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Study and optimization of the recycling process of the *Chlorella vulgaris* culture medium and its effects on the functional properties of biomass for food applications

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Final version

Coimbra, 2020

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Internship report presented to Escola Superior Agrária de Coimbra to fulfill the requirements for obtaining a master degree in Biotechnology

Final version

Coimbra, 2020

"It's not love or money that makes the world go round, it's photosynthesis."

(Masojídek, et al., 2004)

"(…) a world in which food is nutritious and accessible for everyone and natural resources are managed in a way that maintains ecosystem functions to support current as well as future human needs."

(FAO, 2014).

AGRADECIMENTOS

A todos aqueles que de alguma forma contribuíram com o seu tempo para tornar possível a concretização deste trabalho, expresso a minha enorme gratidão.

Começar por agradecer à Joana Laranjeira por abrir portas, pela oportunidade de contribuir, de aprender, e de aplicar aprendizagens na Allmicroalgae. Agradecer também por ter garantido alimentação, EPI's e alojamento para os estagiários, que considero apoios de grande importância neste tipo de colaborações. À professora Marta Henriques, muito obrigada por ter aceitado orientar-me; pelo seu tempo, pela sua sempre célere e pronta ajuda, pelas sugestões e revisão do manuscrito, pela partilha de conhecimentos.

Um enorme obrigada à Margarida, pela sua orientação no terreno ao longo deste ano tão desafiante. As contaminações, as alterações no projeto, as mudanças de instalações, somaram-se aos desafios lançados pela pandemia. Foi um ano absolutamente louco, mas pude sempre contar com as suas ideias, sugestões, revisões, apoio e motivação; que se revelaram basilares para a concretização e apresentação deste projeto. Ainda, agradecer o enorme apoio, e o da Maria Soares, pelas boleias diárias entre Leiria e Pataias. À Maria Soares, agradecer também a partilha de ideias e o entusiasmo das discussões sobre o projeto, a amizade de anos e as idas às camarinhas, aos jogos, passeios entomológicos e afins. Agradeço o apoio extraordinário de toda a equipa da unidade de I&D e realço a disponibilidade de todos na partilha de conhecimentos. À Joana Teles, por fazer sempre mais, pela prontidão com que solucionou problemas e por me ter ajudado a soldar e montar os leds para os ensaios. À Inês, pela vigilância durante a inoculação do ensaio em plenos 40º C, pelas sucessivas ajudas na montagem dos 12 reatores no exterior, pela calma e simpatia. Ao Pedro Cunha, por ser o melhor cortador de sacos, por me ajudar a carregar IBC's juntamente com a Inês, por todo o apoio dado e pela honestidade desmesurada. À Nádia, pela ajuda no laboratório nos mais diversos procedimentos, pelas conversas e pela playlist matinal carregada de energia positiva. Agradecer ainda ao Hugo Galvão por ter contribuído com críticas pertinentes e construtivas ao longo do trabalho.

Agradeço ao Pedro Quelhas, por me ajudar no processo de recolha dos permeados e no entendimento do processo de colheita, bem como a todo o pessoal do processamento que ajudou na recolha. Ao Dinis, pelo esclarecimento de todos os parâmetros relacionados

ao cultivo autotrófico, a nível industrial. À Adriana, à Mafalda, ao Bernardo e à Ana Barros, agradeço por todas as dicas sobre o processo de adaptação da *Chlorella* vinda de heterotrofia, pela paciência, disponibilidade, e fornecimento de inóculo. À Joana G. pelas boleias, à Soraia pela simpatia e por abrir o portão inúmeras vezes. À Joana R. e Inês, agradeço por terem aturado o barulho da bomba de vácuo, a funcionar durante meses. À Mariana, pela dica que salvou a saga de centrifugações. À Tamára, pela ajuda na primeira noite a centrifugar, e pelo apoio dado às análises das minhas amostras no CCMAR. A todos os colegas do turno da noite, que foram vigiando o meu bem-estar aquando as largas noites de trabalho, e às diversas equipas de limpeza, agradeço pela sua boa disposição e simpatia.

Aos colegas de tese, Alexandra, Ana, Gonçalo e Sara, agradeço a troca de ideias, os desabafos, e a ajuda na altura de montar reatores e recolher permeado no exterior. Este ano teria sido ainda mais difícil sem vocês. Um agradecimento muito especial à Alexandra por ter analisado as minhas amostras ao nível da proteína e cinzas, aquando da minha obrigatoriedade de isolamento profilático. À Lara, aos técnicos Isabel Costa, Sandrine e José Viegas agradeço pela ajuda na ESAC.

Aos meus pais, pela força de trabalho, determinação e pensamento positivo que sempre me incutiram. Aos meus irmãos, em especial à minha irmã, pelo apoio imensurável; ao Hugo, pelo pensamento crítico; e aos meus sobrinhos pelo recarregar de energias e alegrias. Foi bom voltar a Leiria para estarmos todos mais próximos, apesar das distâncias impostas.

