250 mL of water as solvent. Two different extraction time were investigated, 2 hours (S-L2) and 17 hours (S-L17). UAE of proteins was carried out at the same extraction condition of c-PC.

4.2.1.5 Analytical methods

The c-phycocyanin content was evaluated by colorimetric method. The absorbance of the crude and purified c-PC extracts was read at 620 nm by UV spectrophotometer (Genova, Jenway, Stone, UK). The standard curve (R2 = 0.9981) showed in equation 4.6 was produced using c-phycocyanin standard.

ABS620 = 1.708 c-PC +0.013 (eq 4.6.)

The protein (PR) concentration was measured by the Bradford assay as explained previously (Section 4.1.1.5)

The c-PC purity was evaluated as ratio between the absorbance at 620 nm (ABS_{620}) characteristic of the c-phycocyanin content and the absorbance at 280 nm (ABS_{280}) representative of total proteins [141].

4.2.1.6 Polyacrylamide gel electrophoresis

Polyacrylamide dodecyl sulfate gel electrophoresis (1D SDS-PAGE) was performed on crude and purified c-PC extracts. Precast polyacrylamide gels, Nu-PAGE™ 4-12% Bis-Tris (Invitrogen, Thermofisher scientific) were used to separate proteins (size from 15 to 250 kDa). 50 µg of protein

sample were mixed with 2.5 µL of Nu-PAGE™ LDS sample buffer, 1 µL of Nu-PAGE™ reducing agent and boiled for 5 minutes in heating block. Then, samples were centrifuged in a microfuge at maximum speed for 5 minutes. Gels were run at 200 V for 50 minutes to ensure the correct proteins fractionalization. Gels were firstly washed three time with milli water, then into Coomassie blue gel satin and left overnight, and de-stained with milli water.

4.2.2 Results and discussion

4.2.2.1 Extraction of c-phycocyanin and improvement of its commercial grade

The c-phycocyanin (c-PC) was extracted from *Arthrospira platensis* dried biomass by ultrasound-assisted extraction using a solution of 1.5% of $CaCl_2$ (w/v) in water as solvent. A concentration of 9.36±0.82 $mg_{PC}/100mg_{AP}$ was obtained by UAE extraction. Figure 4.3. shows the c-PC extract powder obtained after precipitation, with its characteristic blue color. The results were comparable with that obtained by İlter et al. (2018). In this paper, a c-PC concentration from 70.46±1.25 to 102.98±1.25 was obtained by UAE varying homogenization rate (rpm)/amplitude, biomass/solvent ratio [139].

Figure 4.3. powder of c- phycocyanin obtained by UAE extraction.

The purity of c-PC plays a crucial role in commercial applications, and it is generally evaluated as the ratio between ABS_{620}/ABS_{280}. A purity higher than 4.0 is considered analytical grade, from 3.9 to 0.7 reactive grade, and purity of 0.7 is considered a food grade [143]. For c-PC purification several precipitating agents could be used, in which PEG, TCA, ethanol, acetone, and ammonium sulfate are the most commonly used. Between them, ammonium sulfate represents a good candidate for c-phycocyanin purification because is cheap, a reliable method, and also prevents denaturation of protein due to its bacteriostatic effect and low heat of solubilization. The purity of the C-PC was increased from 0.58±0.00 (crude extract) to 1.10 ±0.03 (purified extract) after precipitation with 50% saturated solution of ammonium sulfate for 2 hours of agitation. For instance, the obtained c-PC could be utilized in food applications and as reactive compounds. In

Figure 4.4. the solutions of crude and purified c-CP are shown, both presented the blue typical color of c-phycocyanin.

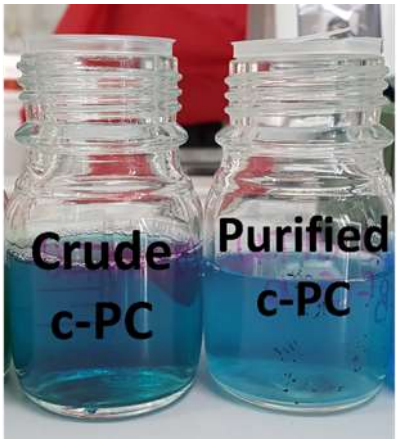

Figure 4.4. Crude and purified c-phycocyanin solutions

The crude and purified c-PC extracts were run into polyacrylamide dodecyl sulfate gel electrophoresis (1D SDS-PAGE) to determine the protein profile after the c-PC extraction and purification. In both lanes of purified (1) and crude (2) c-PC extract (Figure 4.5) it was clearly visible the strong bands associated with two subunits of α and β c-phycocyanin at 17 and 19 kDa, respectively. After the c-PC purification by ammonium sulfate, the two bands resulted stronger in comparison with the crude extract, this was conducible to the increase in c-PC concentration in the extract at the extent of total protein content.

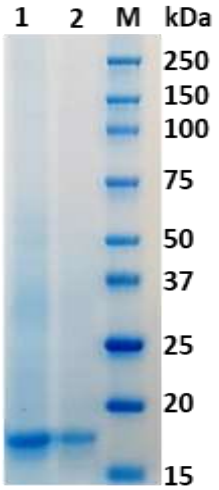

Figure 4.5. Polyacrylamide dodecyl sulfate gel electrophoresis (1D SDS-PAGE) of crude and purified c-PC extracts. 1- Purified extract, 2- Crude extract, 3- Protein marker.

4.2.2.2 Protein extraction from c-phycocyanin residue

A. platensis proteins were furthermore extracted from wet c-PC extraction residue in order to valorize the wastes of the process. Two protein extraction technologies were investigated, solid-liquid extraction, with an extraction time of 2 hours (S-L2) and 17 hours (S-L17) using 250 mL of water as solvent and ultrasound-assisted extraction performed under the same experimental condition as c-PC extraction.

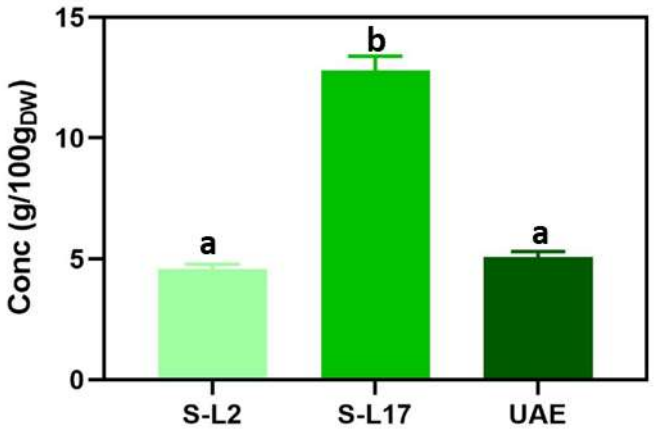

Figure 4.6. Protein concentration obtained by solid liquid and ultrasound assisted extraction. S-L2, solid-liquid extraction for 2 hours, S-L17 solid-liquid extraction for 17 hours, UAE ultrasound-assisted extraction. Different letters (a-b) refer to statistically significant differences among results within columns ($p<0.05$, ANOVA with Tuckey's HSD, post-hoc multiple comparison test).

Figure 4.6. illustrates the protein concentration obtained from wet c-PC extraction residue. A protein concentration of 4.58±0.20, 12.80±0.60, 5.09±0.22 g/100gDW was obtained for S-L2, S-L17 and, UAE, respectively. For solid liquid-extraction, the increase of extraction time from 2 to 17 hours led to an increase in protein content more than double, while, after UAE the obtained protein concentration was comparable with S-L2. Considering the results obtained from the extraction of the direct protein from *A. platensis* (section 4.1) the protein concentration from c-PC was lower. This could be attributed to the previous c-PC extraction a c-PC purification step was needed to separate the other undesired proteins. Moreover, increasing biomass/solvent ratio may

improve the diffusivity of solvent into the cell and increase extracts concentration, but an excess of solvent has been reported to absorb cavitation energy and led to a lower extraction yield [144].

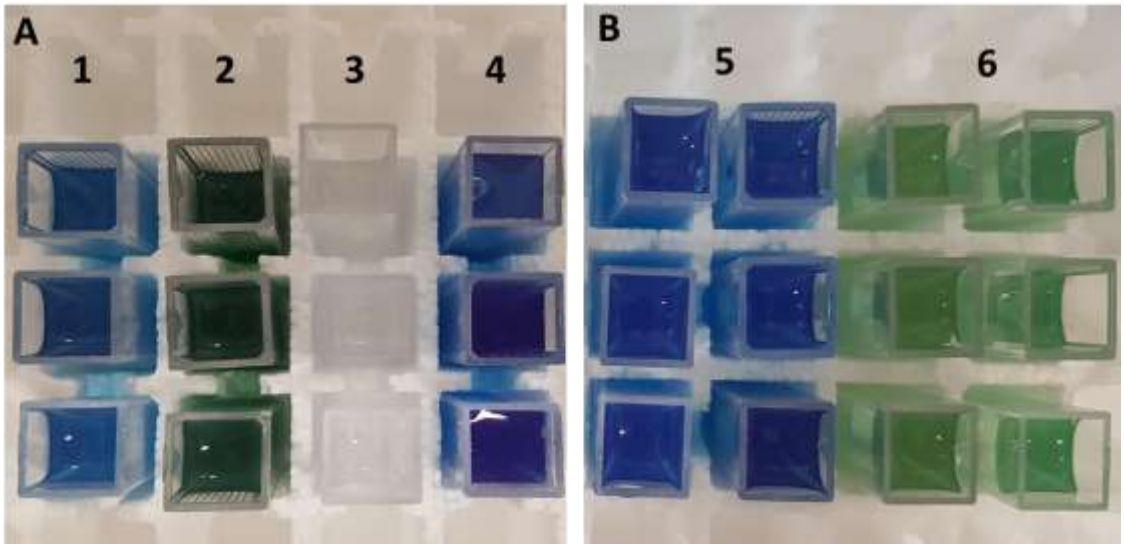

Figure 4.7. Colors of the fractions obtained by the extraction process. A- solid-liquid extraction (17 hour), 1 purified c-PC, 2 protein, 3 supernatant of c-PC purification, 4 crude c-PC, B- ultrasound-assisted extraction, 5 crude c-PC, 6 proteins.

In Figure 4.7. one can see the different fractions obtained c-PC extraction by UAE, and the furthermore protein extraction by solid-liquid extraction (figure 4.7A) and UAE (figure 4.7B). Set 4, 5 represented the c-PC extracted by UAE, set 1 the c-PC after ammonium sulfate purification and, lane 3 the supernatant (containing proteins) after c-PC precipitation. Therefore, the protein fraction obtained by solid-liquid extraction (set 2) was darker compared with UAE (set 6), confirming the results previously discussed.

4.2.3 Conclusions

In this study, the extraction of c-Phycocyanin from *A. platensis* by ultrasound-assisted extraction and the furthermore protein extraction from wet c-phycocyanin extraction residue was investigated. c-Phycocyanin was extracted using 1.5% of $CaCl_2$ as solvent and, a concentration of 9.63 ± 0.82 $mg_{c\text{-}PC}$ /100mg_{Ap} was obtained. Moreover, the c-phycocyanin extracted was purified with $(NH_4)_2SO_4$, the obtained c-phycocyanin was suitable for food applications and, as reactive compound. Moreover, in order to study the possible application of the zero-waste strategy, a

protein-rich extract with a concentration of 12.80 ± 0.80 mg_{PR} /100mg_{Ap} was obtained by solid/liquid extraction for 17 h.

4.3 Protein extraction from co-culture biomass growth in WWW

In this section, the biomass obtained after the wastewater treatment in the different microalgae growth configurations, multitubular photobioreactor, open pond and, column photobioreactor (studied in chapter 2), was extracted by UAE using water as solvent to recover the protein fraction. The differences in term of protein content, protein expression and, cell structure were investigated. Furthermore, the differences of cell resistance to UAE were evaluated in terms of cell rupture and particle size distribution.

This study was carried out in collaboration with The University of Sydney and The University of Technology Sydney under the supervision of Professors Fariba Dehghani and Peter Ralph.

4.3.1 Materials and methods

4.3.1.1 Microalgae strain and culture condition

The microalgae co-culture biomass was obtained from the winery wastewater treatment in the different growth system configurations, multitubular photobioreactor (MTP), open pond (OP) and, column photobioreactor (CP), as reported in Chapter 2, Section 2.3.

4.3.1.2 Ultrasound-assisted extraction (UAE) of water-soluble components

The co-culture biomass obtained after winery wastewater treatment was extracted using water as solvent by ultrasound-assisted extraction. The extraction solvent/mass ratio and ultrasonic power were selected according to our previous study on protein extraction optimization using Box-Behnken Design software (Section 4.1). Briefly, 130 mg of sample was extracted with 10 mL of milli water by the ultrasonic probe (UP400St ultrasonic processor, Hielscher, Germany) at power 30 W and amplitude 100%, at different extraction time (5, 7.5, 10, 30, 60, 90, 120 minutes). The temperature was controlled by an ice bath to guarantee no denaturation of the extracted molecules. After the extraction, solvent (S1) was separated from residual co-culture biomass by centrifugation at 10000 xg for 10 minutes. Proteins were recovered from other water-soluble components (S2) by a precipitation step obtained by acidification of the solution (pH 4) by HCl. Protein pellets (P) were separated from the liquid solution by centrifugation at 10000 xg for 10 minutes and then resuspended into tris-HCl buffer (pH 8.8). The schematic diagram of the water-soluble extraction process was reported in section 4.1.1.4. The water-soluble extraction was performed on the

samples at different extraction times to study the differences in intracell components release by sonication.

4.3.1.2.1 Water-soluble components quantification

Protein concentration in PR and S1 was evaluated by Bradford assay. Briefly, 5 µL of the sample were added to 250 µL of Bradford reagent into a 96-well plate[142]. The plate was shacked for 1 minute and left 10 minutes in dark condition. The absorbance was read at 595 nm by a microplate reader (Spectra max plate reader, Bio strategy, New Zealand). The standard curve (R^2 = 0.9983) shown in equation 4.7 was produced using BSA as standard and the unknown protein concertation (PR) was expressed as g/L.

$$ABS_{595} = 0.3054\ PR - 0.031 \quad \text{(eq 4.7)}$$

Water-soluble carbohydrate concentration in S2 was evaluated by phenol-sulfuric acid method. 150 µL of the sample were put into a 96-well plate with 450 µL of concentrate H_2SO_4 and 90 µL of phenol solution (5% w/v) [145]. The plate was left for 10 minutes and read at 490 nm by a microplate reader (Spectra max plate reader, Bio strategy, New Zealand). The calibration curve was prepared with mannose solution the unknown carbohydrate concentration (CA) concentration estimated by the equation 4.8 (R^2 = 0.9955) and expressed as g/L.

$$ABS_{490} = 0.2268\ CA - 0.022 \quad \text{(eq 4.8)}$$

4.3.1.3 Protein characterization

For protein characterization, 130 mg of sample were extracted with 1 mL Trizol (Roche, Switzerland) by sonication with a Digital Sonifier S-450D (Branson, Danbury, CT (intensity at 40%)) for 30-second intervals (3 min in total) to lyse the cells. Next, 300µL of chloroform was added to the cell lysate and vigorously shaken for 15 s. The sample was left to stand at room temperature for 5 min before being centrifuged at 12,000 ×g for 15 min at 4 °C. The top colorless layer was removed, and 300µL of ethanol was added to resuspend the bottom green layer. The sample was centrifuged at 2000 xg for 5 min at 4 °C and the supernatant was transferred to a clean centrifugation tube before adding 1.5 mL of isopropanol. For precipitation of proteins, the mixture was allowed to stand for 30 min at room temperature, then centrifuged at 14,000 xg for 10 min at 4 °C. The cell pellet obtained was washed with 95% ethanol. To solubilize the protein pellet,

200µL of lysis buffer (7 M urea, 2 M thiourea, 1% C7BzO, 40 mMTris pH 8.7) was added to the pellet.

4.3.1.3.1 1D-SDS PAGE

1D-SDS PAGE was performed to separate the extracted protein by their size (apparent molecular weight). 12% of Acrylamyde separating gels were casted using Bio-rad glass plates. Briefly, 1.25 mL of 1.5 M Tris-HCl pH 8.8., 1.5 mL of 30% acrylamide solution, 0.65 mL of glycerol, l.55 mL of H_2O, 25 µL of 20 % SDS solution, 5 µL of TEMED and 25 µL of 10% (w/v) ammonium persulfate solution (APS) were mixed and gently poured into glass plates. 30 µg of protein sample were mixed with 15 µL of Lamelli buffer and boiled for 5 minutes in heating block. Then, samples were centrifuged at maximum speed for 5 minutes. Gels were run at 150 V for 50 minutes to ensure the correct proteins fractionalization. Gels were firstly poured into Fix solution (40% methanol and 10% acetic acid) for 30 minutes, then into Coomassie blue gel satin and left overnight. Gels were de-stained with milliQ water and then scanned.

4.3.1.4 2D-SDS Page

Two-dimensional electrophoresis was carried out with IPG strips (11 cm, pH 3-10). The IGP strips were rehydrated with 300 µg of proteins and separated by IEF in a Multiphor II unit untill of 100 KV. Strips were then equilibrated and transferred to 10% SDS-PAGE gels (casted as explained for 1D-SDS Page) for the second dimension. Electrophoresis was carried out at 200 V for 1 hour. The gels were firstly poured into Fix solution (40% methanol and 10% acetic acid) for 30 minutes, then into Coomassie blue gel satin and left overnight. Gels were de-stained with milli water and then scanned.

4.3.1.5 Protein identification and quantification by LC/MS/MS

Samples were reduced with 5 mM tributylphosphine,and alkylated with 20 mM acrylamide monomers prior to being digested with trypsin (1:100 ratio) at 37 °C overnight. SiliaPrep XHLB columns, were then used for solid phase extraction (SPE) of peptides. 1 mL of 100% acetonitrile (ACN) was added, followed by 1 mL of 2% ACN, 0.2% trifluoroacetic acid (TFA) to equilibrate the SPE column. Sample was loaded into the column, and then 1 mL of 2% ACN, 0.2% TFA added. 400µL of 75%ACN/ 0.2% TFA load was then added to elute peptides into a clean 2 mL microcentrifuge tube. Samples were placed in a vacuum concentrator to yield a volume of 100µL and evaporate off any ACN.

After the digestion, the reduced and alkylated sample were load to LC/MS/MS. This method was used to detect and identify differentially expressed peptides, and by inference, proteins. This involved analyzing peptide extracts (10 µg) using a Sciex 5600 Triple TOF liquid chromatography tandem mass spectrometry (LC/MS/MS) system. Data were searched by Mascot (version 2.4.1; Matrix Science, London, UK) and searched against by the MSPnr100 database and a database of common contaminants. with the following parameter settings: Peptide scores were deemed significant according to the E-valuen 0.05 and protein matches identified from sequenced genomes of *Arthrospira platensis* and *Chlorella vulgaris*.

4.3.1.6 Co-culture cell rupture by UAE and particle size distribution

The co-culture cell rupture was determined in order to evaluate the difference in terms of cell morphology due to the different growth system and WWW treatment. The cell suspension collected at different extraction time (5, 7.5, 10, 30, 60, 90, 120 minutes) were appropriately diluted for microscopic imaging, placed on a standard Neubauer hemocytometer (10 µl/suspension), left to settle for 15 min and observed under the light microscope (Nikon Eclipse 100) for cell rupture analysis. Eight images of different 0.04 mm^2 hemocytometer grids were captured for each suspension.

The number of particles (cell), the area occupied by the intact cells in each image were evaluated using an automated image algorithm (ImageJ Software). In brief, the algorithm used an in-built size (1-200000) and circularity exclusion (0.85-1) algorithm to distinguish cells from cell debris and other agglomerates, thus enabling the calculation of the apparent area that the cells occupied within the image. The cell count at the different extraction times were normalized by the number of cells counted before the UAE. Moreover, the cells diameter was evaluated by algorithm mentioned above, in order to plot the granulometric particle size distribution.

4.3.2 Results and discussions

4.3.2.1 Water-soluble component concentration

The co-culture biomass obtained after winery wastewater treatment in the different growth configurations was submitted to water-soluble components extraction by UAE to study the difference in cell composition, cell morphology, and response to sonication power. The results obtained from the extraction of the biomass growth in WWW in MTP, OP, and CP were compared with a control run (CTR) using co-culture biomass obtained by growing it in BBM medium. The

water-soluble extraction was carried out at different extraction times, from 5 to 120 minutes to study the influence of extraction time on intracellular components release.

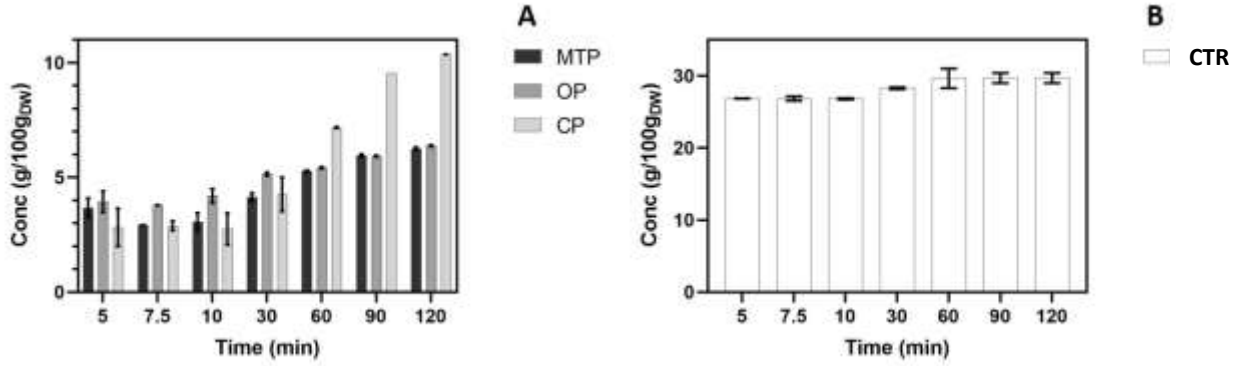

Figure 4.8. Protein concentration obtained by UAE from co-culture biomass in function of extraction time. A-co-culture growth in winery wastewater, B- control run. Multitubular photobioreactor (MTP), Tubular photobioreactor (CP) and, Open pond (OP).

As shown in Figure 4.8, the protein concentration obtained from the CTR was higher compared to that growth in WWW. In fact, a protein concentration of about 25% was obtained since the first 5 minutes of extraction, more than four times higher than that growth in WWW. Moreover, an increase in protein concentration release could be observed from co-culture biomass grown in WWW increasing the extraction time. The protein concentration from the biomass was increased from 3.38 to 6.20 g/100g_{DW} in MTP, from 3.93 to 7.00 g/100g_{DW} and, from 2.82 to 10.95 in CP increasing the extraction time from 5 to 120 minutes.

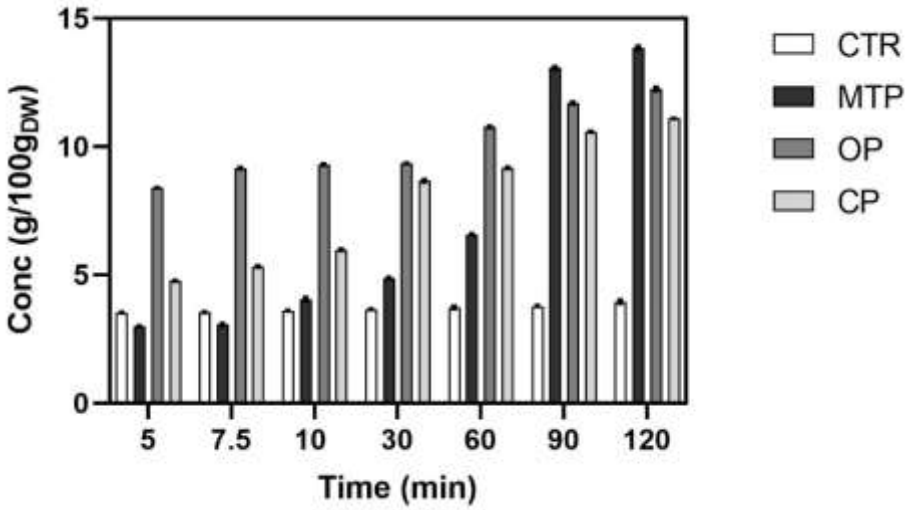

Figure 4.9. Carbohydrate concentration obtained by UAE of co-culture biomass in function of extraction time. Control run (CTR) Multitubular photobioreactor (MTP), Tubular photobioreactor (CP) and, Open pond (OP).

The same behavior in terms of protein release as function of extraction time could be observed for carbohydrate (Figure 4.9). Almost a constant concentration of carbohydrate (3.5 g/100g_{DW}) was obtained from the CTR run at all the extraction times. Instead, an increase in carbohydrate concentration was observed from biomass growth in WWW by increasing the extraction time. A final carbohydrate concentration of 13.8, 12.2 and, 11.0 g/100g_{DW} was obtained from MTP, OP and, CP, respectively.

In general microalgae and cyanobacteria under stress condition tend to accumulate mainly lipid instead of protein when submitted to stress condition [99,146]. Wang et al. (2018) observed a reduction of protein content in *Chlorella pyrenoidosa* from 40.02±1.1 to 22.7 ±1.3 % using tofu whey wastewater with 10 g/L glucose as growth medium instead of regular green microalgae conventional medium (BG-11) [147]. Apandi et al. (2017) grew *Scenedesmus sp.* in presence of different concentration of wet market wastewater, they observed reduction of protein concentration from 50.72 ±6.4 to 37.3 ±1.0 % increasing the wet market wastewater into the medium from 10 to 25% [148].

4.3.2.2 Protein fractions characterization

To evaluate the quantitative estimation of the obtained protein expression by growing the co-culture in the presence of winery wastewater and different photobioreactor configuration 1D-SDS PAGE and 2D-SDS PAGE were carried out.

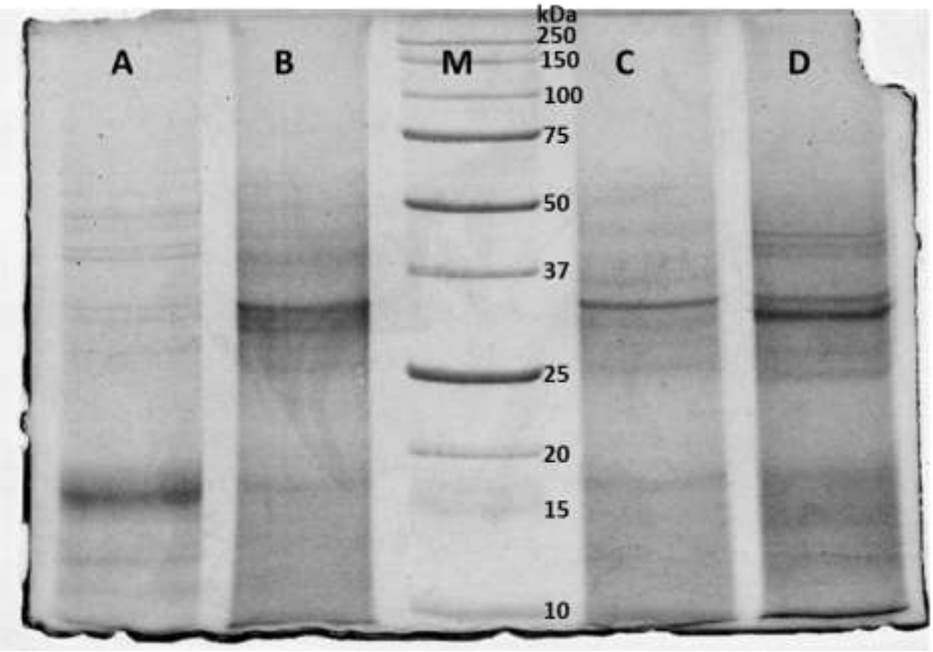

Figure 4.10. 1D-SDS Page: M- Marker, A-Control, B- Co-culture growth in multitubular photobioreactor, C- Co-culture growth in open pond, D- Co-culture growth in column photobioreactor.

Differences in terms of protein composition (Figure 4.10) could be observed for the co-culture grown autotrophically (lane A) and grown in presence of winery wastewater (lanes B, C, and D). In lane A a more expressed band in molecular weight from 15 to 20 kDa was mainly identified. Furthermore, other bands were identified in a molecular weight range from 25 to 50 kDa and from 10 to 15 kDa. It is well known that the most abundant pigment in *A. platensis* is phycocyanin, representing around 15%. The c-PC consists of two subunits, α, and β with a molecular weight of 15-19 kDa and 18 to 24 kDa, respectively [149]. The same bands could be observed for the co-culture growth in WWW but in a lower concentration. The most abundant expressed proteins from the co-culture grown in WWW were observed in the molecular weight region from 25 to 37 kDa. In lanes B and D two clear strong bands were observed, while in lane C only one. Moreover, other bands were observed in the molecular weight region from 25 to 50 kDa, and from 10 to 20 kDa. Similar results in terms of protein expression from pure *A. platensis* were reported by Seghiri et al. [150], and from *C. vulgaris* by Ursu et al. [151] and Sharma et al. [152]. Moreover, the variability in band intensity could be attributed to an effect of applied treatment on the expression of regulatory genes. These results suggest that proteins were affected by environmental conditions not only quantitatively, as previously discussed but also qualitatively. Khairy et al. also observed

a difference in protein expression when growing *Chlorella vulgaris* under autotrophic and heterotrophic conditions [153].

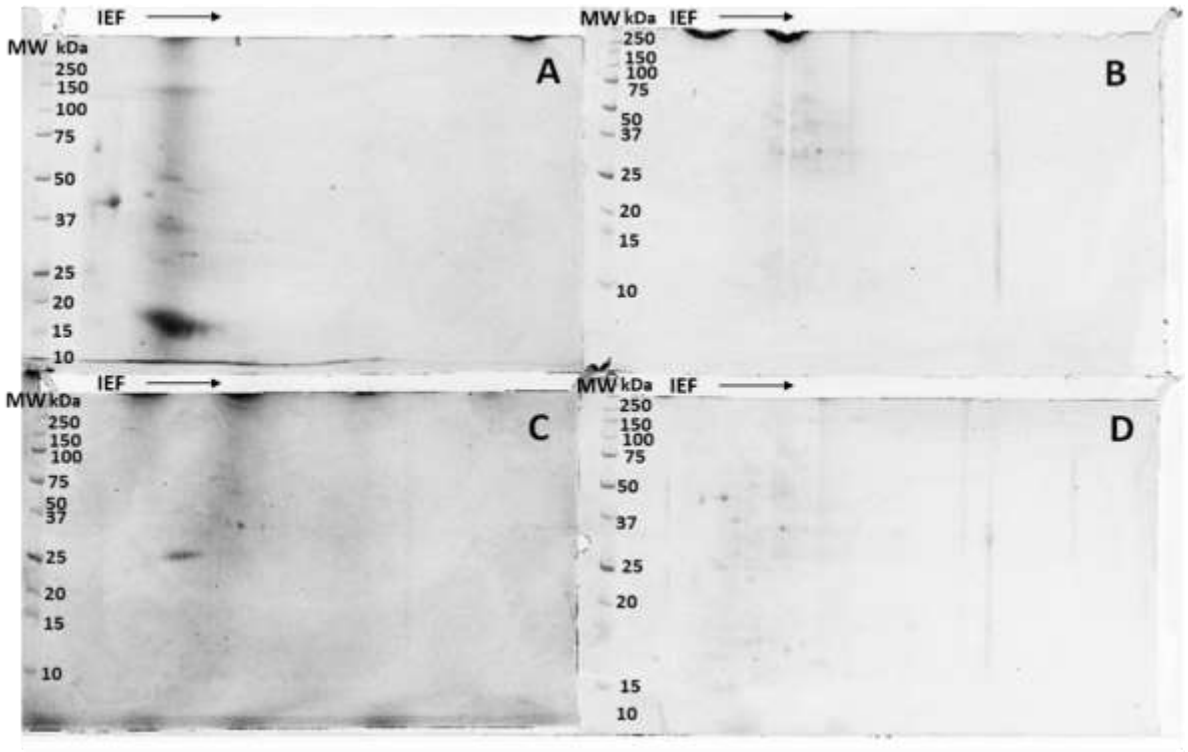

Figure 4.11. 2D-SDS Page: M- Marker, A-Control, B- Co-culture growth in multitubular photobioreactor, C- Co-culture growth in open pond, D- Co-culture growth in column photobioreactor.

The 2-DE profile of different sample proteins (Figure 4.11) confirmed was already discussed for 1D-SDS page. Differences in terms of protein composition profile were observed in the different samples. In gel A, the major proteins were separated at pH around 4, and a big spot at molecular weight from 15 to 20 kDa was observed. In gel B, two groups of proteins have been identified: the main group in pH range of 5-6 and a molecular weight from 25 to 50 kDa, and a minor group at pH around 9. In gel C a main group was observed at pH around 5, while gel D showed the same protein profile of gel B. The differences in terms of protein profile of the different samples could be attributed to the different co-culture metabolism during the winery wastewater treatment. Moreover, as observed in the previous chapter the co-culture grown differently in the investigated growth system configurations obtaining differences in terms of biomass concentration and

productivity. In this contest, it is well known that the microalgae cell composition is strongly influenced by the growth condition and the culture media in which they are grown.

4.3.2.3 Cell rupture and particle size distribution

Cell rupture of microalgae grown in winery wastewater in the different growth systems (MTP, OP and, CP) was modeled as a function of extraction time, and the decay profiles were compared to the control run (growth in BBM) (Figure 4.12). The cell rupture by UAE was determined in terms of cell counting using a standard Neubauer hemocytometer and the collected images were analyzed using an automated image algorithm (ImageJ Software).

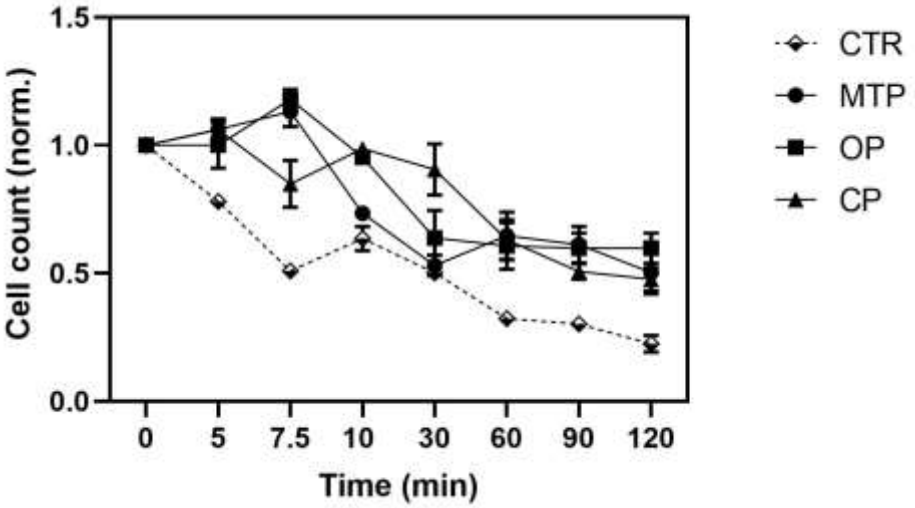

Figure 4.12. Cell rupture by UAE at different extraction times. Control run (CTR) Multitubular photobioreactor (MTP), Tubular photobioreactor (CP) and, Open pond (OP).

The cell count of the control run is quickly reduced up to 50% in the first 7.5 minutes of water-soluble extraction, then a continuous slower reduction of cell count was observed until 75% at 120 minutes. Instead, the cell count trend obtained by the co-culture grown in WWW followed a different trend. Before the 7.5 minutes of extraction a drastic reduction of cell count occurred, then after 10 minutes of extraction, a slow reduction was observed for all the samples, until reduction values around 50, 40, and 52 % for MTP, OP, and CP respectively. These results could explain the different behaviors in water-soluble components release as function of extraction time discussed in the previous section. In fact, regarding the biomass obtained from WWW treatment an increase of the extraction time led to the reduction of cell count corresponding to an increase in

the release of intracellular components. On the other hand, for the biomass grown in BBM, the quick reduction of cell count corresponded to faster release of the intracellular components. While, for the biomass growth in BBM, the quick reduction of cell count corresponded to faster release of the intracellular components. The cell area and the correspondent cell diameter were evaluated from the collected images. The collected values of cell diameter were used to analyze the particle size distribution of the different samples obtained at different extraction times.