O maior agradecimento devo-o ao João, que ficou sozinho nos meses em que estive em Leiria, pelo apoio, incentivo, amor e carinho ao longos destes meses tão desgastantes. Obrigada por ser o melhor companheiro, no trabalho de equipa que é a vida a dois.

O caminho faz-se caminhando. Obrigada a todos os que tornaram esta caminhada mais leve!

ABSTRACT

Microalgae are considered a sustainable solution to meet the high demand for food and feed worldwide in a near future. However, its cultivation requires a considerable amount of water (fresh or marine), threatening the sustainability of its production. With a certified organic cultivation mode, *Chlorella vulgaris* is the core business of Allmicroalgae, being sold mainly for food applications. To reduce the water footprint of the process, the permeate resulting from the biomass harvesting, through tangential flow filtration membranes, was used and tested to regrow *C. vulgaris* autotrophically on a laboratory and pilot-scale, for three consecutive reuses (R1, R2, and R3). The permeate was supplemented with ammonium and other nutrients and tested as a recycled culture medium (RCM) at three different concentrations, 100% (fully reused – P100), 70%, and 50% (P70 and P50). Growth assessment was evaluated and the physicochemical and technofunctional properties of the obtained biomass were evaluated. At the laboratory-scale, no significant differences were observed between the productivity and the specific growth rate of the control and the conditions tested. At the pilot scale, 3 consecutive reuses were performed and *C. vulgaris* was successfully cultivated under RCM with no differences between fresh medium (control) and RCM. An exception was observed for the case of 100% permeate in the growth of *C. vulgaris* in the third reuse, where the productivity was significantly lower than the control. After evaluating the total protein content, ash, pigments and technofunctional properties of the biomass, it was found that only the chlorophyll content of the P100 cultivation was negatively affected by the third reuse of the RCM (R3). These results showed the possibility of reusing RCM 3 times up to 70% of permeate, at least, without reducing the productivity and quality of the biomass compared to the control.

Keywords *Chlorella vulgaris*, culture medium recycling, pilot-scale, sustainability, nutritional composition

RESUMO

As microalgas são consideradas uma solução sustentável global que responderá às necessidades alimentares, humanas e animais, num futuro próximo. No entanto, o seu cultivo requer um uso considerável de água (doce ou salgada), ameaçando a sustentabilidade da sua produção. Produzida em modo biológico, *Chlorella vulgaris* é comercializada para fins alimentares como negócio principal da Allmicroalgae. De forma a reduzir a pegada hídrica do processo, o permeado, resultante da colheita de biomassa através do processo de filtração de fluxo tangencial, foi reutilizado e testado por 3 vezes consecutivas (R1, R2 e R3) para o cultivo autotrófico de *C. vulgaris*, às escalas laboratorial e piloto. O permeado foi suplementado com amónia e outros nutrientes e testado como meio de cultura reciclado (RCM) para ser reutilizado em três concentrações diferentes, 100% (reutilização total – P100), 70% e 50% (P70 e P50). Comparou-se o crescimento das culturas e as propriedades físico-químicas e funcionais da biomassa foram avaliadas. À escala laboratorial, não foram observadas diferenças significativas nas produtividades e taxa especifica de crescimento entre o controlo e as condições testadas. À escala piloto, foram realizadas 3 reutilizações consecutivas, nas quais *C. vulgaris* cresceu com sucesso e não apresentou diferenças de crescimento entre meio fresco (controlo) e RCM. Observou-se uma exceção na terceira reutilização, no caso de 100% de permeado, onde a produtividade foi significativamente mais baixa do que no controlo. Após avaliar a composição de proteína, cinzas, pigmentos e as propriedades funcionais da biomassa, concluiu-se que apenas o conteúdo em clorofila no cultivo P100 foi afetado negativamente aquando da terceira reutilização de RCM (R3). Estes resultados mostram a possibilidade de reutilizar RCM 3 vezes, até, pelo menos, aos 70 % de permeado, sem reduzir a produtividade e qualidade da biomassa ao comparar com o controlo.

Palavras chave *Chlorella vulgaris*, reciclagem meio cultura, escala piloto, sustentabilidade, composição nutricional

ABBREVIATIONS LIST

BC	bubble column
DOM	dissolved organic matter
EAI	emulsifying activity index
ESI	emulsifying stability index
EU	European Union
FAO	food and agriculture organization of the United Nations
FC	foaming capacity
FCM	fresh culture medium
FM	fresh medium
FP	flat panel
FS	foaming stability
GRAS	generally recognized as safe
OHC	oil holding capacity
P100	one hundred percent of permeate
P50	fifty percent of permeate
P70	seventy percent of permeate
PBR	photobioreactor
R1	first reuse
R2	second reuse
R3	third reuse

RCM	recycled culture medium
T	temperature
TFF	tangential flow filtration
WBCSD	world business council for sustainable development
WHC	water holding capacity

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HOST INSTITUTION: ALLMICROALGAE

Located in the small town of Pataias, close to Leiria, Allmicroalgae (Figure 1) is a Portuguese joint venture by Secil – the biggest Portuguese cement company, and the French Biotechnological company GreenTech. Its industrial microalgae production unit was established in 2013, right next to the Cibra-Pataias cement factory with the main goal of offsetting the greenhouse gas emissions produced.