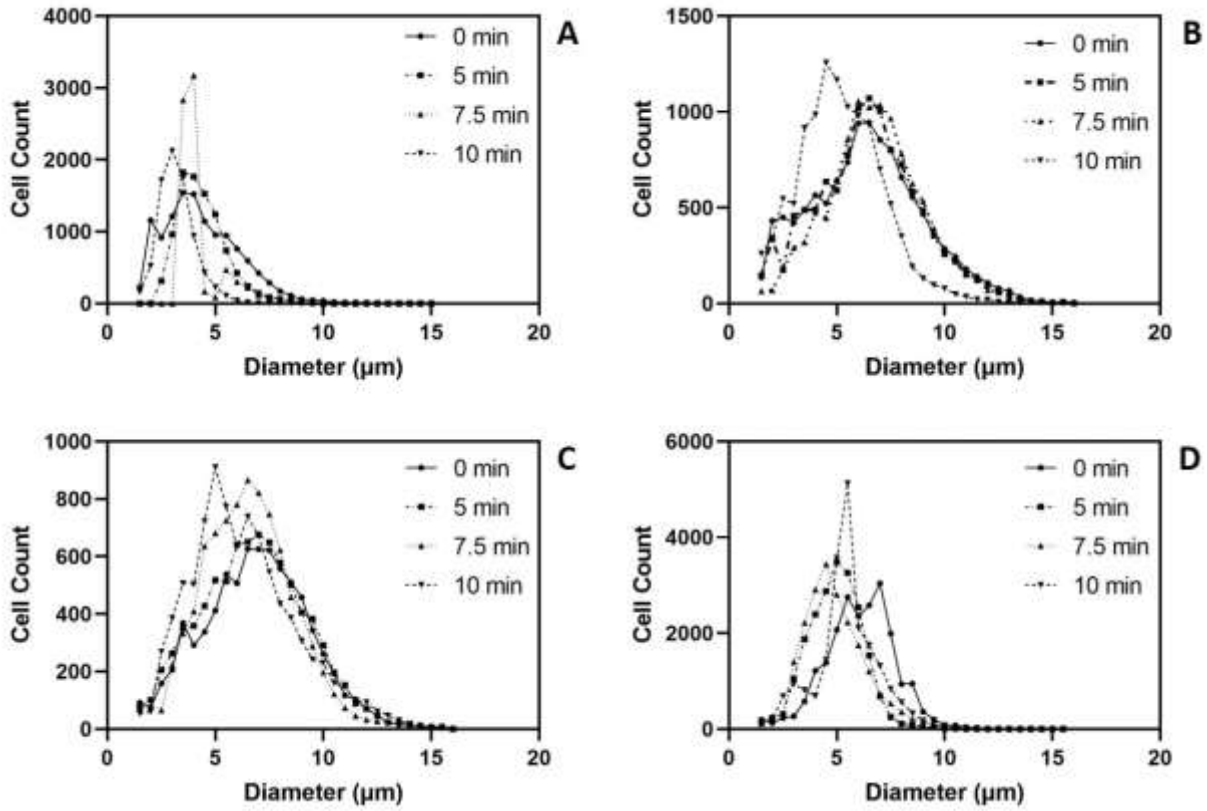

Figure 4.13. Particle size distribution (PSD) of A- Control, growth in WWW in B- MTP, C- OP and, D-CP at different water-soluble extraction time (0, 5, 7.5, 10 min).

The particle size distributions (PSD) of the untreated biomasses and after 5, 7.5 and, 10 minutes of UAE extraction were shown in Figure 4.13. A decrease in particle size after UAE extraction was observed for all the biomasses by increasing the extraction time. Moreover, the cell diameter of co-culture growth in BBM was lower compared with that grown in WWW. In fact, a cell diameter lower than 4 µm was observed for the greater part of the cells grown in BBM, while a cell diameter of about 7 µm was calculated for the biomass grown in WWW and in all the reactor configurations. Yap et al. (2016) studied the influence of nitrogen deprivation on cell size, cell

strength and resistance to mechanical disruption. They observed that submitting *Nannochloropsis sp.* and *Chlorococcum sp.* to nitrogen stress condition the cell diameter was increased by more than 25%. Also, an increase of cell wall thickness was observed by nitrogen deprivation. Moreover, they evaluated the Young module of the samples by AFM, and observed an increase in this parameter by more than 30% after nitrogen deprivation [154]. The increase of cell wall thickness and Young module of cells growth under stress condition could explain the more difficult release of water-soluble components by sonication of the samples obtained after WWW treatment.

4.3.3 Conclusion

Protein extraction was carried out on the biomass obtained after WWW treatment in the three different growth systems studied (multitubular photobioreactor, open pond, column photobioreactor). The differences in terms of protein content, protein expression, cell structure were investigated. Furthermore, the differences of cell resistance to UAE were investigated in terms of cell rupture and particle size distribution. Winery wastewater treatment by co-culture affected strongly intracellular components composition, cell morphology, and resistivity to cell rupture. The protein concentration after WWW was lower compared to the control run. Moreover, differences in terms of protein expression were observed. The co-culture cells after WWW resulted more resistive to rupture by UAE and with higher diameter.

5 Valorization of exhausted co-culture biomass by pyrolysis process

Pyrolysis is a thermochemical process that can efficiently recover most of the energy available in biomass, in which chemicals, in the absence of oxygen, are converted into bio-oil, biogas, and bio-char [59]. The pyrolysis process is characterized by complex mechanisms, in which decarboxylation, dehydration, cracking, dehydrogenation, and rearrangement reactions take place. Pyrolysis bio-oil is generally composed of a wide range of different compounds including hydrocarbons, acids, alcohols, polyaromatics, nitrogenated compounds, indole, and carbonyls, with molecular weight from 18 to 5000 g/mol [13-14]. The presence of oxygenated and nitrogenated compounds in the bio-oil leads to few undesirable properties such as low heating value and high viscosity, which do not allow miscibility with fossil fuels[60]. Some studies have shown that pyrolysis bio-oils from microalgae have better properties than those from lignocellulosic biomass, being more stable and having higher heating value and lower oxygen content[159].

In this chapter, the thermal pyrolysis of co-culture (*Chlorella vulgaris* and *Arthrospira platensis*) biomass obtained after winery wastewater treatment in membrane photobioreactor (chapter 3) in comparison with co-culture grown in Bold Basal's medium and pure *Arthrospira platensis* was investigated. The influence of reaction time and temperature on product yield and composition were studied.

5.1 Material and Methods

5.1.1 Materials

Co-culture of *Arthrospira platensis* and *Chlorella vulgaris* biomass was grown in membrane photobioreactor configuration (Chapter 3). Briefly, co-culture was grown in winery wastewater (WWW) treatment and, in Bold Basal's Medium (BBM, control run), the obtained biomass (MWW and MIX, respectively) was used for thermal pyrolysis treatment. *Arthrospira platensis* (AP) was grown in a tubular photobioreactor in autotrophic metabolism (chapter 2, section 2.3.1.5).

5.1.2 Biomass characterization

Moisture and ash contents of AP, MIX, and MWW biomasses were quantified according to AOAC methods (AOAC, 2000). While the calorific value of the biomasses was also determined with a

calorimetric bomb (C200, IKA, Staufen, Germany). The elemental composition of AP, MIX, and MWW was performed with a CHNS-O elemental analyzer (FLASH EA1112, ThermoQuest, Cleveland, USA). Carbon, hydrogen, nitrogen and sulfur percentages were evaluated directly from the sample combustion (950 °C), while the oxygen amount was evaluated theoretically considering ash and moisture content. Lipids were extracted and quantified as described in the previous sections (section 2.1.1.3 for AP and, section 3.1.4 for MIX and MWW), while protein content was evaluated using the nitrogen to a protein conversion factor of 6.25 according to Yamaguchi (1992) [160].

Biomasses were also characterized by Fourier Transform Infrared Spectroscopy (FTIR) using a Nicolet 380 FT-IR Spectrometer (Thermo Scientific, Madison, WA, USA). All the spectra were elaborated using the Omnic Lite Software (Thermo Electron Corporation, Madison, WA, USA).

5.1.3 Pyrolysis reaction system

AP, MIX and, MWW were used as raw materials for thermal pyrolysis, which was performed in a tubular quartz reactor connected to a condenser able to separate the liquid from gaseous products (Figure 5.1.). Briefly, about 10 g of dryed sample were charged into the reactor, and then the system was purged with nitrogen. At first, the differences in terms of product yields and composition in the function of the different biomasses were investigated. The reactor was put into an oven (Carbolite, MTF 10/25/130, Pocklington, UK), and the reaction temperature was set at 450 °C for 1 h, according to our previous works [118,138]. Then, the influence of reaction temperature was investigated on thermal pyrolysis of MWW biomass for 1 h, at 400-425-450-500 °C. At least, the influence of reaction time was studied performing the process on MWW biomass at 400 and 425 °C for 1 and 2h.

The reaction system was provided with an integrated condenser for the separation of reaction vapors. Incondensable gases (RG) were collected in a latex balloon, while the liquid (L), was collected in a flask. After pyrolysis, the solid residue present in the reactor was collected and washed with acetone to separate the liquid residue (LR) from the solid residue (SR).

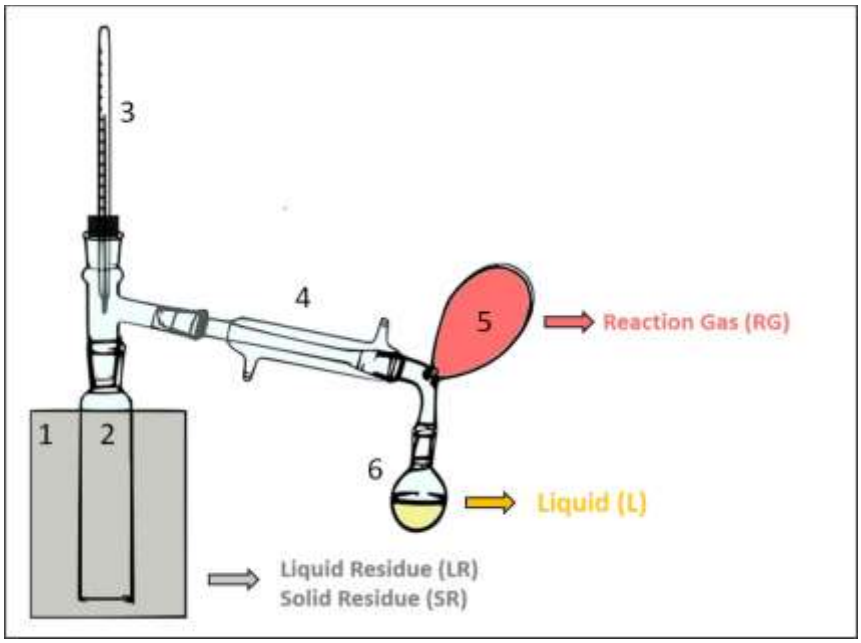

Figure 5.1. Schematic catalytic pyrolysis setup. 1-oven, 2-quartz tubular reactor, 3-thermometer, 4-condenser, 5-latex balloon, 6-flask.

5.1.4 Pyrolysis products characterization

Reaction products of AP, MIX and, MWW thermal pyrolysis (RG, L, LR and, SR) were analyzed by Fourier Transform Infrared Spectroscopy (FTIR). To analyze solid samples, samples and KBr were mixed (1:50 w/w) and pressed, while liquid samples were deposited on a KBr pressed disk. Moreover, L samples were diluted with $CHCl_3$ up to a ratio of 1:10 (v/v) and injected in a gas chromatography-mass spectrometer (GC-MS), model Focus-ISQ (Thermo Scientific, Milan, Italy). Results were expressed as percentages of the areas of peaks detected for the individual compounds with respect to the total peak area.

5.2 Results and discussions

5.2.1 Biomass characterization

The biomasses used for thermal pyrolysis treatment were characterized in terms of moisture content, calorific value, and elemental composition as showed in Table 5.1. The moisture content of MWW (3.70 $g/100g_{DB}$) was significantly lower compared with AP and MIX (around 8 $g/100g_{DB}$), this could be attributed to the different techniques used for drying the biomass. AP and MIX were dried by freeze dryer, while MWW by an oven. Moreover, the elemental composition of the biomass is quite different. The elemental composition of co-culture grown in both WWW and BBM showed lower content of carbon and nitrogen and higher content of oxygen compared

to AP. This is due to the presence of *C. vulgaris*, in fact, the elemental composition of MIX and MWW is more similar to that of *C. vulgaris*. An example of *C. vulgaris* elemental composition was reported by Adamkis et al. (2018), they observed content of carbon around 46.66%, hydrogen 6.58%, nitrogen 6.12% and, oxygen 40.64% [161].

Table 5.1. Quantification of components of biomasses, AP pure *Artrospira platensis*, MIX co-culture of *Chlorella vulgaris* and *Arthrospira platensis* growth in BBM and, MWW *Chlorella vulgaris* and *Arthrospira platensis* growth in WWW.

	AP	MIX	MWW
Moisture content (g/100g_{DB})	8.00±0.00	8.20±0.00	3.70±0.00
Calorific value (kJ/g)	19.70±0.00	17.53±0.00	20.00±0.00
C (g/100g_{DB})	53.01±1.02	38.43±1.08	39.18±0.04
H (g/100g_{DB})	7.86±0.12	2.93±0.43	4.47±0.06
N (g/100g_{DB})	11.19±0.58	6.30±0.30	4.52±0.14
S (g/100g_{DB})	0.18±0.00	n.d.[a]	n.d.[a]
O (g/100g_{DB})	27.76*	46.36*	46.82*
Lipid (g/100g_{DB})	12.70±3.90	11.00±1.11	27.80±1.14
Protein (g/100g_{DB})	69.93±3.62	39.37±1.87	28.25±0.37

[a]Not determined. * Data were theoretically obtained.

In Figure 5.2 we reported the FTIR spectra of the three biomasses.

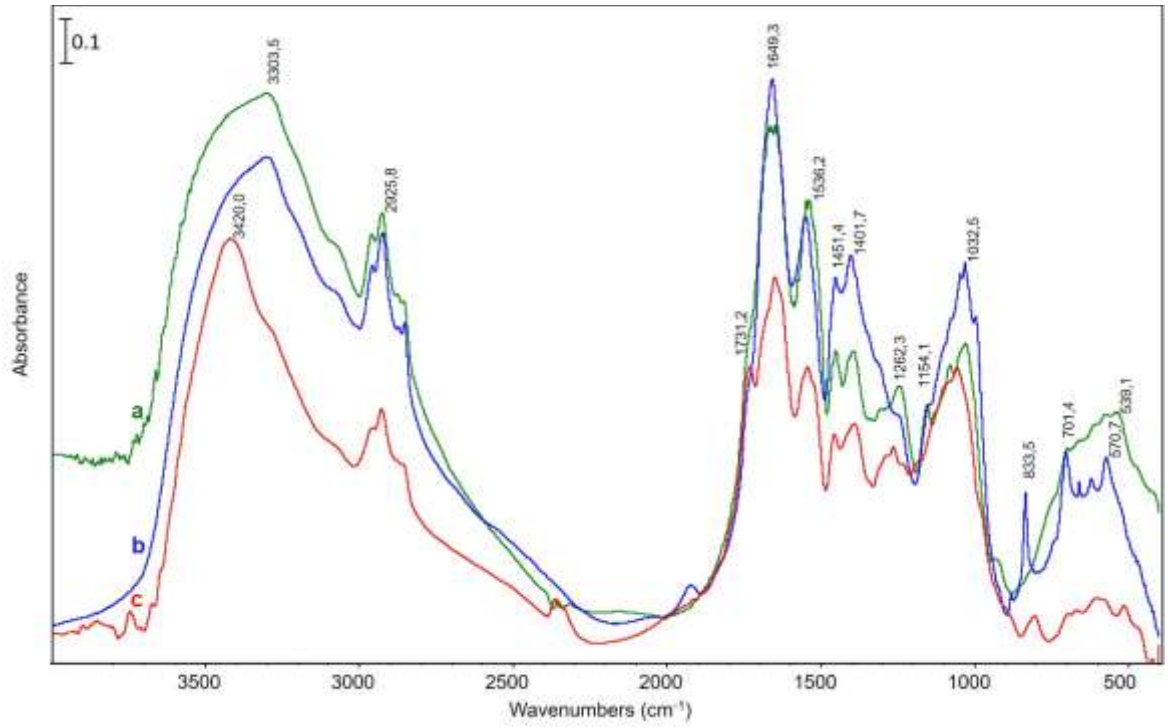

Figure 5.2. FTIR spectra of a) *A. platensis* biomass, b) co-culture growth in BBM and c) co-culture growth in WWW.

The FTIR of the three biomasses showed almost the same characteristic bands. All the investigated biomasses are made mainly by carbohydrates, proteins, and lipids and even if their proportion in the biomasses the corresponding FTIR bands are the same. In the frequency region between 3500 to 3300 cm^{-1} the band characteristic of N-H and O-H stretching was clearly visible, which points out the presence of proteins, lipids, phenolics, and alcohols. On the other hand, the region between 2960 and 2850 cm^{-1} corresponds to valence vibration of C-H, in particular at 2959 cm^{-1} the asymmetrical stretching and at 2875 cm^{-1} the symmetrical stretching of $-CH_3$ bonds and, at 2925 and 2862 cm^{-1} the asymmetrical and symmetrical stretching of $-CH_2$ bonds, respectively. The band at 1731 cm^{-1} was attributed to C=O stretching, this band is presented only in MWW spectrum and may be attributed to the higher amount of lipids in the biomass. The band at 1469 cm^{-1} was attributed to N-H bending vibration of amine I. The band at 1536 cm^{-1} corresponds to the aromatic stretching of C=C bond, while those at 1451, 1401 and, 1154 cm^{-1} to N-C bond of amide III. Moreover, the bands at 1262 and 1032 cm^{-1} were correlated to the C (O)-O stretching vibration and -OH in plane vibration. The region between 970 and 920 cm^{-1} and from 780 to 700 cm^{-1} were attributed to trans and cis =C-H out of plane banding, respectively. Moreover, the S-O stretching at 700-600 cm^{-1} points out the presence of sulfonic acid, sulfated polysaccharides, glycolipids and, sulfolipids.

5.2.2 Influence of biomass on reaction products composition

The thermal pyrolysis was carried out at 450 °C for 1 h on the three biomasses (AP, MIX, and MWW) to study the differences in terms of products yield and composition. The reaction product were classified as RG, a liquid fraction (L) composed of two immiscible fractions (lipophilic and hydrophilic), liquid residue (LR), and solid residue (SR).

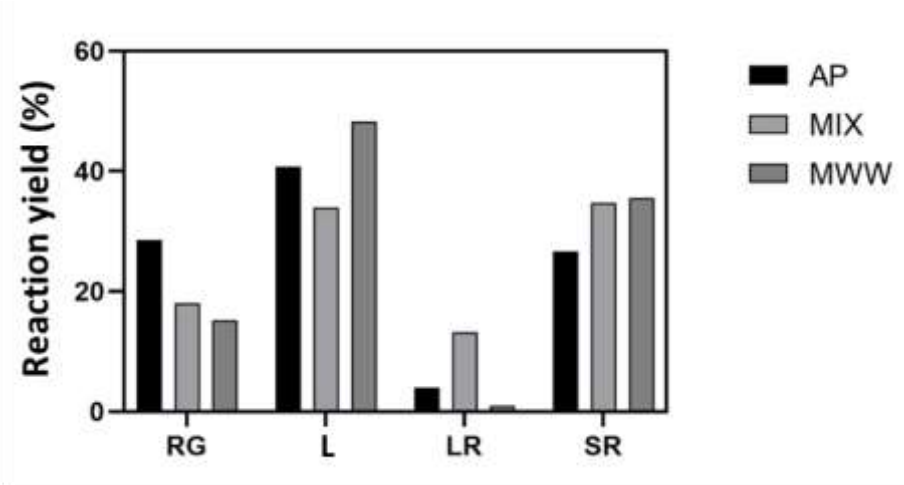

Figure 5.3. Percentage distribution (%) of products from thermal pyrolysis at 450 °C of pure of *Arthrospira platensis* biomass (AP), co-culture growth in BBM (MIX) and in winery wastewater (MWW). RG, reaction gas; LR, liquid residue; SR, solid residue.

In Figure 5.3. it is shown the product distribution resulting from thermal pyrolysis of the three biomasses. A higher amount of bio-oil (48.3%) was obtained using MWW as raw material, while with AP and MIX a percentage of about 40.7 and 33.9% was obtained, respectively. In contrast, the highest content of reaction gases (28.6%) was obtained using pure AP as biomass, and around 17% using MIX and MWW. Instead, thermal pyrolysis of MIX produced a higher content of liquid residue (12%) in comparison with AP and MWW (3.9 and 0.97%, respectively). The solid residue obtained from MIX and MWW was around 35%, while from AP around 27%.

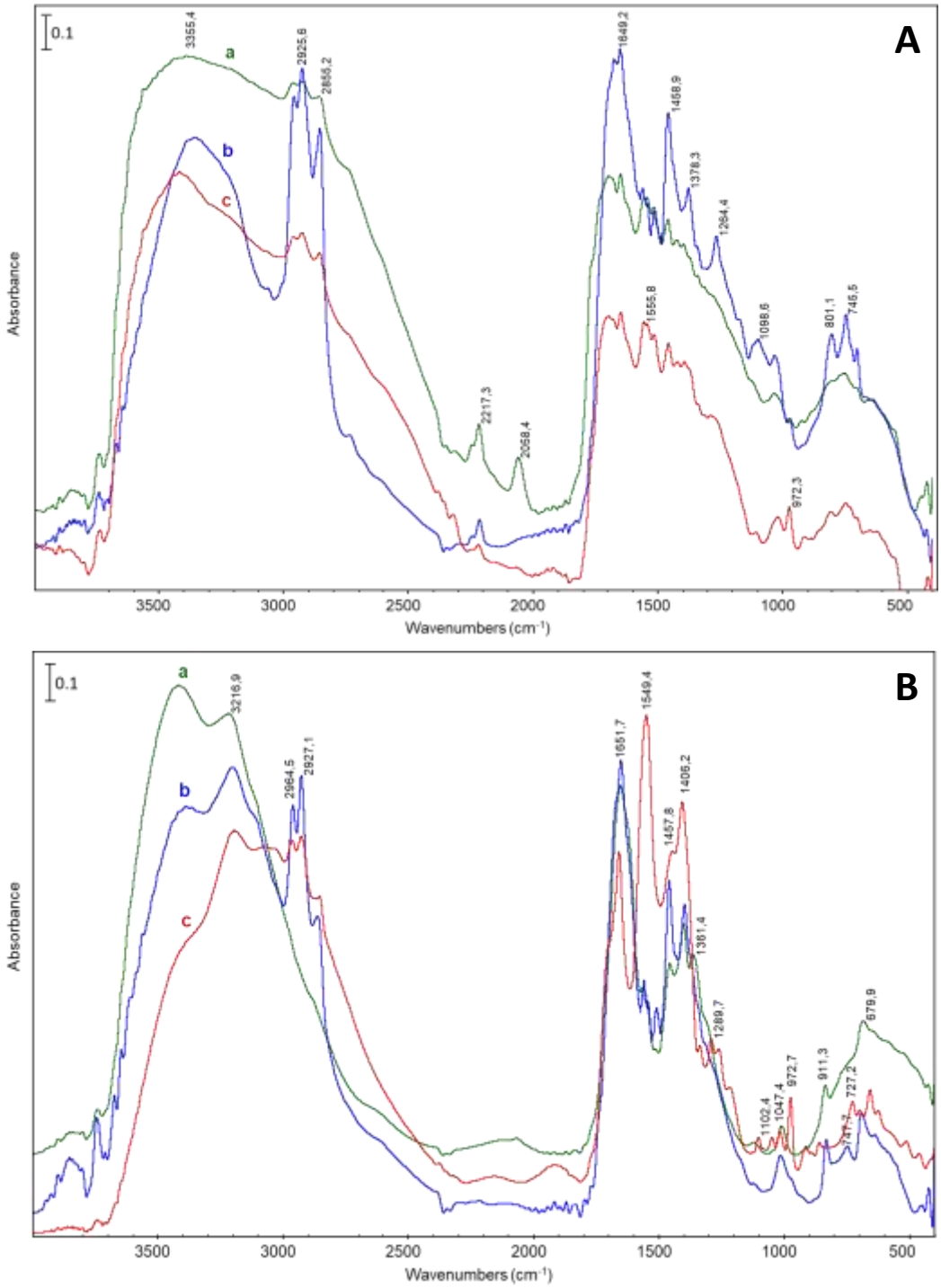

Figure 5.4. FTIR analysis of bio-oil of a-pure *Arthrospira platensis* biomass, b-co-culture grown in BBM, c-co-culture grown in winery wastewater. A- lipophilic fraction and B-hydrophilic fraction.

Bio-oil was made by two immiscible fractions, a lipophilic phase, and a hydrophilic phase. The two fractions were characterized separately by FTIR, as shown in Figure 5.4. The FTIR spectra of the lipophilic phase of L obtained from the three biomasses are shown in Figure 5.4A, in which the characteristic bands of the characteristic of N-H and O-H stretching in the region from 3500 to

3300 cm^{-1} are visible. On the other hand, in the region between 2960 and 2850 cm^{-1} were presented the bands corresponding to valence vibration of C-H, in particular at 2959 cm^{-1} the asymmetrical stretching and at 2875 cm^{-1} the symmetrical stretching of -CH_3 bonds and, at 2925 and 2862 cm^{-1} the asymmetrical and symmetrical stretching of -CH_2 bonds, respectively, while from 2500 and 1700 cm^{-1} the overtones bands of arenes. Moreover, the bands at 1649, 1555, and 1458 cm^{-1} were correlated to =C-H bond of amide I, N-H and C-N bonds of amide II and C-N bond of amide III, respectively. The band at 1378 cm^{-1} was associated with in plane scissoring of -CH (CH_3) bond cm^{-1}, and those at 1264 and 1098 cm^{-1} to C (O)-O and C-O, C-C and C-O-C bonds, respectively. The band of 973 cm^{-1}, present only in MIX spectra, was attributed to out of plane -OH banding. Moreover, the region from 800 to 600 cm^{-1} corresponded to -CH of aromatics and N-H out of plane bending. The bio-oil hydrophilic spectra (Figure 5.4B) showed some of the bands explained before for the lipophilic fraction with the addition of other bands. Among them, at 1406 cm^{-1} the band of in plane scissoring of (CH) C=CH_2 and C-N, at 1361 cm^{-1} the in plane scissoring of (C-H) CH_3 bond, at 1280 cm^{-1} the C-O bond stretching, at 1102 cm^{-1} the stretching od C-N bond and, at 1047 cm^{-1} the stretching of C-O bond.

The lipophilic fraction of L was also analyzed by GC-MS in order to evaluate the main compounds of this fraction and observe the differences among the different biomasses. In Figure 5.5 their classification into main classes of compounds: HC-hydrocarbons, O-oxygenates, N-nitrogenates, N/O- complex molecules containing oxygen and nitrogen atoms, and others- molecules also containing sulfur.

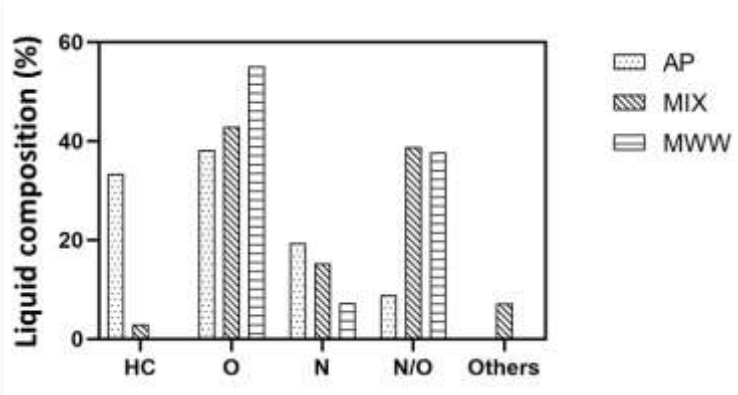

Figure 5.5. Composition of lipophilic fraction of L determined by GC-MS, resulting from thermal pyrolysis of pure *Arthrospira platensis* biomass (AP), co-culture grown in BBM (MIX), co-culture grown in winery wastewater (MWW). HC-hydrocarbons; O-oxygenates; N-nitrogenates; N/O, complex oxygenates/nitrogenates.

The hydrocarbon fraction was present principally in L obtained from the thermal pyrolysis of pure AP (33.41%). The hydrocarbons include both aromatic and aliphatic hydrocarbons, the former fraction increasing and improving the octane number, while the latter being important for its use as a transportation fuel. In the thermal pyrolysis process, the hydrocarbons were mainly produced by decarboxylation, deamination, and fragmentation of lipids or by thermal degradation of proteins [34]. The aliphatic ones included long chain alkanes and alkenes, among which from AP biomass 1,6-heptadien-3-yne and eicosane were found in the highest percentages (16.89 and 12.17%, respectively). Instead, the only hydrocarbon found in bio-oil from MIX biomass was 2,4,6-tris(cyclohexenyl)hept-1-ene (3.04%).

The oxygenates fraction in bio-oil consisted of aldehydes, ketones, alcohols, phenols, esters, and ethers. Even if the oxygenated compounds reduced the quality of bio-oil, phenols and their derivates are considered high added-value chemicals, and their contents can help the process to become more economically feasible [163]. The higher oxygenates fraction was obtained from the pyrolysis of MWW (55.07%), followed by MIX and AP pyrolysis with a percentage of 42.89 and 38.21%, respectively. The main oxygenate compound found in bio-oil from AP was 9-octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester, cis- with a percentage of about 15.05% and in MIX bio-oil the 10.61% of cyclohexane, (ethoxymethoxy)-. Moreover, in bio-oil from MWW, the principal oxygenated compounds were 2-furanmethanol and, 3-butenoic acid in thepercentage of 13.31 and 10.98% respectively.

The nitrogenates compounds present in bio-oil were principally amine, indole, and pyrrole, whose presence in bio-oil would lead, during combustion reaction, to the production of nitrogen oxides. These compounds found in the liquid fraction were mainly produced by protein decarboxylation and CO_2 or water elimination with the production of dipeptides, while the presence of N-heterocycles was due to the Maillard reaction that occurred from the interaction between sugars or other carbonyl compounds with amino acid [30]. The highest percentage of nitrogenates was obtained from thermal pyrolysis of pure AP (19.45%), followed by MIX and MWW (15.30 and 7.26%, respectively). The main nitrogenated compounds in bio-oil from the pyrolysis of AP biomass was hexadecanenitrile (4.82%), from MIX biomass were benzene, 1-isocyano-4-methyl- and pyridine, 2,3,5-trimethyl- (7.29 and 5.24 %, respectively) and from MWW biomass hydrazine, 1-(5-hexenyl)-1-methyl- (5.30%).

The complex oxygenates/nitrogenates mainly consisted of amides or fatty acid amides produced by the interaction between lipids and protein derivates. The thermal pyrolysis of co-culture grown

both in BMM and winery wastewater produced a high amount of complex oxygenates/nitrogenates compounds, 38.77 and 37.66%, respectively, while a concentration (8.92%) considerably lower was obtained from pyrolysis of pure AP. The principal complex oxygenated/nitrogenated compound found in bio-oil from AP biomass was hexadecanamide (6.77%), from MIX biomass was carbamic acid, methyl-, phenyl ester (8.50%) and, from MWW biomass butanamide, 3-methyl- (4.13%).

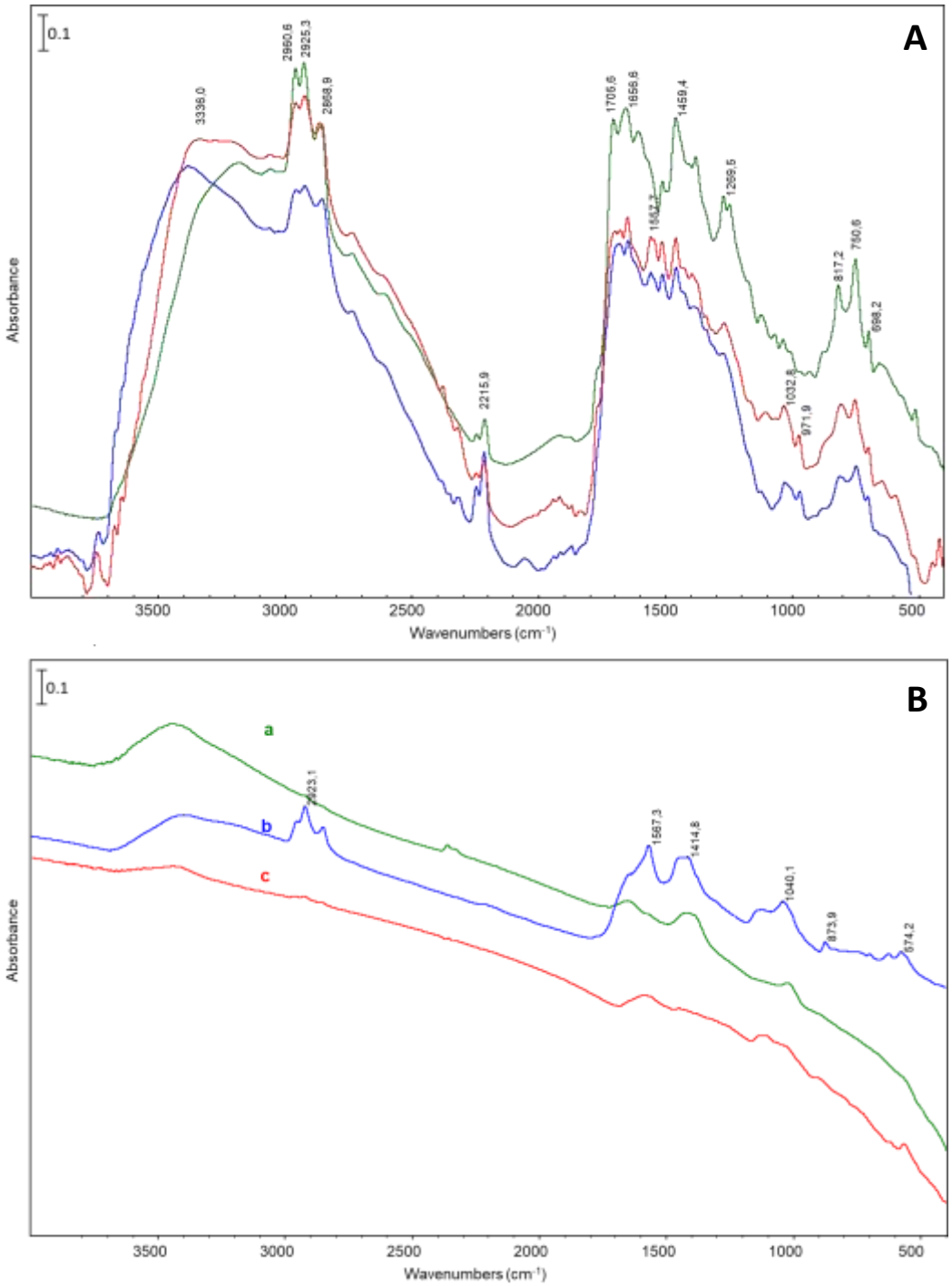

Figure 5.6. FTIR analysis of liquid and solid residue of a-*Arthrospira platensis* biomass, b-co-culture grown in BBM, c-co-culture grown in winery wastewater. A- liquid residue (LR) and B- Solid residue (SR).

The thermal pyrolysis residue is the solid that remained in the reactor after the reaction, which was collected and washed with acetone to separate the liquid residue (LR), mainly made up of high molecular weight components, from the solid one (SR). Figure 5.6A shows the spectra of LR obtained from the thermal pyrolysis of AP, MIX, and MWW. The presented bands, as expected,

were coincident with those present in bio-oil spectra, which confirms that this fraction is made by the same component classes (hydrocarbon, oxygenated, nitrogenated and oxygenated/nitrogenated complex compounds) but with higher boiling point. Moreover, the solid residue (SR) obtained from thermal pyrolysis of AP, MIX and, MWW was analyzed by FTIR as well (Figure 5.6B). The characteristic bands at 3340 and 1420 cm^{-1} that are representative of -OH stretching and C-C deformation, respectively. The spectra from MIX pyrolysis showed more bands in comparison with AP and MWW spectra, which could be attributed to the non-complete conversion of the starting biomass. The solid residue from pyrolysis, also called char, could find several applications in different fields. For instance, it can be used as carbon-based biofuel, because of a higher calorific value than that of starting biomass [17,26], or even as fertilizer, being able to improve the soil texture releasing nutrients [166].

5.2.3 Influence of pyrolysis temperature on reaction products composition

The thermal pyrolysis was carried out from co-culture biomass obtained after winery wastewater treatment (MWW) at different temperatures 400, 425, 450, and 500 °C to study the influence of this parameter on reaction product composition and yields. In Figure 5.7 the product distribution obtained at the different temperatures is shown.