The microalgae produced are mainly for food and feed and as a result of the company's commitment to constantly improve the cultivation of microalgae and to seek new market opportunities, Allmicroalgae has its research and development unit. In this way, Allmicroalgae participates in several national and international research projects while hosting students for the development of new projects. The present work results from the development of one of those projects.

Figure 1. Aerial photograph of Allmicroalgae and cement factory (**Pontos de vista, 2017**).

1. INTRODUCTION

1.1. FOOD INDUSTRY TRENDS AND NOVEL FOODS

Consumers are increasingly demanding and the food industry is forced to deliver food products that, besides being tasty, must be also nutritious, healthy, and sustainable, responding to those looking for healthier lifestyles with pleasant diets (WBCSD, 2018). Evidence shows the need to change eating behavior and diets worldwide to reduce the risk of cardiometabolic disorders (Imamura, et al., 2015), since the leading cause of diet-related deaths is attributed to cardiovascular disease (GBD, 2019). Alternative sources of nutrients can help to fight this problem (Gouveia, et al., 2008). At the same time that billions of people around the world still suffer from deficiencies in proteins and micronutrients (FAO, 2018), it is possible to observe this trend of healthier eating, strongly driven by the millennial generation[1], where there is a demand for natural, organic, simple, and less processed foods (WBCSD, 2018). A trend observed in almost all developed countries paired with the search for unique food experiences, new ingredients, and flavors, and a plant-based diet. In addition to these niche markets, the main trend and concern is to find affordable food options that are also safe and nutritious (WBCSD, 2018).

In the European Union (EU), a *novel food product* is defined as "food and food ingredient that had not been consumed to a significant degree by the human in the EU before 15 May 1997 (…) and that can be newly developed, innovative food, food produced using new technologies and production processes, as well as food which is or has been traditionally eaten outside of the EU" (EU, 2020). According to the regulation (EU) 2015/2283 on novel foods, those include "Foods consisting of or isolated from microorganisms, fungi or algae".

Although microalgae have been used for centuries, their industrial cultivation is relatively recent (Gouveia, et al., 2008). Used alone, as a food or food supplement or other combinations, due to their nutritional properties microalgae are considered one of the

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[1] generation of anyone born between 1981 and 1996, according to Pew research center (2019).

sustainable solutions of the future to meet the high demand for food and feed (Torres-Tiji, et al., 2020; Probst, et al., 2015).

1.2. MICROALGAE

Microalgae is a group of highly diverse microscopic photosynthetic organisms, including not only eukaryotic but also prokaryotic, such as cyanobacteria. As primary producers, microalgae can be found in almost any environment, but mainly in water, whether marine, freshwater, brackish, or even in wastewater (Lee, 2008). Microalgae may be either benthic, living in the upper layer of shelf sediments (Pinckney, 2018), or planktonic living in the water column. They can occur in different forms of cellular organization such as unicellular (flagellate or non-flagellate), colonial (flagellate or non-flagellate), and filamentous (Tomaselli, 2004). These microorganisms use different metabolic pathways, mainly autotrophy that requires light and an inorganic carbon source, but also heterotrophy that allows them to grow in the absence of light when supplemented with an organic carbon source. Mixotrophic growth can also be found and is characterized as a mixture of the two types of metabolism (Meng, et al., 2020).

Photosynthesis occurs in the chloroplasts, where light-dependent and light-independent reactions are involved. Light is absorbed by photopigments, *e.g.* chlorophylls, and several redox reactions take place. Sunlight energy uses water as a substrate to convert it into oxygen (O_2) and chemical energy which in turn is used to assimilate CO_2. In general, sunlight energy is used to synthesize oxygen and organic compounds from carbon dioxide and water (Moroney & Somanchi, 1999; Masojídek, et al., 2004; Blankenship, 2014; Johnson, 2016) . Part of the released O_2 is used again by microalgae through metabolic reactions such as photorespiration or dark respiration (Masojídek, et al., 2004). Through dark respiration, O_2 and carbohydrates are consumed, resulting in the formation of CO_2, water, and energy. The resulting energy is of great importance since it is used by microalgae for cell maintenance and biosynthetic processes contributing to cell growth (Geider & Osborne, 1989; Edmundson & Huesemann, 2015). Dark respiration rates are higher in the darkness which can result in some losses of biomass and productivity during the dark period (Dinghui, et al., 2011; Edmundson & Huesemann, 2015).