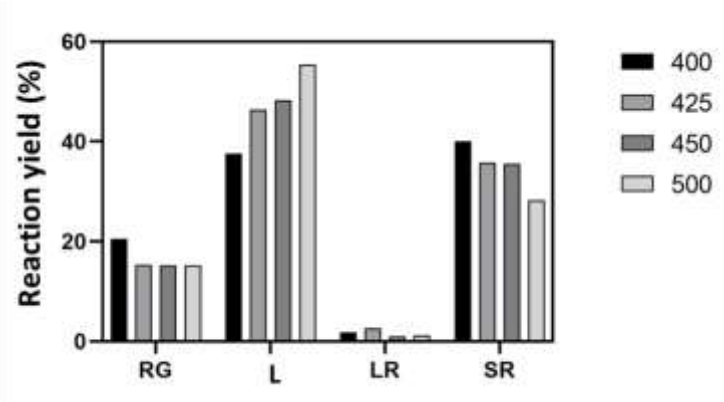

Figure 5.7. Percentage distribution (%) of products from thermal pyrolysis of co-culture grown in winery wastewater (MWW) at 400, 425, 450, and 500 °C. RG, reaction gas; LR, liquid residue; SR, solid residue.

The concentration of reaction gas (RG) and liquid residue (LR) remained almost constant increasing reaction temperature. A concentration of about 15% of RG was obtained at all the tested temperatures, the only exception was observed at 400 °C, while a contraction from 0.97 to 2.6% of liquid residue was obtained at all the temperatures. Instead, an increase of bio-oil composition

from 37.54 to 55.39% and a reduction of solid residue from 40.03 to 28.26% were obtained increasing the reaction temperature from 400 to 500 °C.

As discussed previously, L was made by two immiscible fractions, a lipophilic and a hydrophilic phase. The two fractions were characterized separately by FTIR, as showed in Figure 5.8. The FTIR spectra of the lipophilic and hydrophilic phases of L obtained from thermal pyrolysis of MWW at 400, 425, 450, and 500 °C presented the same bands illustrated in Section 5.2.2.

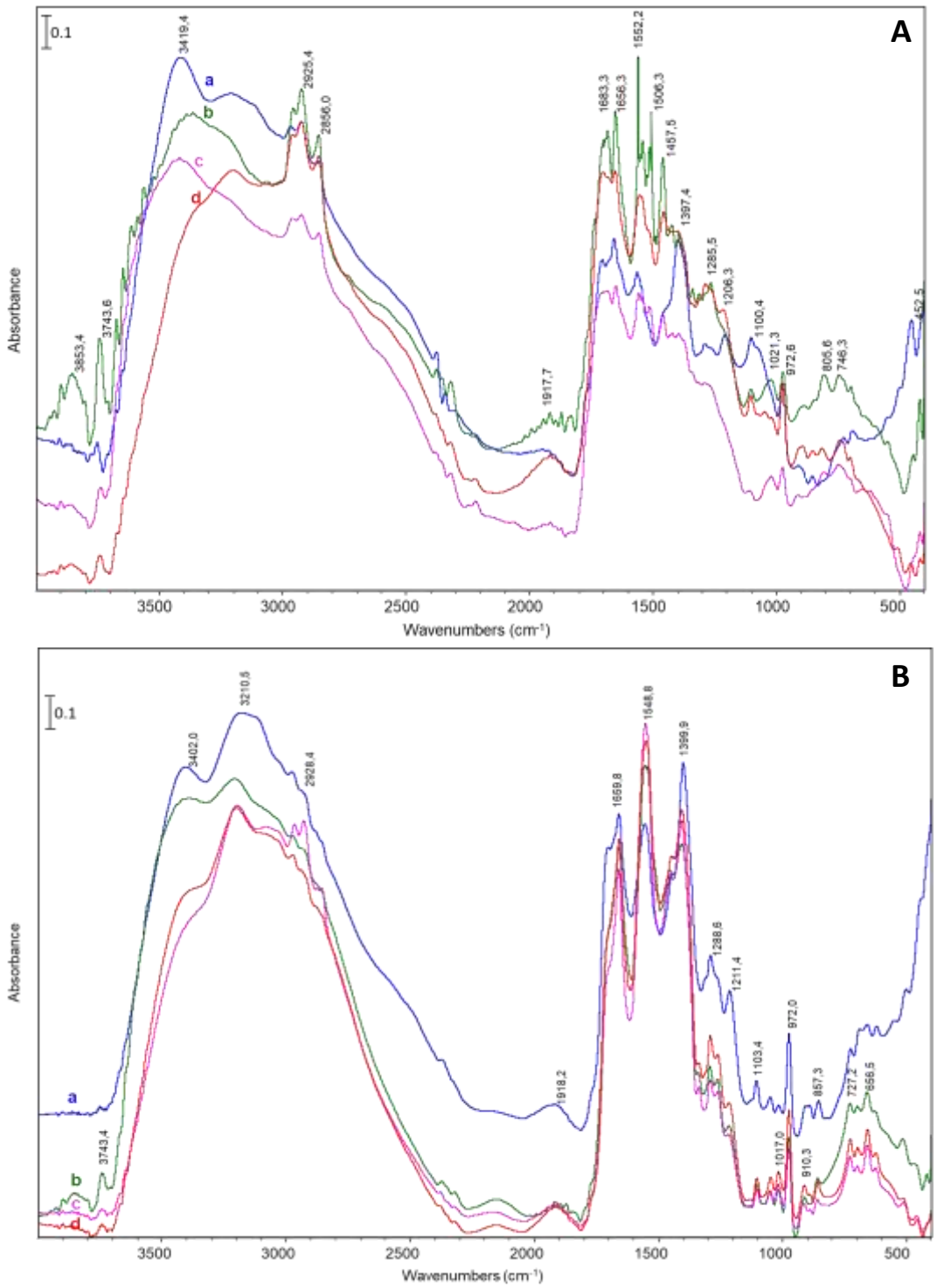

Figure 5.8. FTIR analysis of L fraction of co-culture grown in winery wastewater at a) 400 °C, b) 425 °C, c) 450 °C and d) 500 °C. A- lipophilic fraction and B-hydrophilic fraction.

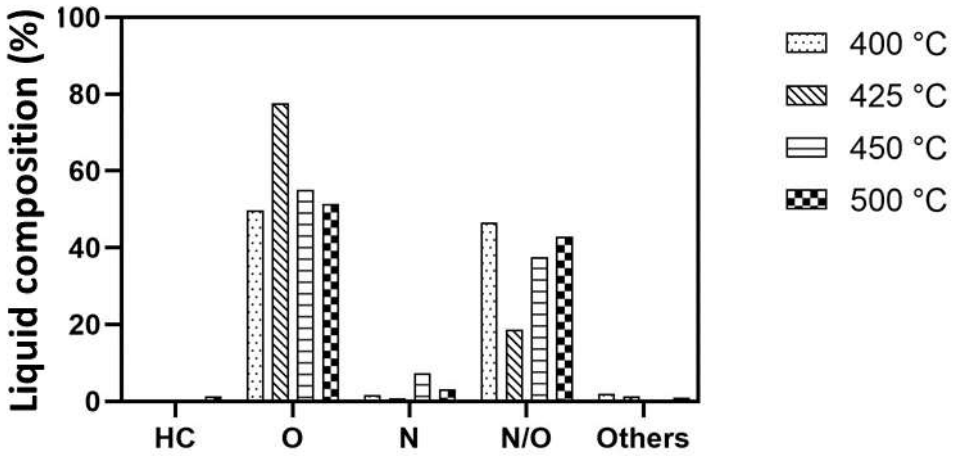

Figure 5.9. Composition of lipophilic fraction of L determined by GC-MS, resulting from thermal pyrolysis of pure of co-culture grown in winery wastewater (MWW) at 400, 425, 450, and 500 °C. HC-hydrocarbons; O-oxygenates; N-nitrogenates; N/O, complex oxygenates/nitrogenates.

The lipophilic fraction L was also analyzed by GC-MS in order to evaluate the main compounds of this fraction and observe the effect of reaction temperature. In Figure 5.9 their classification into main classes of compounds, as done previously in section 5.2.2.

The hydrocarbon and nitrogenated fractions obtained by thermal pyrolysis at all the tested temperatures were very low. In fact, the only hydrocarbon found in bio-oil was cyclohexane, 1,1'-(2-propyl-1,3-propanediyl)bis- (1.37%) obtained at 500°C, while the highest nitrogenates concentration was obtained from the reaction carried out at 450 °C, in which the main compound was hydrazine, 1-(5-hexenyl)-1-methyl- at a concentration of 5.30 %. The highest fraction of bio-oil was constituted by oxygenated, among which the bio-oil obtained by thermal pyrolysis at 425 °C was made by 77.47.45% of oxygenated, followed by that performed at 450, 500 and, 400 °C with an oxygenated content of 55.07, 51.45, and 49.67%, respectively. The main oxygenated compound of bio-oil obtained by thermal pyrolysis at 400 and 500 °C was isocrotonic acid in percentage of 29.89 and 13.90%, respectively, while in the bio-oil obtained at 420 °C the main compounds were crotonic and isocrotonic acids (25,23 and 21.79%, respectively), and in that obtained at 450 °C 2-furanol and 3-butanoic acid (13.31 and 10.98%, respectively). The complex nitrogenated/oxygenated compounds were produced in high concentration by thermal pyrolysis of MWW at all the tested temperatures (46.58, 37.66, and 42.95%, at 400, 450, and 500°C, respectively), the only exception occurring at 425 °C (18.67%). The main complex oxygenated/nitrogenated compound in bio-oil obtained at 400°C was 2,6-piperidinedione, 3-ethyl-

(14.17%), at 425 °C carbamic acid, methyl-, phenyl ester (4.70%) and, at 450 and 500 °C butanamide, 3-methyl- or 2-methyl- in the concentration of about 4.13 and 31.17%, respectively.

As previously described, the thermal pyrolysis residue was composed of a liquid fraction (LR), extracted by acetone, and a solid fraction (SR). The FTIR spectra of these products were shown in Figure 5.10, panel A for the liquid residue and panel B for the solid residue.

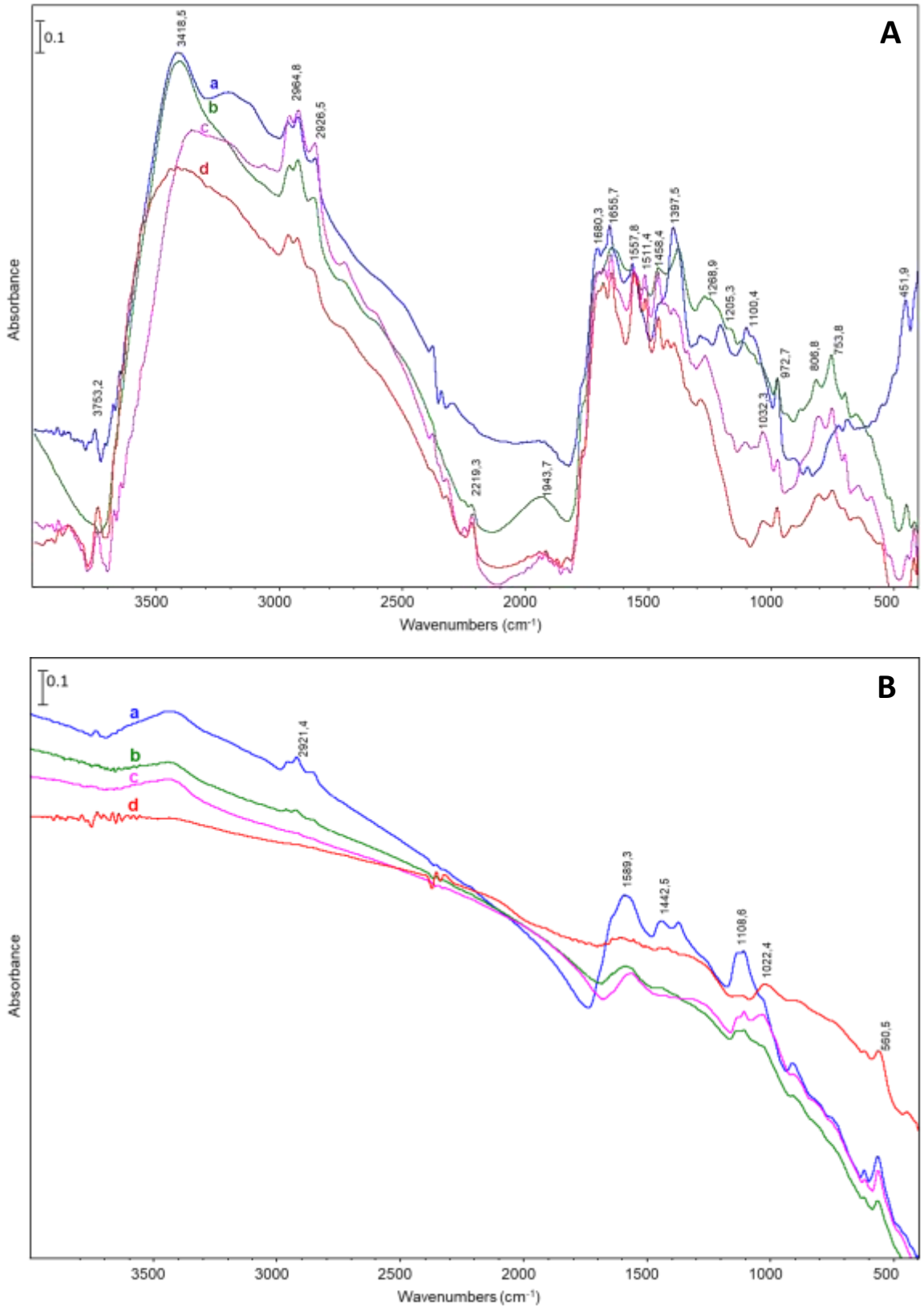

Figure 5.10. FTIR analysis of liquid and solid residue of c-co-culture grown in winery wastewater (MWW) at a) 400 °C, b) 425 °C, c) 450 °C and d) 500 °C. A- liquid residue (LR) and B-Solid residue (SR)

The characteristic bands of the liquid and solid residue were explained previously in section 5.2.2. The spectra of solid residue showed, as expected, that increasing the reaction temperature from 400 to 500 °C the presented bands were reduced, thus the conversion of the starting biomass was increased.

5.2.4 Influence of pyrolysis time on reaction products composition

After the evaluation of the differences of thermal pyrolysis product yields and composition as functions of starting biomass (AP, MIX and, MWW) and reaction temperature (400, 425, 450, and 500°C), the influence of reaction time was investigated. The thermal pyrolysis of MWW was carried out at 400 and 425 °C for 1 and 2 hours. In Figure 5.11 we can see the reaction product yield obtained under the tested conditions.

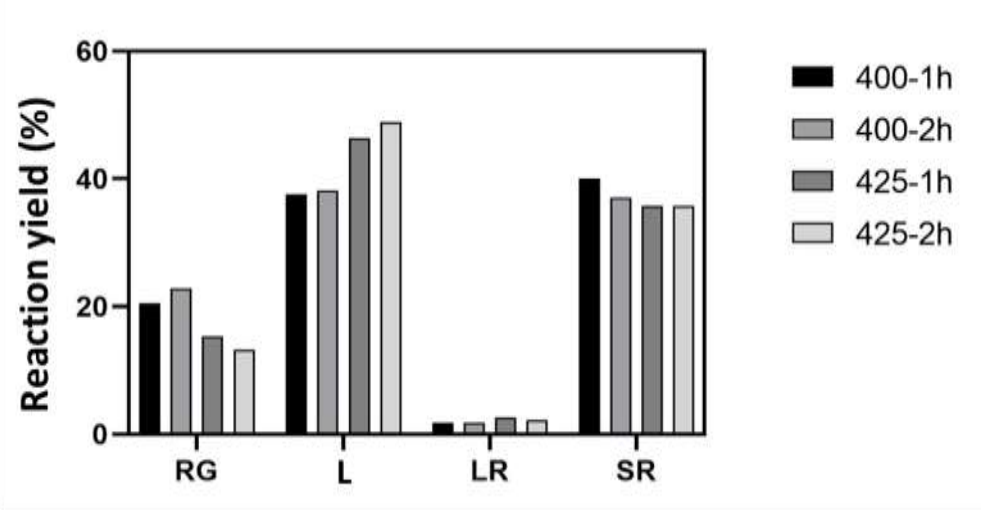

Figure 5.11. Percentage distribution (%) of products from thermal pyrolysis of co-culture grown in winery wastewater (MWW) at 400 and, 425 °C for 1h and 2h. RG, reaction gas; LR, liquid residue; SR, solid residue.

The thermal pyrolysis reaction time did not strongly affect the distribution of reaction products. The reaction gas was increased from 20.58 to 22.89% and reduced from 15.31 to 13.16% increasing reaction time from 1 to 2 hours at 400 and 425°, respectively. L content remained almost constant increasing reaction time at 400°C (around 38%), while a slight increase from 46.35 to 48.92% occurred at 425°. The liquid residue was produced in a small amount at all reaction times and temperatures, from 1.80 to 2.20%. Moreover, the solid residue was reduced from 40.03 to 37.08% at 400°C increasing the extraction time, while remained constant in concentration around 35% at 425°C.

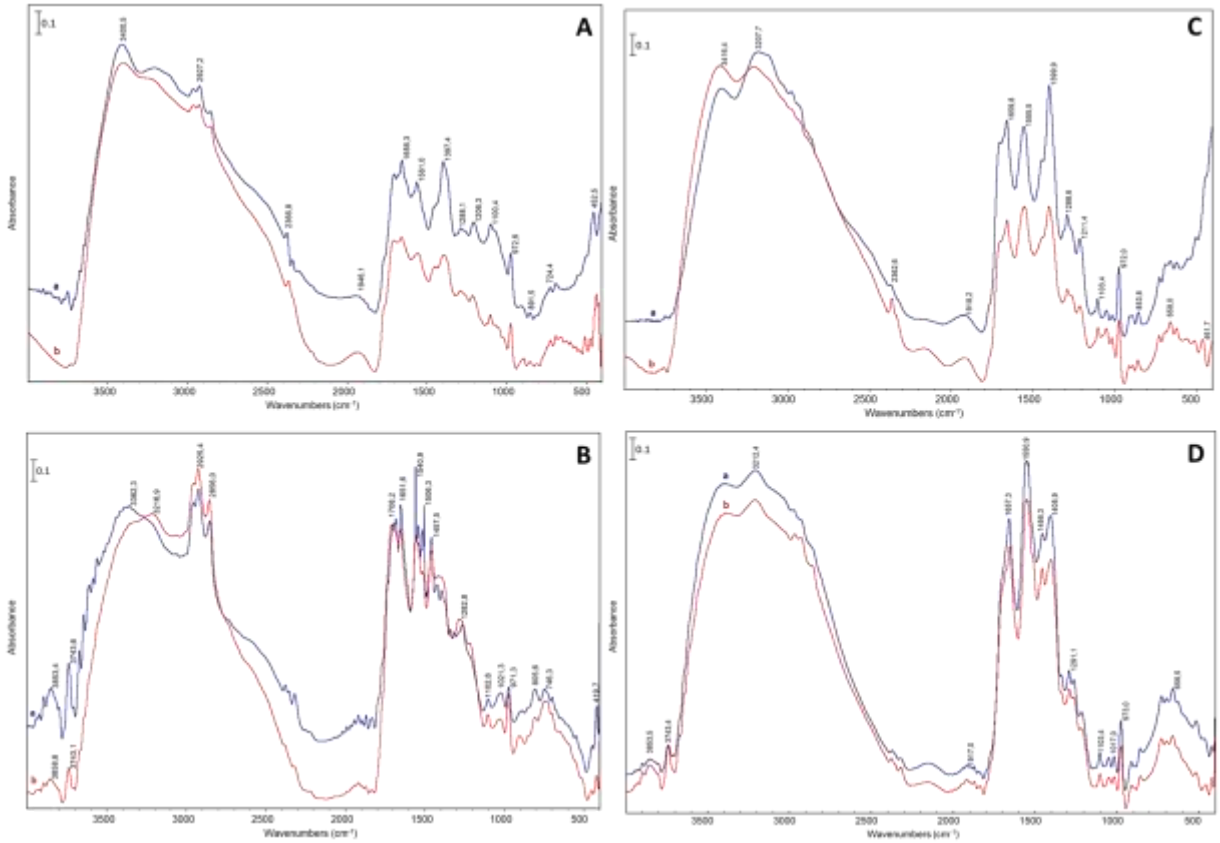

Figure 5.12. FTIR analysis of L fraction of co-culture grown in winery wastewater (MWW) at 400 °C panel A and C, 425 °C panel B and D. A, B bio-oil lipophilic fraction and C, D bio-oil hydrophilic fraction. Where, a) 1h of reaction time and b) 2h of reaction time.

As discussed previously, bio-oil was made by two immiscible fractions, a lipophilic and a hydrophilic phase. The two fractions were characterized separately by FTIR, as shown in Figure 5.12. The FTIR spectra of the lipophilic and hydrophilic phases of bio-oil obtained by thermal pyrolysis of MWW at 400, 425, °C for 1 and 2 hours presented the same bands illustrated in section 5.2.2. No significant differences were observed in the spectra bands increasing the reaction temperature.

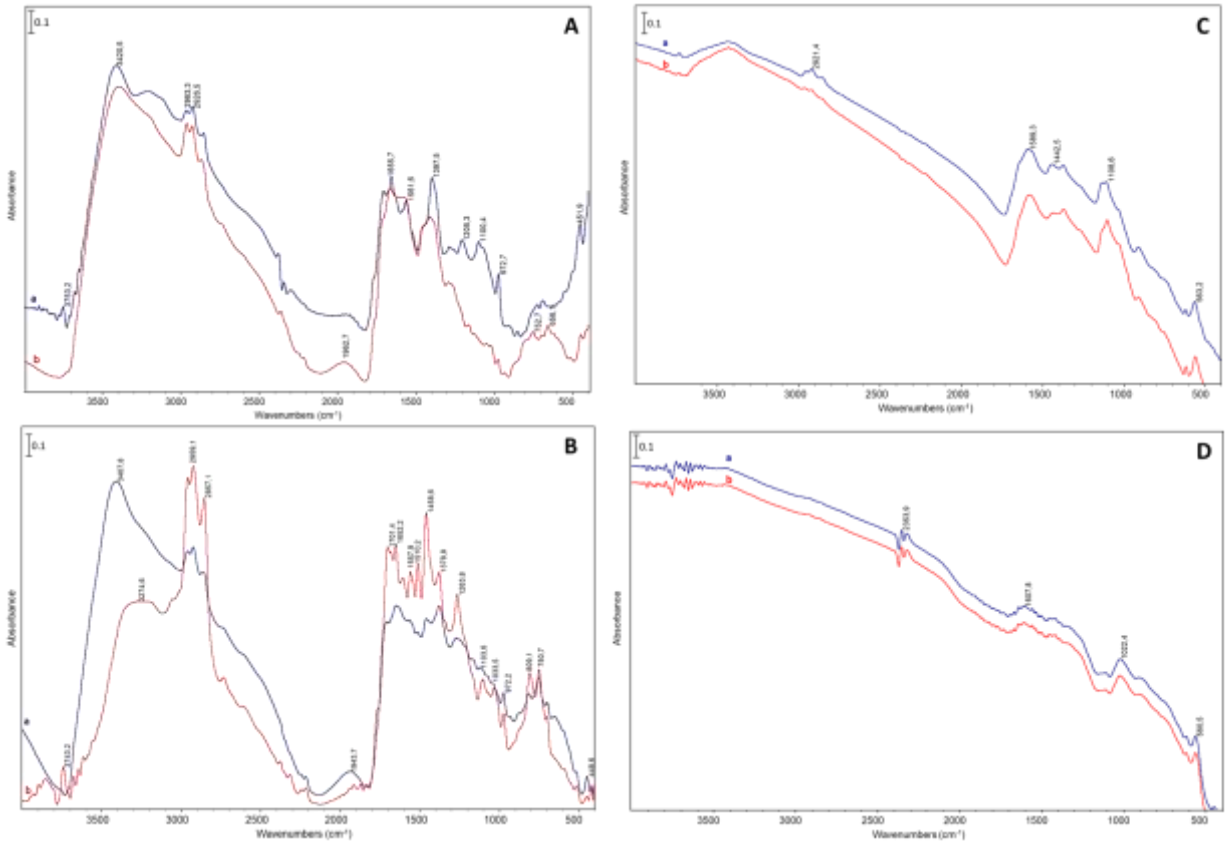

Figure 5.13. FTIR analysis of thermal pyrolysis residues of co-culture grown in winery wastewater (MWW) at 400 °C panel A and C, 425 °C panel B and D. A, B liquid residue and C, D solid residue fraction. Where, a) 1h of reaction time and b) 2h of reaction time.

Moreover, as previously described, the thermal pyrolysis residue was composed of LR and SR. The FTIR spectra of these products are shown in Figure 5.13. The characteristic bands of the liquid and solid residue were explained previously in section 5.2.2. As observed in bio-oil spectra, the increase in reaction temperature did not affect significantly the spectra bands.

5.3 Conclusions

Thermal pyrolysis of biomass obtained after winery wastewater treatment in membrane photobioreactor was investigated in comparison with pure *Arthrospira platensis* and co-culture growth in Bold Basal's medium. The influence of reaction time and temperature on product yield and composition were studied. Starting biomass and reaction temperature significantly impacted the reaction products composition and yield, instead of reaction time, that did not affect considerably thermal pyrolysis reaction. The grater L production (55.39%) was obtained by

thermal pyrolysis of co-culture growth in winery wastewater at 500°C. The bio-oil produced was made by a mixture of oxygenates, nitrogenated and complex oxygenates/nitrogenates compounds. Even if these compounds reduce the quality of liquid fraction for fuel application, they are suitable as chemical intermediates helping to make the pyrolysis treatment economically feasible. Moreover, the solid residue (around 30%), a by-product of thermal pyrolysis, could be used as a carbon-based fuel or as an adsorbent material. This study suggested that thermal pyrolysis may be a good strategy to recover the energetic potential of microalgae co-culture used to purify wastewaters.

6 General Conclusions

In this study, the development of a biorefinery from microalgae was investigated in order to make the microalgae production and the commercial utilization of their products (high-added-value components and biofuels) economically feasible. The first step concerned the investigation of microalgae medium to led to the drastic reduction of microalgae production cost. The best solution is represented by the use of wastewater as a growth medium, thanks to the exploitation of pollutant molecules as nutrient sources for the microalgae metabolism and no necessity to provide CO_2. For this reason, winery wastewaters deriving from different steps of wine making process were used as a growth medium for the co-culture of *Arthrospira platensis* and *Chlorella vulgaris*. The influence of winery wastewater concentration, the light conditions, and the growth system configurations on co-culture biomass concentration and productivity and, the removal of pollution impacts were investigated. This study demonstrated that the co-culture has been effectively able to grow in winery wastewater in several reactor configurations reaching concentration and biomass productivity remarkably higher compared to cultures in the conventional medium (5.5, 3.9 and, 2.2 $g/100g_{DW}$ in wastewater and, 3.7, 2.9 and, 1.1 $g/100g_{DW}$ in conventional medium in multi-tubular photobioreactor, column photobioreactor and, open pond, respectively). Moreover, the pollutant impact of the wastewaters was highly reduced by the co-culture in a shorter time in comparison to conventional methods (about 95% in 5 days).

The obtained low-cost biomass was used for the extraction of proteins and the production of biofuels and compounds useful as green chemical intermediates by thermal pyrolysis. The optimization of protein extraction by ultrasound-assisted extraction was carried out as a function of the main operative conditions that influence the extraction processes, the volume of solvent, solid/liquid ratio, and extraction time. A maximum concentration of 48.8 $g/100g_{DW}$ was obtained using 75 mL of solvent, 1 g of biomass and 30 minutes of total extraction time. Moreover, the c-phycocyanin considering its pharmacological properties was selected as a high-added value component with the greater market value. For this reason, the c-phycocyanin was extracted with a solution of calcium chloride and purified with ammonium sulfate, obtaining a product suitable for food applications and as a reactive compound (concentration of c-PC obtained 9.6 $mg_{c\text{-}PC}/100\ mg_{AP}$).

The use of wastewater as a nutrient source for the co-culture metabolism affected the morphology and the composition of the cells. An increase in lipid and carbohydrate contents and a decrease in protein content were observed due to the stress conditions in which the co-culture was submitted; also, protein expression and lipid accumulation were affected by reactor configuration. Moreover, the co-culture cells showed a larger diameter and a higher resistivity to cell rupture by mechanical disruption techniques.

The energetical recovery of the co-culture biomass was performed by thermal pyrolysis. The influence of starting biomass (grown in winery wastewater or conventional medium), the reaction temperature, and the reaction time were investigated on the yield and composition of the products. The biomass grown in winery wastewater produced a liquid fraction (from 35 to 55%) rich in nitrogenated, oxygenated, and complex oxygenated / nitrogenated compounds. For this reason, the application of the produced liquid as biofuel is very complex because it requires an upgrading process. However, it contains compounds useful as green chemical intermediates (such as isocrotonic and butanoic acids, phenol, and its derivates). Moreover, the reaction gas (around 17%) and the solid residue (from 40 to 30 %) can be used for several applications. The first can directly burn to furnish the heat necessary for the thermal pyrolysis reaction, and the latter as carbon-based biofuels or fertilizer.

In conclusion, this study demonstrates a possible application of a zero-waste strategy on microalgae cultivation and the production of high added-value components and biofuels in a biorefinery concept.

References

[1] IEA, IEA bioenergy Task 42 on biorefineries: co-production of fuels, chemicals, power and materials from biomass. In: Minutes of the third Task meeting,Copenhagen, Denmark, 25–26 March 2007 <http://www.biorefinery.nl/ieabioenergy-task42/> (2008).

[2] C.T. Weber, L.F. Trierweiler, J.O. Trierweiler, Food waste biorefinery advocating circular economy: Bioethanol and distilled beverage from sweet potato, J. Clean. Prod. 268 (2020). doi:10.1016/j.jclepro.2020.121788.

[3] F. Cherubini, The biorefinery concept: Using biomass instead of oil for producing energy and chemicals, Energy Convers. Manag. 51 (2010) 1412–1421. doi:10.1016/j.enconman.2010.01.015.

[4] J. Gravitis, M. Suzuki, Biomass Refinery - A Way to Produce Value Added Products and Base for Agricultural Zero Emissions System, Proc. 99 Int. Conf. Agric. Eng. (1999) III: 9-22. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.202.7106&rep=rep1&type=pdf.

[5] R. Katakojwala, S.V. Mohan, A critical view on the environmental sustainability of biorefinery systems, Curr. Opin. Green Sustain. Chem. 27 (2021) 100392. doi:10.1016/j.cogsc.2020.100392.

[6] A.T. Ubando, C.B. Felix, W.H. Chen, Biorefineries in circular bioeconomy: A comprehensive review, Bioresour. Technol. 299 (2020). doi:10.1016/j.biortech.2019.122585.

[7] H.P. Vu, L.N. Nguyen, M.T. Vu, M.A.H. Johir, R. McLaughlan, L.D. Nghiem, A comprehensive review on the framework to valorise lignocellulosic biomass as biorefinery feedstocks, Sci. Total Environ. 743 (2020) 140630. doi:10.1016/j.scitotenv.2020.140630.

[8] V.L. Pachapur, S. Kaur Brar, Y. Le Bihan, Integrated wood biorefinery: Improvements and tailor-made two-step strategies on hydrolysis techniques, Bioresour. Technol. 299 (2020) 122632. doi:10.1016/j.biortech.2019.122632.

[9] M.K. Islam, H. Wang, S. Rehman, C. Dong, H.Y. Hsu, C.S.K. Lin, S.Y. Leu, Sustainability metrics of pretreatment processes in a waste derived lignocellulosic biomass biorefinery, Bioresour. Technol. 298 (2020) 122558. doi:10.1016/j.biortech.2019.122558.

[10] C. Caldeira, A. Vlysidis, G. Fiore, V. De Laurentiis, G. Vignali, S. Sala, Sustainability of food waste biorefinery: A review on valorisation pathways, techno-economic constraints, and environmental assessment, Bioresour. Technol. 312 (2020) 123575. doi:10.1016/j.biortech.2020.123575.

[11] B. Khoshnevisan, N. Duan, P. Tsapekos, M.K. Awasthi, Z. Liu, A. Mohammadi, I. Angelidaki, D.C. Tsang, Z. Zhang, J. Pan, L. Ma, M. Aghbashlo, M. Tabatabaei, H. Liu, A critical review on livestock manure biorefinery technologies: Sustainability, challenges, and future perspectives, Renew. Sustain. Energy Rev. 135 (2021) 110033. doi:10.1016/j.rser.2020.110033.

[12] A. Giwa, Comparative cradle-to-grave life cycle assessment of biogas production from marine algae and cattle manure biorefineries, Bioresour. Technol. 244 (2017) 1470–1479. doi:10.1016/j.biortech.2017.05.143.

[13] J.M. Greene, J. Gulden, G. Wood, M. Huesemann, J.C. Quinn, Techno-economic analysis and global warming potential of a novel offshore macroalgae biorefinery, Algal Res. 51 (2020). doi:10.1016/j.algal.2020.102032.

[14] A.R. Rudke, C.J. de Andrade, S.R.S. Ferreira, *Kappaphycus alvarezii* macroalgae: An

unexplored and valuable biomass for green biorefinery conversion, Trends Food Sci. Technol. 103 (2020) 214–224. doi:10.1016/j.tifs.2020.07.018.
[15] L. Fan, H. Zhang, J. Li, Y. Wang, L. Leng, J. Li, Y. Yao, Q. Lu, W. Yuan, W. Zhou, Algal biorefinery to value-added products by using combined processes based on thermochemical conversion: A review, Algal Res. 47 (2020) 101819. doi:10.1016/j.algal.2020.101819.
[16] S.A. Razzak, M.M. Hossain, R.A. Lucky, A.S. Bassi, H. De Lasa, Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing - A review, Renew. Sustain. Energy Rev. 27 (2013) 622–653. doi:10.1016/j.rser.2013.05.063.
[17] M. Yamamoto, M. Fujishita, A. Hirata, S. Kawano, Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris (Chlorophyta, Trebouxiophyceae*), J. Plant Res. 117 (2004) 257–264. doi:10.1007/s10265-004-0154-6.
[18] J.M. Lv, L.H. Cheng, X.H. Xu, L. Zhang, H.L. Chen, Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions, Bioresour. Technol. 101 (2010) 6797–6804. doi:10.1016/j.biortech.2010.03.120.
[19] H.J. Morris, A. Almarales, O. Carrillo, R.C. Bermúdez, Utilisation of *Chlorella vulgaris* cell biomass for the production of enzymatic protein hydrolysates, Bioresour. Technol. 99 (2008) 7723–7729. doi:10.1016/j.biortech.2008.01.080.
[20] S. Lordan, R.P. Ross, C. Stanton, Marine bioactives as functional food ingredients: Potential to reduce the incidence of chronic diseases, Mar. Drugs. 9 (2011) 1056–1100. doi:10.3390/md9061056.
[21] H.C. Kwang, H.J. Lee, S.Y. Koo, D.G. Song, D.U. Lee, C.H. Pan, Optimization of pressurized liquid extraction of carotenoids and chlorophylls from *Chlorella vulgaris*, J. Agric. Food Chem. 58 (2010) 793–797. doi:10.1021/jf902628j.
[22] Vonshak, *Spirulina platensis (Arthrospira)*: physiology, cell-biology and biotechnology, (1st ed.), Taylor and Francis Ltd., London, 1997. http://library1.nida.ac.th/termpaper6/sd/2554/19755.pdf.
[23] S. Venkata Mohan, M. V. Rohit, P. Chiranjeevi, R. Chandra, B. Navaneeth, Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: Progress and perspectives, Bioresour. Technol. 184 (2015) 169–178. doi:10.1016/j.biortech.2014.10.056.
[24] C.Y. Chen, K.L. Yeh, R. Aisyah, D.J. Lee, J.S. Chang, Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review, Bioresour. Technol. 102 (2011) 71–81. doi:10.1016/j.biortech.2010.06.159.
[25] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: A review, Renew. Sustain. Energy Rev. 14 (2010) 217–232. doi:10.1016/j.rser.2009.07.020.
[26] K. Chojnacka, A. Zielińska, Evaluation of growth yield of *Spirulina (Arthrospira) sp.* in photoautotrophic, heterotrophic and mixotrophic cultures, World J. Microbiol. Biotechnol. 28 (2012) 437–445. doi:10.1007/s11274-011-0833-0.
[27] C. Posten, Design principles of photo-bioreactors for cultivation of microalgae, Eng. Life Sci. 9 (2009) 165–177. doi:10.1002/elsc.200900003.
[28] K. Kumar, C.N. Dasgupta, B. Nayak, P. Lindblad, D. Das, Development of suitable photobioreactors for CO_2 sequestration addressing global warming using green algae and cyanobacteria, Bioresour. Technol. 102 (2011) 4945–4953. doi:10.1016/j.biortech.2011.01.054.
[29] J.N. Rogers, J.N. Rosenberg, B.J. Guzman, V.H. Oh, L.E. Mimbela, A. Ghassemi, M.J. Betenbaugh, G.A. Oyler, M.D. Donohue, A critical analysis of paddlewheel-driven raceway ponds for algal biofuel production at commercial scales, Algal Res. 4 (2014) 76–88.