Despite the existence of mandatory photoautotrophic microalgae, many species switch to heterotrophy: the metabolism (dark respiration) doesn't fix carbon through photosynthesis, but consumes organic carbon, while O_2 is used and CO_2 released (Khan, et al., 2016). In heterotrophy, organic substrates like glucose, acetate, ethanol, glycerol, etc., are the most commonly used to provide energy and carbon to cells (Silva, et al., 2019) and each has its associated metabolic pathway (Khan, et al., 2016). Depending on the composition of the culture medium and the photobioreactor, microalgae can carry mixotrophy: simultaneously metabolizing inorganic and organic carbon sources through photo-autotrophy and heterotrophy, respectively (Silva, et al., 2019).

1.3. BIOTECHNOLOGICAL APPLICATIONS

When artificially cultivated in an aerated liquid culture media, autotrophic microalgae can easily have access to light, CO_2, and nutrients. Thus, its simple unicellular structure, ability to grow all year in saline and non-fertile conditions, and rapid growth, give these organisms the capacity to be more efficient than plants in terms of converting solar energy to biomass and fixing CO_2 (Benedetti, et al., 2018). When exposed to environmental changes, microalgae can adapt their metabolism (Driver, et al., 2015). This flexibility of carbon metabolism and energy conversion can promote efficiency and sustainability in microalgae bioproducts production platforms (Sun, et al., 2018). Therefore, there is growing interest in the field of microalgae cultivation and biotechnological research to understand all possible applications of microalgae biomass and its valuable compounds, according to each cultivated specie, strain, and metabolism (Pulz, 2004; Benedetti, et al., 2018).

1.3.1. FOOD AND NUTRITIONAL APPLICATIONS

Microalgae produce a variety of bioproducts with interesting characteristics for the food industry: pigments, lipids, proteins, vitamins, antioxidants, polysaccharides, and bioactive compounds (Khan, et al., 2018). Due to its nutritional properties, microalgae powder or its bioproducts are marketed as food supplements or incorporated into functional food or feed, to increase its nutritional value (Probst, et al., 2015; Khan, et al., 2018).

Although they are often sold in the form of capsules, tablets, or dry powder, the consumer demands for innovative food products and, therefore, the functional properties of microalgae must be studied to better understand how they can be used in the food industry. Whether due to their sensory, nutritional, physicochemical or kinetics properties (Rahman & McCarthy, 1999), microalgae can effectively contribute to increasing the quality of a food product (Caporgno & Mathys, 2018; Gouveia, et al., 2006; Gouveia, et al., 2008).

The high quality and protein content in microalgae is promising for functional food formulations as an alternative to conventional protein sources, contributing to meet the population protein demands by having all essential amino acids (Kose, et al., 2017; Caporgno & Mathys, 2018).

The functional properties of microalgae proteins make them competitive against commercially available ingredients for emulsifying (Ursu, et al., 2014), coloring, and flavoring, among others (Guil-Guerrero, et al., 2004) (Waghmare, et al., 2016).

Photosynthetic pigments such as chlorophylls, carotenoids (β-carotene, astaxanthin), and phycobiliproteins can be added in emulsions to replace synthetic food dyes and preservatives as well as antioxidant and antimicrobial (Shakeri, et al., 2018; Gouveia, et al., 2006; Metsoviti, et al., 2020). Volatile compounds may be used for sensorial purposes (Durme, et al., 2013). Polyunsaturated fatty acids like long-chain ω-3 and ω-6 are essential lipids in healthy human nutrition. The intake of ω-3 is related to the reduction of chronic and inflammatory diseases (Lane, et al., 2014) and, therefore, used as a nutritional supplement (Grima, et al., 2004) (Andrade, et al., 2018) (Metsoviti, et al., 2020). Evidences show that the intake of algae oils increases plasma DHA (a ω-3) and blood erythrocytes, reducing the risk for coronary heart diseases (Bucher, et al., 2012). DHA is also required for brain function (Lane, et al., 2014). Microalgae are pointed as a good source of polyunsaturated fatty acids (better than some seed oils), especially for people following vegetarian diets, since the main source of ω-3 remains fish oils (Lane, et al., 2014). At the bottom of the food chain, microalgae are the primary producers of polyunsaturated fatty acids (Adarme-Vega, et al., 2012). By consuming microalgae, the environmental impact is been reduced (Keller, et al., 2017) as well as the bioaccumulation

of toxic metals associated with higher consumption of trophic levels (Avagyan, 2008; Souza, et al., 2012).

Microalgae also produce vitamins, with A being the most abundant (Solomons, 2012) followed by vitamins C, B1, B2, B3, B5, B6, B9, E, and K, which are used for human metabolism in different function: to strengthen the immune system, cells regeneration, detoxication, anti-inflammatory processes, among others (Andrade, et al., 2018). Vitamin content enhances the nutritional value of microalgae, although it is strongly dependent on factors of cultivation, harvesting, and drying methods (Andrade, et al., 2018).

Certain species (*e.g. Arthrospira* spp.) may increase the viability of the probiotics when supplemented with fermented dairy products (Varga, et al., 2002; Caporgno & Mathys, 2018). Aligned with that, polysaccharides and oligosaccharides are also capturing some attention as potential prebiotic compounds (Andrade, et al., 2018; Hadebe, 2016) which positively affects gut health, improving general health (Caporgno & Mathys, 2018; Andrade, et al., 2018).

Despite all the potential health benefits, only a few microalgae species are well studied and have undergone a safety assessment. Among these scarce species, *Chlorella vulgaris* is recognized as safe (GRAS status) and therefore can be used as a food ingredient and as part of a balanced diet (Andrade, et al., 2018; Caporgno & Mathys, 2018; Barkia, et al., 2019).

1.3.2. PHARMACEUTICAL APPLICATIONS

Several bioactive compounds can be extracted from microalgae as a healthy ingredient for therapeutic purposes. The microalgae potential in the pharmaceutical industry is enormous due to antioxidant, antiviral, anticancer, antitumor, antiobesity, neuroprotective, antimicrobial, antinociceptive, and anti-inflammatory activities (Ahmadi, et al., 2015; Barkia, et al., 2019).

The antioxidant capacity of microalgae phenolic compounds is highly appreciated by the pharmaceutical industry for application in diseases associated to oxidation (Coêlho, et al., 2019; Andrade, et al., 2018). Polysaccharides like the β-1,3-glucan, present in *Chlorella* sp., have a very high nutritional value and works as an active

immunostimulator, antioxidant, and are reported to have antitumor effects (Iwamoto, 2004; Tanaka, et al., 1984). Nostoflan, a polysaccharide present in a blue-green alga, has antiviral activity against the Herpes simplex virus (Kanekiyo, et al., 2007) and other polysaccharides with antiviral activity exist, having different viruses as a target (Ahmadi, et al., 2015). Microalgae peptides have been also associated to antioxidative, antihypertensive, immunomodulatory, anticancer, hepato-protective, and anticoagulant activities (Caporgno & Mathys, 2018). The anti-inflammatory activity of aqueous algal extracts (Guzmán, et al., 2001), β-carotene, and phycocyanin has been reported (Andrade, et al., 2018).

A trend in biopharmaceuticals production is emerging where genetic transformation is enabling the use of microalgae as a bioreactor to produce recombinant proteins for different purposes (Yan, et al., 2016; Ahmad, et al., 2020). Ingestion of microalgae pills containing an antigen is a possibility for the future of oral vaccines (Yan, et al., 2016), being one of the many examples of applications of microalgae in this field.

1.3.3. Wastewater treatment, Bioremediation, and CO_2 mitigation

Wastewater treatment (WWT) aims to return the polluted water and soil to their original state. Rich in nutrients like N and P, wastewater is a promising and sustainable platform to support microalgae growth bringing benefits to WWT once they oxygenate water while removing the excess of nutrients (Rawat, et al., 2011). Unlike chemical treatment, it does not add secondary pollution to the process, since the produced biomass can be reused for different purposes, such as food, feed, biofertilizers, biofuel, etc. The possibility of microalgae commercialization is, in this case, a win-win situation (Rawat, et al., 2011; Ferreira, et al., 2019).

Heavy metals continue to be released by different industries into the environment and microalgae can absorb these contaminants from wastewater for what is called a bioremediation process (Guevara-García, et al., 2017). High concentrations of metals can lead to cellular death and the possibility of using genetically modified microalgae with increased tolerance to heavy metals has been discussed throughout the literature (Rugnini, et al., 2017; Hallmann, 2019).

While consuming nutrients and absorbing contaminants from wastewater, microalgae consume carbon from the organic matter dissolved in wastewater, or inorganic from CO_2 (Arbib, et al., 2014). To offset carbon emissions, some highly polluting industries, such as power plants or cement industries *e.g.* CMP - Cimentos Maceira e Pataias have implemented ways to cultivate photoautotrophic organisms like microalgae that can transform a pollutant into a financial revenue while contributing to CO_2 mitigation (Farrelly, et al., 2013).

1.3.4. BIOFUELS

Microalgae can be used for the production of biofuel, reducing the environmental impact compared to fossil fuels, which is why researchers point out the potential of this renewable resource for the production of bioethanol biodiesel (Carneiro, et al., 2017). The high content of carbohydrates present in microalgae can be used as a substrate to produce bioethanol by fermenting microorganisms (Özçimen & İnan, 2015; Silva & Bertucco, 2019). On the other hand, the extraction and transesterification of energy-rich oils, *e.g* lipids, can result in biodiesel production, but species chosen must have a high lipid content, high productivity, and low-cost production (Faried, et al., 2017; Metsoviti, et al., 2020). Process optimization and technology development are still needed to fulfill its potential and reduce the high production costs still associated with it.