doi:10.1016/j.algal.2013.11.007.
[30] S.H. Al-lwayzy, T. Yusaf, R.A. Al-Juboori, Biofuels from the fresh water microalgae *Chlorella vulgaris* (FWM-CV) for diesel engines, Energies. 7 (2014) 1829–1851. doi:10.3390/en7031829.
[31] S. Cho, T.T. Luong, D. Lee, Y.K. Oh, T. Lee, Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production, Bioresour. Technol. 102 (2011) 8639–8645. doi:10.1016/j.biortech.2011.03.037.
[32] O. Perez-Garcia, Y. Bashan, M. Esther Puente, Organic carbon supplementation of sterilized municipal wastewater is essential for heterotrophic growth and removing ammonium by the microalga *Chlorella vulgaris*, J. Phycol. 47 (2011) 190–199. doi:10.1111/j.1529-8817.2010.00934.x.
[33] Y. Li, Y.F. Chen, P. Chen, M. Min, W. Zhou, B. Martinez, J. Zhu, R. Ruan, Characterization of a microalga *Chlorella sp.* well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production, Bioresour. Technol. 102 (2011) 5138–5144. doi:10.1016/j.biortech.2011.01.091.
[34] S.R. Subashchandrabose, B. Ramakrishnan, M. Megharaj, K. Venkateswarlu, R. Naidu, Consortia of cyanobacteria/microalgae and bacteria: Biotechnological potential, Biotechnol. Adv. 29 (2011) 896–907. doi:10.1016/j.biotechadv.2011.07.009.
[35] N. Jagmann, B. Philipp, Reprint of Design of synthetic microbial communities for biotechnological production processes, J. Biotechnol. 192 (2014) 293–301. doi:10.1016/j.jbiotec.2014.11.005.
[36] R. Muñoz, B. Guieysse, Algal-bacterial processes for the treatment of hazardous contaminants: A review, Water Res. 40 (2006) 2799–2815. doi:10.1016/j.watres.2006.06.011.
[37] L.B. Bacellar Mendes, A.B. Vermelho, Allelopathy as a potential strategy to improve microalgae cultivation, Biotechnol. Biofuels. 6 (2013) 1–14. doi:10.1186/1754-6834-6-152.
[38] E.M. Gross, Allelopathy of aquatic autotrophs, CRC. Crit. Rev. Plant Sci. 22 (2003) 313–339. doi:10.1080/713610859.
[39] F.M.I. Natrah, P. Bossier, P. Sorgeloos, F.M. Yusoff, T. Defoirdt, Significance of microalgal-bacterial interactions for aquaculture, Rev. Aquac. 6 (2014) 48–61. doi:10.1111/raq.12024.
[40] J. Lian, R.H. Wijffels, H. Smidt, D. Sipkema, The effect of the algal microbiome on industrial production of microalgae, Microb. Biotechnol. 11 (2018) 806–818. doi:10.1111/1751-7915.13296.
[41] N. Renuka, A. Sood, R. Prasanna, A.S. Ahluwalia, Phycoremediation of wastewaters: a synergistic approach using microalgae for bioremediation and biomass generation, Int. J. Environ. Sci. Technol. 12 (2015) 1443–1460. doi:10.1007/s13762-014-0700-2.
[42] I. De Godos, C. González, E. Becares, P.A. García-Encina, R. Muñoz, Simultaneous nutrients and carbon removal during pretreated swine slurry degradation in a tubular biofilm photobioreactor, Appl. Microbiol. Biotechnol. 82 (2009) 187–194. doi:10.1007/s00253-008-1825-3.
[43] M.A. Bustamante, R. Moral, C. Paredes, A. Pérez-Espinosa, J. Moreno-Caselles, M.D. Pérez-Murcia, Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry, Waste Manag. 28 (2008) 372–380. doi:10.1016/j.wasman.2007.01.013.
[44] A. Eusébio, M. Petruccioli, M. Lageiro, F. Federici, J.C. Duarte, Microbial characterisation of activated sludge in jet-loop bioreactors treating winery wastewaters, J. Ind. Microbiol. Biotechnol. 31 (2004) 29–34. doi:10.1007/s10295-004-0111-3.

[45] M. Petruccioli, J.C. Duarte, F. Federici, High-rate aerobic treatment of winery wastewater using bioreactors with free and immobilized activated sludge, J. Biosci. Bioeng. 90 (2000) 381–386. doi:10.1016/S1389-1723(01)80005-0.
[46] R. Mosteo, J. Sarasa, M.P. Ormad, J.L. Ovelleiro, Sequential solar photo-fenton-biological system for the treatment of winery wastewaters, J. Agric. Food Chem. 56 (2008) 7333–7338. doi:10.1021/jf8005678.
[47] L.A. Ioannou, G.L. Puma, D. Fatta-Kassinos, Treatment of winery wastewater by physicochemical, biological and advanced processes: A review, J. Hazard. Mater. 286 (2015) 343–368. doi:10.1016/j.jhazmat.2014.12.043.
[48] X. Melamane, R. Tandlich, J. Burgess, Anaerobic digestion of fungally pre-treated wine distillery wastewater, African J. Biotechnol. 6 (2007) 1990–1993. doi:10.5897/ajb2007.000-2305.
[49] C.I. Nair, K. Jayachandran, S. Shashidhar, Biodegradation of phenol, African J. Biotechnol. 7 (2008) 4951–4958. doi:10.4018/978-1-5225-8903-7.ch045.
[50] S. Casani, M. Rouhany, S. Knøchel, A discussion paper on challenges and limitations to water reuse and hygiene in the food industry, Water Res. 39 (2005) 1134–1146. doi:10.1016/j.watres.2004.12.015.
[51] M. Geetha Devi, J.J. Dumaran, S. Feroz, Dairy Wastewater Treatment Using Low Molecular Weight Crab Shell Chitosan, J. Inst. Eng. Ser. E. 93 (2012) 9–14. doi:10.1007/s40034-012-0005-2.
[52] P. Amaresh, Applications of membrane separation technology in food industry, (2020).
[53] P.J. Strong, J.E. Burgess, Treatment methods for wine-related and distillery wastewaters: A review, Bioremediat. J. 12 (2008) 70–87. doi:10.1080/10889860802060063.
[54] A.A. Casazza, P.F. Ferrari, B. Aliakbarian, P. Perego, Microalgae Growth using Winery Wastewater for Energetic and Environmental Purposes, Chem. Eng. Trans. 49 (2016) 565–570. doi:10.3303/CET1649095.
[55] https://www.iea.org/data-and-statistics
[56] M. Lisý, H. Lisá, D. Jecha, M. Baláš, P. Križan, Characteristic properties of alternative biomass fuels, Energies. 13 (2020) 1–17. doi:10.3390/en13061448.
[57] H. Chowdhury, B. Loganathan, 3rd generation biofuels from microalgae: A review, Curr. Opin. Green Sustain. Chem. (2019). doi:10.1016/j.cogsc.2019.09.003.
[58] D.F. Correa, H.L. Beyer, H.P. Possingham, S.R. Thomas-Hall, P.M. Schenk, Biodiversity impacts of bioenergy production: Microalgae vs. first generation biofuels, Renew. Sustain. Energy Rev. 74 (2017) 1131–1146. doi:10.1016/j.rser.2017.02.068.
[59] E. Sannita, B. Aliakbarian, A.A. Casazza, P. Perego, G. Busca, Medium-temperature conversion of biomass and wastes into liquid products, a review, Renew. Sustain. Energy Rev. 16 (2012) 6455–6475. doi:10.1016/j.rser.2012.06.017.
[60] N.H. Zainan, S.C. Srivatsa, F. Li, S. Bhattacharya, Quality of bio-oil from catalytic pyrolysis of microalgae Chlorella vulgaris, Fuel. 223 (2018) 12–19. doi:10.1016/j.fuel.2018.02.166.
[61] A.A. Casazza, B. Aliakbarian, A. Lagazzo, G. Garbarino, M.M. Carnasciali, P. Perego, G. Busca, Pyrolysis of grape marc before and after the recovery of polyphenol fraction, Fuel Process. Technol. 153 (2016) 121–128. doi:10.1016/j.fuproc.2016.07.014.
[62] V. Anand, V. Sunjeev, R. Vinu, Catalytic fast pyrolysis of Arthrospira platensis (spirulina) algae using zeolites, J. Anal. Appl. Pyrolysis. 118 (2016) 298–307. doi:10.1016/j.jaap.2016.02.013.
[63] A.P. Pinheiro Pires, J. Arauzo, I. Fonts, M.E. Domine, A. Fernández Arroyo, M.E. Garcia-Perez, J. Montoya, F. Chejne, P. Pfromm, M. Garcia-Perez, Challenges and opportunities

for bio-oil refining: A review, Energy and Fuels. 33 (2019) 4683–4720. doi:10.1021/acs.energyfuels.9b00039.
[64] Z. Du, X. Ma, Y. Li, P. Chen, Y. Liu, X. Lin, H. Lei, R. Ruan, Production of aromatic hydrocarbons by catalytic pyrolysis of microalgae with zeolites: Catalyst screening in a pyroprobe, Bioresour. Technol. 139 (2013) 397–401. doi:10.1016/j.biortech.2013.04.053.
[65] A. Maadane, N. Merghoub, T. Ainane, H. El Arroussi, R. Benhima, S. Amzazi, Y. Bakri, I. Wahby, Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content, J. Biotechnol. 215 (2015) 13–19. doi:10.1016/j.jbiotec.2015.06.400.
[66] R.S. Parmar, C. Singh, A comprehensive study of eco-friendly natural pigment and its applications, Biochem. Biophys. Reports. 13 (2018) 22–26. doi:10.1016/j.bbrep.2017.11.002.
[67] H. Begum, F.M.D. Yusoff, S. Banerjee, H. Khatoon, M. Shariff, Availability and Utilization of Pigments from Microalgae, Crit. Rev. Food Sci. Nutr. 56 (2016) 2209–2222. doi:10.1080/10408398.2013.764841.
[68] S. Takaichi, Carotenoids in algae: Distributions, biosyntheses and functions, Mar. Drugs. 9 (2011) 1101–1118. doi:10.3390/md9061101.
[69] D.B. Rodrigues, C.R. Menezes, A.Z. Mercadante, E. Jacob-Lopes, L.Q. Zepka, Bioactive pigments from microalgae Phormidium autumnale, Food Res. Int. 77 (2015) 273–279. doi:10.1016/j.foodres.2015.04.027.
[70] R.R. Sonani, Recent advances in production, purification and applications of phycobiliproteins, World J. Biol. Chem. 7 (2016) 100. doi:10.4331/wjbc.v7.i1.100.
[71] E. Christaki, P. Florou-Paneri, E. Bonos, Microalgae: A novel ingredient in nutrition, Int. J. Food Sci. Nutr. 62 (2011) 794–799. doi:10.3109/09637486.2011.582460.
[72] S. Bleakley, M. Hayes, Algal Proteins: Extraction, Application, and Challenges Concerning Production, Foods. 6 (2017) 33. doi:10.3390/foods6050033.
[73] F. Depeint, W.R. Bruce, N. Shangari, R. Mehta, P.J. O'Brien, Mitochondrial function and toxicity: Role of the B vitamin family on mitochondrial energy metabolism, Chem. Biol. Interact. 163 (2006) 94–112. doi:10.1016/j.cbi.2006.04.014.
[74] F. Watanabe, S. Takenaka, H. Kittaka-Katsura, S. Ebara, E. Miyamoto, Characterization and bioavailability of vitamin B12-compounds from edible algae, J. Nutr. Sci. Vitaminol. (Tokyo). 48 (2002) 325–331. doi:10.3177/jnsv.48.325.
[75] K.W. Chew, J.Y. Yap, P.L. Show, N.H. Suan, J.C. Juan, T.C. Ling, D.J. Lee, J.S. Chang, Microalgae biorefinery: High value products perspectives, Bioresour. Technol. 229 (2017) 53–62. doi:10.1016/j.biortech.2017.01.006.
[76] S. Topdag, A. Aslaner, C. Tataroglu, Z. Ilce, Evaluation of antioxidant capacity in lung carcinoma, Indian J. Thorac. Cardiovasc. Surg. 21 (2005) 269–271. doi:10.1007/s12055-005-0004-8.
[77] O. Pulz, W. Gross, Valuable products from biotechnology of microalgae, Appl. Microbiol. Biotechnol. 65 (2004) 635–648. doi:10.1007/s00253-004-1647-x.
[78] J. Azmir, I.S.M. Zaidul, M.M. Rahman, K.M. Sharif, A. Mohamed, F. Sahena, M.H.A. Jahurul, K. Ghafoor, N.A.N. Norulaini, A.K.M. Omar, Techniques for extraction of bioactive compounds from plant materials: A review, J. Food Eng. 117 (2013) 426–436. doi:10.1016/j.jfoodeng.2013.01.014.
[79] L.P. Gomez, C. Alvarez, M. Zhao, U. Tiwari, J. Curtin, M. Garcia-Vaquero, B.K. Tiwari, Innovative processing strategies and technologies to obtain hydrocolloids from macroalgae for food applications, Carbohydr. Polym. 248 (2020) 116784. doi:10.1016/j.carbpol.2020.116784.

[80] F. Chemat, M.A. Vian, G. Cravotto, Green extraction of natural products: Concept and principles, Int. J. Mol. Sci. 13 (2012) 8615–8627. doi:10.3390/ijms13078615.
[81] W. Levasseur, P. Perré, V. Pozzobon, A review of high value-added molecules production by microalgae in light of the classification, Biotechnol. Adv. 41 (2020) 107545. doi:10.1016/j.biotechadv.2020.107545.
[82] R. Sathasivam, R. Radhakrishnan, A. Hashem, E.F. Abd_Allah, Microalgae metabolites: A rich source for food and medicine, Saudi J. Biol. Sci. 26 (2019) 709–722. doi:10.1016/j.sjbs.2017.11.003.
[83] M.P. Caporgno, A. Mathys, Trends in Microalgae Incorporation Into Innovative Food Products With Potential Health Benefits, Front. Nutr. 5 (2018) 1–10. doi:10.3389/fnut.2018.00058.
[84] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach, Renew. Sustain. Energy Rev. 55 (2016) 909–941. doi:10.1016/j.rser.2015.11.026.
[85] F. Guihéneuf, D.B. Stengel, Towards the biorefinery concept: Interaction of light, temperature and nitrogen for optimizing the co-production of high-value compounds in Porphyridium purpureum, Algal Res. 10 (2015) 152–163. doi:10.1016/j.algal.2015.04.025.
[86] M.A.H. S.M. Phang, M.S. Miah, B.G. Yeoh, *Spirulina* cultivation in digested sago starch factory wastewater, J. Appl. Phycol. 12 (2000) 395–400.
[87] M.G. Saad, N.S. Dosoky, M.S. Zoromba, H.M. Shafik, Algal biofuels: Current status and key challenges, Energies. 12 (2019). doi:10.3390/en12101920.
[88] M. Song, H. Pei, The growth and lipid accumulation of *Scenedesmus quadricauda* during batch mixotrophic/heterotrophic cultivation using xylose as a carbon source, Bioresour. Technol. 263 (2018) 525–531. doi:10.1016/j.biortech.2018.05.020.
[89] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach, Renew. Sustain. Energy Rev. 55 (2016) 909–941. doi:10.1016/j.rser.2015.11.026.
[90] R. Sathasivam, R. Radhakrishnan, A. Hashem, E.F. Abd_Allah, Microalgae metabolites: A rich source for food and medicine, Saudi J. Biol. Sci. 26 (2019) 709–722. doi:10.1016/j.sjbs.2017.11.003.
[91] M.K. Lam, K.T. Lee, Microalgae biofuels: A critical review of issues, problems and the way forward, Biotechnol. Adv. 30 (2012) 673–690. doi:10.1016/j.biotechadv.2011.11.008.
[92] J. Hu, D. Nagarajan, Q. Zhang, J.S. Chang, D.J. Lee, Heterotrophic cultivation of microalgae for pigment production: A review, Biotechnol. Adv. 36 (2018) 54–67. doi:10.1016/j.biotechadv.2017.09.009.
[93] C.M. Gasol, J. Rieradevall, L. Ruggieri, E. Cadena, J. Martı, X. Gabarrell, T. Gea, X. Sort, A. Sa, C. Valle, Recovery of organic wastes in the Spanish wine industry . Technical , economic and environmental analyses of the composting process, 17 (2009) 830–838. doi:10.1016/j.jclepro.2008.12.005.
[94] R.L.S. and R.P. Singh, Advances in Biological Treatment of Industrial Waste Water and their Recycling for a Sustainable Future, 2019. doi:10.1007/978-981-13-1468-1.
[95] S. Mohana, B.K. Acharya, D. Madamwar, Distillery spent wash : Treatment technologies and potential applications, 163 (2009) 12–25. doi:10.1016/j.jhazmat.2008.06.079.
[96] W.E. Federation, Standard Methods for the Examination of Water and Wastewater Part 1000 Standard Methods for the Examination of Water and Wastewater, Am. Public Heal. Assoc. Washington, DC, USA. (1999).