1.4. *CHLORELLA VULGARIS*

Chlorella vulgaris is a unicellular and non-motile microalga that belongs to the phylum Chlorophyta, class Trebouxiophyceae, order Chlorellales, and family Chlorophyceae (Bock, et al., 2011). Chlorophyta is also known as the green algae group, mostly because of its chlorophyll content (Masojídek, et al., 2004).

Chlorella vulgaris is spherical in shape and its cell composition (figure 1.1), covered by a cellulosic cell wall (Safi, et al., 2014), includes lipids, chlorophylls, fibers, carbohydrates, minerals, and can contain 38 to 58% of protein (Gouveia, et al., 2008). Its vegetative reproduction occurs through autospores: having the same shape as the mother cell, the first generation of cells, formed in a multiple of two inside the mother cell, are released after the maturation and cell wall rupture of the mother cell (Yamamoto, et al., 2004) (Lee, 2008) (Tomaselli, 2004). The cells from the first generation start to grow,

and the cell volume increases to prepare again for division. Thus, *C. vulgaris* diameter can vary from 2.5 to 10 µm depending on the growth phase (Grooth, et al., 1985) (Yamamoto, et al., 2004).

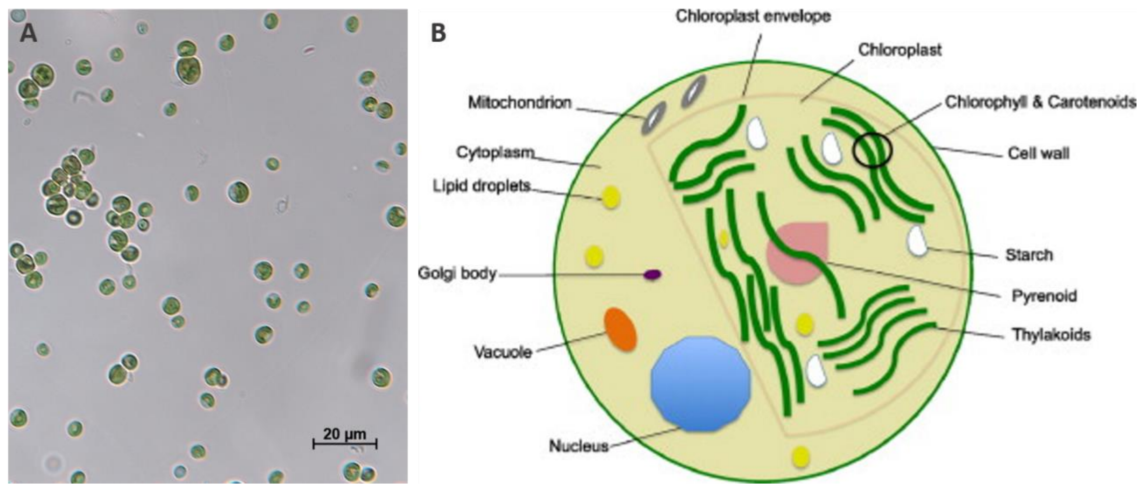

Figure 1.1 *Chlorella vulgaris* cells. Microscopically observed under a bright-field at 400X magnification (A) and its schematic morphologic structure from Safi, et al **(2014)** (B).

This organism grows in freshwater with great flexibility for trophic modes of cultivation (mixotrophic, heterotrophic, and autotrophic) and conditions, showing viable growth from 20 ºC to 28 ºC (Serra-Maia, et al., 2016), and from pH 4 to 10. While pH 10 is pointed out by Gong (2014) and Ihnken et al. (Ihnken, et al., 2014) as optimal for maximum cell densities, Rajanren & Ismail (2016) pointed out pH 7 as ideal pH value. Some species of the genus *Chlorella* are considered to be the most promising for the production of valuable compounds and useful for several biotechnological applications (Benedetti, et al., 2018).

Depending on its cultivation conditions, *Chlorella vulgaris* may produce different amounts of these valuable compounds, but it always synthesizes essential and non-essential amino acids, resulting in a high-quality protein for human consumption (Safi, et al., 2014) with a high rate of digestibility (Kose, et al., 2017). Those proteins have been reported to have good functional properties whereas used from biomass suspension (Raymundo, et al., 2005) or extracted protein (Ursu, et al., 2014). Under nitrogen limitation, *C. vulgaris* accumulates more lipids, suitable for biodiesel production instead of for human consumption (Rajanren & Ismail, 2016). Having favorable growth conditions, the lipids are composed of healthy polyunsaturated fatty acids (Safi, et al.,

2014). Polysaccharides, like β1→3 glucan, found in *Chlorella* sp., or chlorophylls and carotenoids, represent health benefits associated with the prevention of cardiovascular disease and stimulation of the immune system (Safi, et al., 2014). *Chlorella* sp. biomass also contains essential minerals and vitamins, an important key for human nutrition (Solomons, 2012; Safi, et al., 2014).

1.5. MICROALGAE CULTIVATION

Autotrophy, photoheterotrophy, heterotrophy, and mixotrophy are cultivation modes with different requirements in terms of energy and carbon sources. Heterotrophy requires energy and organic carbon sources, in photoheterotrophy the energy source from light is mandatory in addition to the previous requirements, and mixotrophic organisms can utilize both previous mode and autotrophy (Perez-Garcia & Bashan, 2015). This research work focuses on autotrophy as a cultivation mode, on which the microalgae uses sunlight to process CO_2, producing organic compounds (Uggetti, et al., 2018) either in open or closed bioreactors.

1.5.1. GROWTH CONDITIONS

Autotrophic microalgae industrial cultivation is carried in liquid environments where the culture growth is influenced by different factors: the organism life cycle, temperature, light intensity, supply of CO_2, pH, nutrients concentrations, the bioreactor configuration, and the mixing/agitation conditions (Olaizola & Grewe, 2019). When the characteristics of the culture medium are no longer able to sustain the culture growth, a death phase of the cells can be observed. Depletion of substrates in the culture medium or the presence of excreted autoinhibitory compounds may also be some of the causes for death (Vonshak, 1985; Lee & Shen, 2004).

1.5.1.1. Light

Light intensity is one of the most important parameters for microalgae cultivation. However, it is highly dependent on outdoor conditions, such as season, time of day, and weather events. As photosynthesis is light-dependent, it affects biomass composition and culture productivity, the regulation of biological processes, and consequently growth rate. Light is a driver for nutrients and CO_2 demand (Olaizola & Grewe, 2019). Even though

microalgae growth increases with the increase of light intensity, very high light intensity can result in stressful conditions thus contributing to photoinhibition and a drop in the growth rate and biomass productivity (Metsoviti, et al., 2020).

The importance of the light intensity and time of exposure on biomass growth has been reported throughout the literature. *Chlorella vulgaris* has faster growth and higher lipid content when exposed to higher light intensities (Meseck, et al., 2005; Umorin & Lind, 2005; Metsoviti, et al., 2020).

1.5.1.2. Nutritional requirements

Along with Carbon (C), assimilated through photosynthesis, nitrogen (N) and phosphorus (P) are very important macronutrients for microalgae growth. Being involved in several cellular reactions (Grobbelaar, 2004), the depletion of one can compromise the assimilation of the other (Flynn, 1990), accordingly to the optimal Redfield ratio of 106C:16N:1P (Redfield, et al., 1963).

Nitrogen is essential for amino acids formation and, consequently, protein metabolism. The nitrate (NO_3^-) is taken to the cell, reduced to nitrite by the enzyme nitrate reductase, and then transported to the chloroplast stroma where it is reduced to ammonium (NH_4^+) to be incorporated into the C skeletons, giving rise to several amino acids. Thus, NH_4^+ is the preferred source of N when possibly competing with NO_3^- in the culture medium (Flynn, 1990; Sanz-Luque, et al., 2015). Phosphorus is also an essential nutrient, being part of the lipids, carbohydrates, or many cellular processes such as energy transfer and biosynthesis of nucleic acids and DNA. However, it easily forms salts when in solution with other ions, leading to precipitation, turning P into a growth-limiting factor (Cembella, et al., 1984; Solovchenko, et al., 2019).

Nevertheless, other nutrients are also essential for the growth of microalgae, to participate in enzymatic reactions and the biosynthesis of compounds, but generally required in lower concentration and always depending on the composition of each species. Micronutrients as iron (Fe), magnesium (Mg), sulfur (S), potassium (K), sodium (Na), calcium (Ca), boron (B), copper (Cu), manganese (Mn), zinc (Zn), molybdenum (Mo), cobalt (Co), vanadium (V), selenium (Se) and silicon (Si) are required for proper cell

growth and high biomass yield (Tokusoglu & Unal, 2013; Quigg, 2016), but in vestigial concentrations. Vitamin supplementation might also be necessary (Grobbelaar, 2004).

1.5.1.3. Gas exchange

Incorporation of CO_2 into the microalgae cultures is as necessary as it is the O_2 removal. While the first is of major importance for nutrition and pH regulation through the inorganic carbon cycle, higher concentrations of O_2 can result in productivity losses due to photorespiration. In photorespiration, O_2 is used to convert organic carbon into CO_2, serine, and ammonia without metabolic gains. This way is important to maintain a low O_2/CO_2 ratio to favor carboxylation. To achieve this ratio of O_2/CO_2, the feeding air must be enriched with CO_2 (Masojídek, et al., 2004; Olaizola & Grewe, 2019). The system used for CO_2 incorporation also promotes the mixing of the culture, which is a very important factor to promote uniform light exposure for all cells within the bioreactor (Masojídek, et al., 2004).