[97] E.Y. Ortiz Montoya, A.A. Casazza, B. Aliakbarian, P. Perego, A. Converti, J.C.M. De Carvalho, Production of *Chlorella vulgaris* as a source of essential fatty acids in a tubular photobioreactor continuously fed with air enriched with CO_2 at different concentrations, Biotechnol. Prog. 30 (2014) 916–922. doi:10.1002/btpr.1885.
[98] A. Brezoiu, C. Matei, M. Deaconu, A. Stanciuc, A. Trifan, A. Gaspar-pintiliescu, D. Berger, Polyphenols extract from grape pomace . Characterization and valorisation through encapsulation into mesoporous silica-type matrices, Food Chem. Toxicol. 133 (2019) 110787. doi:10.1016/j.fct.2019.110787.
[99] V. Ganeshkumar, S.R. Subashchandrabose, R. Dharmarajan, K. Venkateswarlu, R. Naidu, M. Megharaj, Use of mixed wastewaters from piggery and winery for nutrient removal and lipid production by Chlorella sp. MM3, Bioresour. Technol. 256 (2018) 254–258. doi:10.1016/j.biortech.2018.02.025.
[100] H. Sati, M. Mitra, S. Mishra, P. Baredar, Microalgal lipid extraction strategies for biodiesel production: A review, Algal Res. 38 (2019) 101413. doi:10.1016/j.algal.2019.101413.
[101] G.F. Ferreira, L.F. Ríos Pinto, R. Maciel Filho, L. V. Fregolente, A review on lipid production from microalgae: Association between cultivation using waste streams and fatty acid profiles, Renew. Sustain. Energy Rev. 109 (2019) 448–466. doi:10.1016/j.rser.2019.04.052.
[102] H.S. Kwak, J.Y.H. Kim, H.M. Woo, E.S. Jin, B.K. Min, S.J. Sim, Synergistic effect of multiple stress conditions for improving microalgal lipid production, Algal Res. 19 (2016) 215–224. doi:10.1016/j.algal.2016.09.003.
[103] L. Malandra, G. Wolfaardt, A. Zietsman, M. Viljoen-Bloom, Microbiology of a biological contactor for winery wastewater treatment, Water Res. 37 (2003) 4125–4134. doi:10.1016/S0043-1354(03)00339-7.
[104] R. Torrijos, M and Moletta, Winery wastewater depollution, Wat. Shi. Tech. 35 (1997) 249–257.
[105] A.V. Lindner, D. Pleissner, Utilization of phenolic compounds by microalgae, Algal Res. 42 (2019) 101602. doi:10.1016/j.algal.2019.101602.
[106] C.E. Cerniglia, D.T. Gibson, C. Van Baalen, Oxidation of naphthalene by cyanobacteria and microalgae, J. Gen. Microbiol. 116 (1980) 495–500. doi:10.1099/00221287-116-2-495.
[107] G. Pinto, A. Pollio, L. Previtera, F. Temussi, Biodegradation of phenols by microalgae, (2002) 2047–2051.
[108] A. Papazi, A. Ioannou, M. Symeonidi, A.G. Doulis, K. Kotzabasis, Bioenergetic strategy of microalgae for the biodegradation of tyrosol and hydroxytyrosol, Zeitschrift Fur Naturforsch. - Sect. C J. Biosci. 72 (2017) 227–236. doi:10.1515/znc-2016-0214.
[109] R. Ferrarini, A. Versari, S. Galassi, A preliminary comparison between nanofiltration and reverse osmosis membranes for grape juice treatment, J. Food Eng. 50 (2001) 113–116. doi:10.1016/S0260-8774(00)00199-0.
[110] M. Huy, G. Kumar, H.W. Kim, S.H. Kim, Photoautotrophic cultivation of mixed microalgae consortia using various organic waste streams towards remediation and resource recovery, Bioresour. Technol. 247 (2018) 576–581. doi:10.1016/j.biortech.2017.09.108.
[111] A.A. Casazza, P.F. Ferrari, B. Aliakbarian, A. Converti, P. Perego, Effect of UV radiation or titanium dioxide on polyphenol and lipid contents of *Arthrospira (Spirulina) platensis*, Algal Res. 12 (2015) 308–315. doi:10.1016/j.algal.2015.09.012.
[112] M. Pettinato, B. Aliakbarian, A.A. Casazza, P. Perego, Encapsulation of antioxidants from Spent coffee ground extracts by spray drying, Chem. Eng. Trans. 57 (2017) 1219–1224. doi:10.3303/CET1757204.
[113] G. Pinto, A. Pollio, L. Previtera, M. Stanzione, F. Temussi, Removal of low molecular

weight phenols from olive oil mill wastewater using microalgae, Biotechnol. Lett. 25 (2003) 1657–1659. doi:10.1023/A:1025667429222.
[114] W.S. Chai, W.G. Tan, H.S. Halimatul Munawaroh, V.K. Gupta, S.H. Ho, P.L. Show, Multifaceted roles of microalgae in the application of wastewater biotreatment: A review, Environ. Pollut. 269 (2021) 116236. doi:10.1016/j.envpol.2020.116236.
[115] B. Miyawaki, A.B. Mariano, J.V.C. Vargas, W. Balmant, A.C. Defrancheschi, D.O. Corrêa, B. Santos, N.F.H. Selesu, J.C. Ordonez, V.M. Kava, Microalgae derived biomass and bioenergy production enhancement through biogas purification and wastewater treatment, Renew. Energy. 163 (2021) 1153–1165. doi:10.1016/j.renene.2020.09.045.
[116] J. Arun, K. Panchamoorthy, P. Sundarrajan, V. Felix, M. Joselynmonica, R. Malolan, Bioresource Technology Reports A conceptual review on microalgae biorefinery through thermochemical and biological pathways : Bio-circular approach on carbon capture and wastewater treatment, Bioresour. Technol. Reports. 11 (2020) 100477. doi:10.1016/j.biteb.2020.100477.
[117] E. Spennati, A.A. Casazza, P. Perego, C. Solisio, A. Converti, Microalgae Growth in Winery Wastewater under Dark Conditions, 74 (2019) 1471–1476. doi:10.3303/CET1974246.
[118] A.A. Casazza, E. Spennati, A. Converti, G. Busca, Production of carbon-based biofuels by pyrolysis of exhausted *Arthrospira platensis* biomass after protein or lipid recovery, Fuel Process. Technol. 201 (2020) 106336. doi:10.1016/j.fuproc.2020.106336.
[119] E. Spennati, A.A. Casazza, A. Converti, Winery wastewater treatment by microalgae to produce low-cost biomass for energy production purposes, Energies. 13 (2020). doi:10.3390/en13102490.
[120] Â.P. Matos, R. Coeli, D.O. Torres, L. Rodrigo, I. Morioka, E. Helena, S. Moecke, K.B. França, E. Sebastião, S. Anna, Growing *Chlorella vulgaris* in Photobioreactor by Continuous Process Using Concentrated Desalination : Effect of Dilution Rate on Biochemical Composition, Int. J. Chem. Eng. 2014 (2014).
[121] C.E.N. Sassano, L.A. Gioielli, L.S. Ferreira, M.S. Rodrigues, S. Sato, A. Converti, J.C.M. Carvalho, Evaluation of the composition of continuously-cultivated *Arthrospira (Spirulina) platensis* using ammonium chloride as nitrogen source, Biomass and Bioenergy. 34 (2010) 1732–1738. doi:10.1016/j.biombioe.2010.07.002.
[122] P. Praveen, J.Y.P. Heng, K.C. Loh, Tertiary wastewater treatment in membrane photobioreactor using microalgae: Comparison of forward osmosis & microfiltration, Bioresour. Technol. 222 (2016) 448–457. doi:10.1016/j.biortech.2016.09.124.
[123] Y. Luo, P. Le-Clech, R.K. Henderson, Simultaneous microalgae cultivation and wastewater treatment in submerged membrane photobioreactors: A review, Algal Res. 24 (2017) 425–437. doi:10.1016/j.algal.2016.10.026.
[124] J. González-Camejo, R. Barat, D. Aguado, J. Ferrer, Continuous 3-year outdoor operation of a flat-panel membrane photobioreactor to treat effluent from an anaerobic membrane bioreactor, Water Res. 169 (2020). doi:10.1016/j.watres.2019.115238.
[125] F. Gao, Z.H. Yang, C. Li, Y. jie Wang, W. hong Jin, Y. bing Deng, Concentrated microalgae cultivation in treated sewage by membrane photobioreactor operated in batch flow mode, Bioresour. Technol. 167 (2014) 441–446. doi:10.1016/j.biortech.2014.06.042.
[126] G. Singh, P.B. Thomas, Bioresource Technology Nutrient removal from membrane bioreactor permeate using microalgae and in a microalgae membrane photoreactor, Bioresour. Technol. 117 (2012) 80–85. doi:10.1016/j.biortech.2012.03.125.
[127] L. Marbelia, M.R. Bilad, I. Passaris, V. Discart, D. Vandamme, A. Beuckels, K. Muylaert, I.F.J. Vankelecom, Membrane photobioreactors for integrated microalgae cultivation and

nutrient remediation of membrane bioreactors effluent, Bioresour. Technol. 163 (2014) 228–235. doi:10.1016/j.biortech.2014.04.012.
[128] B.E. Kates, M., Volcani, Lipid components of diatoms, Biochim. Biophys. Acta - Lipids Lipid Metab. Volume 116 (1966) 264–278.
[129] P. Zunin, P. Salvadeo, R. Boggia, F. Evangelisti, Sterol oxidation in meat- and fish-based homogenized baby foods containing vegetable oils, J. AOAC Int. 89 (2006) 441–446.
[130] I. Rawat, R. Ranjith Kumar, T. Mutanda, F. Bux, Biodiesel from microalgae: A critical evaluation from laboratory to large scale production, Appl. Energy. 103 (2013) 444–467. doi:10.1016/j.apenergy.2012.10.004.
[131] M.R. Andrade, J.A. V Costa, Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate, 264 (2007) 130–134. doi:10.1016/j.aquaculture.2006.11.021.
[132] T. Heredia-arroyo, W. Wei, R. Ruan, B. Hu, Mixotrophic cultivation of *Chlorella vulgaris* and its potential application for the oil accumulation from non-sugar materials, 5 (2011). doi:10.1016/j.biombioe.2011.02.036.
[133] V. Andreotti, A. Solimeno, S. Rossi, E. Ficara, F. Marazzi, V. Mezzanotte, J. García, Bioremediation of aquaculture wastewater with the microalgae Tetraselmis suecica: Semi-continuous experiments, simulation and photo-respirometric tests, Sci. Total Environ. 738 (2020) 139859. doi:10.1016/j.scitotenv.2020.139859.
[134] X.B. Tan, X.C. Zhao, Y.L. Zhang, Y.Y. Zhou, L. Bin Yang, W.W. Zhang, Enhanced lipid and biomass production using alcohol wastewater as carbon source for *Chlorella pyrenoidosa* cultivation in anaerobically digested starch wastewater in outdoors, Bioresour. Technol. 247 (2018) 784–793. doi:10.1016/j.biortech.2017.09.152.
[135] M. Moon, C.W. Kim, W. Farooq, W.I. Suh, A. Shrivastav, M.S. Park, S.K. Mishra, J.W. Yang, Utilization of lipid extracted algal biomass and sugar factory wastewater for algal growth and lipid enhancement of *Ettlia sp.*, Bioresour. Technol. 163 (2014) 180–185. doi:10.1016/j.biortech.2014.04.033.
[136] P.L. Gupta, H.J. Choi, R.R. Pawar, S.P. Jung, S.M. Lee, Enhanced biomass production through optimization of carbon source and utilization of wastewater as a nutrient source, J. Environ. Manage. 184 (2016) 585–595. doi:10.1016/j.jenvman.2016.10.018.
[137] A. Anbalagan, S. Schwede, C.F. Lindberg, E. Nehrenheim, Influence of hydraulic retention time on indigenous microalgae and activated sludge process, Water Res. 91 (2016) 277–284. doi:10.1016/j.watres.2016.01.027.
[138] E. Spennati, A.A. Casazza, A. Converti, G. Busca, Thermocatalytic pyrolysis of exhausted arthrospira platensis biomass after protein or lipid recovery, Energies. 13 (2020). doi:10.3390/en13205246.
[139] I. İlter, S. Akyıl, Z. Demirel, M. Koç, M. Conk-Dalay, F. Kaymak-Ertekin, Optimization of phycocyanin extraction from *Spirulina platensis* using different techniques, J. Food Compos. Anal. 70 (2018) 78–88. doi:10.1016/j.jfca.2018.04.007.
[140] W. Pan-utai, S. Iamtham, Physical extraction and extrusion entrapment of C-phycocyanin from *Arthrospira platensis*, J. King Saud Univ. Sci. 31 (2019) 1535–1542. doi:10.1016/j.jksus.2018.05.026.
[141] G. Patil, S. Chethana, A.S. Sridevi, K.S.M.S. Raghavarao, Method to obtain C-phycocyanin of high purity, J. Chromatogr. A. 1127 (2006) 76–81. doi:10.1016/j.chroma.2006.05.073.
[142] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976 May 7;72:248-54. doi: 10.1006/abio.1976.9999., (n.d.).
[143] S.P. Kamble, R.B. Gaikar, R.B. Padalia, K.D. Shinde, Extraction and purification of C-phycocyanin from dry *Spirulina* powder and evaluating its antioxidant , anticoagulation and

prevention of DNA damage activity, 3 (2013) 149–153. doi:10.7324/JAPS.2013.3826.
[144] S.T. Silveira, J.F.M. Burkert, J.A. V Costa, C.A. V Burkert, S.J. Kalil, Optimization of phycocyanin extraction from *Spirulina platensis* using factorial design, 98 (2007) 1629–1634. doi:10.1016/j.biortech.2006.05.050.
[145] T. Masuko, A. Minami, N. Iwasaki, T. Majima, S.I. Nishimura, Y.C. Lee, Carbohydrate analysis by a phenol-sulfuric acid method in microplate format, Anal. Biochem. 339 (2005) 69–72. doi:10.1016/j.ab.2004.12.001.
[146] M. Wan, P. Liu, J. Xia, J.N. Rosenberg, G.A. Oyler, M.J. Betenbaugh, Z. Nie, G. Qiu, The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in *Chlorella sorokiniana*, Appl. Microbiol. Biotechnol. 91 (2011) 835–844. doi:10.1007/s00253-011-3399-8.
[147] S.K. Wang, X. Wang, J. Miao, Y.T. Tian, Tofu whey wastewater is a promising basal medium for microalgae culture, Bioresour. Technol. 253 (2018) 79–84. doi:10.1016/j.biortech.2018.01.012.
[148] N.M. Apandi, R.M.S. Radin Mohamed, N.A.A. Latiffi, N.F.M. Rozlan, A.A.S. Al-Gheethi, Protein and Lipid Content of Microalgae *Scenedesmus sp.* Biomass Grown in Wet Market Wastewater, MATEC Web Conf. 103 (2017). doi:10.1051/matecconf/201710306011.
[149] M. Dejsungkranont, H. Chen, Enhancement of antioxidant activity of C-phycocyanin of *Spirulina* powder treated with supercritical fl uid carbon dioxide, Agric. Nat. Resour. 51 (2017) 347–354. doi:10.1016/j.anres.2017.12.001.
[150] R. Seghiri, J. Legrand, R. Hsissou, A. Essamri, Comparative study of the impact of conventional and unconventional drying processes on phycobiliproteins from *Arthrospira platensis*, Algal Res. 53 (2021) 102165. doi:10.1016/j.algal.2020.102165.
[151] A.V. Ursu, A. Marcati, T. Sayd, V. Sante-Lhoutellier, G. Djelveh, P. Michaud, Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*, Bioresour. Technol. 157 (2014) 134–139. doi:10.1016/j.biortech.2014.01.071.
[152] R. Sharma, Effects of Culture Conditions on Growth and Biochemical Profile of *Chlorella Vulgaris*, J. Plant Pathol. Microbiol. 03 (2012). doi:10.4172/2157-7471.1000131.
[153] H.M. Khairy, E.M. Ali, S.M. Dowidar, Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of Chlorella vulgaris Beijerinck, African J. Biotechnol. 10 (2011) 13514–13519. doi:10.5897/ajb11.1931.
[154] B.H.J. Yap, S.A. Crawford, R.R. Dagastine, P.J. Scales, G.J.O. Martin, Nitrogen deprivation of microalgae: effect on cell size, cell wall thickness, cell strength, and resistance to mechanical disruption, J. Ind. Microbiol. Biotechnol. 43 (2016) 1671–1680. doi:10.1007/s10295-016-1848-1.
[155] L. Soto-sierra, P. Stoykova, Z.L. Nikolov, Extraction and fractionation of microalgae-based protein products, Algal Res. 36 (2018) 175–192. doi:10.1016/j.algal.2018.10.023.
[156] J. Cheng, R. Huang, T. Yu, T. Li, J. Zhou, K. Cen, Biodiesel production from lipids in wet microalgae with microwave irradiation and bio-crude production from algal residue through hydrothermal liquefaction, Bioresour. Technol. 151 (2014) 415–418. doi:10.1016/j.biortech.2013.10.033.
[157] B.P. Nobre, F. Villalobos, B.E. Barragán, A.C. Oliveira, A.P. Batista, P.A.S.S. Marques, R.L. Mendes, H. Sovová, A.F. Palavra, L. Gouveia, A biorefinery from *Nannochloropsis sp.* microalga - Extraction of oils and pigments. Production of biohydrogen from the leftover biomass, Bioresour. Technol. 135 (2013) 128–136. doi:10.1016/j.biortech.2012.11.084.
[158] S.D. Stefanidis, K.G. Kalogiannis, E.F. Iliopoulou, A.A. Lappas, P.A. Pilavachi, In-situ upgrading of biomass pyrolysis vapors: Catalyst screening on a fixed bed reactor, Bioresour.

Technol. 102 (2011) 8261–8267. doi:10.1016/j.biortech.2011.06.032.
[159] F. Li, S.C. Srivatsa, S. Bhattacharya, A review on catalytic pyrolysis of microalgae to high-quality bio-oil with low oxygeneous and nitrogenous compounds, Renew. Sustain. Energy Rev. 108 (2019) 481–497. doi:10.1016/j.rser.2019.03.026.
[160] Yamaguchi, Determination of the Nitrogen-to-Protein Conversion Factor in Cereals. In: Linskens H.F., Jackson J.F. (eds) Seed Analysis. Modern Methods of Plant Analysis, vol 14. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-01639, (1992).
[161] I.D. Adamakis, P.A. Lazaridis, E. Terzopoulou, S. Torofias, M. Valari, P. Kalaitzi, V. Rousonikolos, D. Gkoutzikostas, A. Zouboulis, G. Zalidis, K.S. Triantafyllidis, Cultivation, characterization, and properties of Chlorella vulgaris microalgae with different lipid contents and effect on fast pyrolysis oil composition, Environ. Sci. Pollut. Res. 25 (2018) 23018–23032. doi:10.1007/s11356-018-2368-5.
[162] Z. Du, B. Hu, X. Ma, Y. Cheng, Y. Liu, X. Lin, Y. Wan, H. Lei, P. Chen, R. Ruan, Catalytic pyrolysis of microalgae and their three major components: Carbohydrates, proteins, and lipids, Bioresour. Technol. 130 (2013) 777–782. doi:10.1016/j.biortech.2012.12.115.
[163] S.R.A. Kersten, W.P.M. van Swaaij, L. Lefferts, K. Seshan, Options for Catalysis in the Thermochemical Conversion of Biomass into Fuels, Catal. Renewables From Feed. to Energy Prod. (2007) 119–145. doi:10.1002/9783527621118.ch6.
[164] S.C. Moldoveanu, Analytical pyrolysis of natural organic polymers, 1998. doi:10.1017/CBO9781107415324.004.
[165] J. Yanik, R. Stahl, N. Troeger, A. Sinag, Pyrolysis of algal biomass, J. Anal. Appl. Pyrolysis. 103 (2013) 134–141. doi:10.1016/j.jaap.2012.08.016.
[166] K.Y. Chan, Z. Xu, Biochar: Nutrient properties and their enhancement, Biochar Environ. Manag. Sci. Technol. (2012) 67–84. doi:10.4324/9781849770552.