1.5.1.4. Temperature

Each organism has a tolerated temperature range. It is crucial to choose species adapted to the climate conditions of interest since the temperature can affect productivity in terms of reaction equilibrium, enzymatic processes, protein degradation, and cell viability (Serra-Maia, et al., 2016). Depending on the cultivation bioreactor, culture temperature can be controlled even outdoors (Olaizola & Grewe, 2019). On the other hand, the temperature can be used as a factor of stress to induce the production of valuable metabolites (Khan, et al., 2018).

1.5.2. Industrial autotrophic cultivation

The type of bioreactor is one of the most decisive aspects of microalgae production. Both open and closed systems have advantages and disadvantages, so it is important to deeply understand how they work and made the selection according to the performance of the microalgae strain to be used and the purpose of the process avoiding unnecessary costs (Murthy, 2011).

Open systems are widely used due to their low investment and operating costs for large-scale outdoor production. Open ponds, tanks, raceway ponds, and cascade systems

are examples of open systems (Figure 1.2). In this case, the microalgae under cultivation are exposed to external environmental conditions, promoting gas exchange, but making it a system more susceptible to contaminations than in a closed system (Narala, et al., 2016). A strategy to avoid contamination is to cultivate microalgae with high specificity in medium conditions, such as high pH or salinity (Narala, et al., 2016), making open systems suitable for extremophilic microalgae (Varshney, et al., 2015).

Figure 1.2 Thin-layer cascade system (A) and a pilot raceway (B) are the two open systems at Allmicroalgae facilities.

Indoor or outdoor, flat panels and tubular (vertical or horizontal) photobioreactors (PBR) are the most common closed systems (figure 1.3) (Acién, et al., 2017). Requiring higher initial investment, energy consumption, and a specialized workforce, closed systems for industrial cultivation end up becoming more expensive. However, it allows better control of contamination and growth conditions, such as nutrients availability, temperature, pH, O_2, and CO_2 concentrations. In a closed reactor, the release of O_2 can be challenging. On the other hand, CO_2 losses through evaporation do not occur as much as in open systems. Closed reactor configurations usually allow better sunlight input compared to open ponds (Olaizola & Grewe, 2019; Chu & Phang, 2019), but the thin-layer of a cascade PBR has been pointed as having high productivity at very low costs (Apel, et al., 2017).

A hybrid system combining open and closed systems can result in better biomass productivities, especially when compared to open systems since the combination of open ponds with closed PBR's promotes better efficiency in the use of light (Narala, et al.,

2016; Liu, et al., 2018). This can be a cost-effective solution to improve cultivation systems productivity while reducing the environmental impact (Adesanya, et al., 2014).

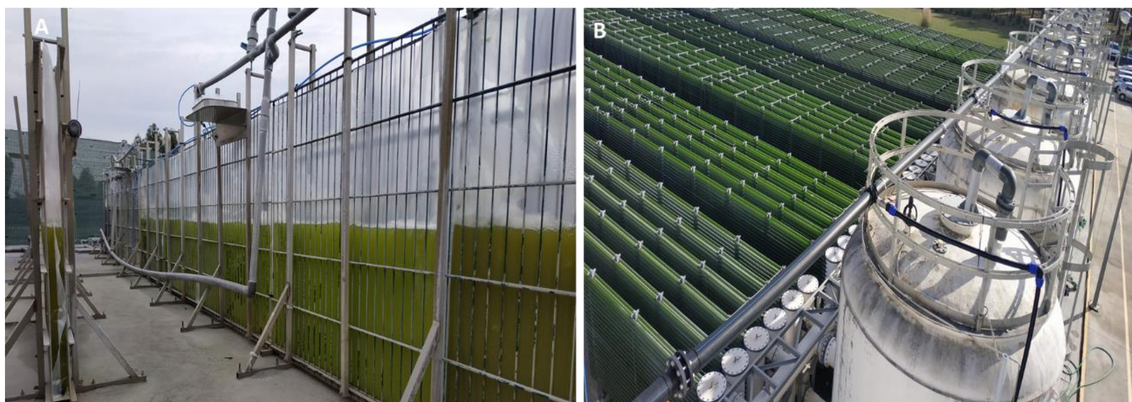

Figure 1.3 Flat panels (A) and horizontal tubular (B) photobioreactors at Allmicroalgae facilities.

1.5.3. INDUSTRIAL HETEROTROPHIC CULTIVATION

Heterotrophic growth occurs in the dark and for this reason light isn't a limiting growth factor as it is in autotrophy, in which bioreactors are built to optimize sunlight exposure (Perez-Garcia, et al., 2011). For this reason, industrial heterotrophic bioreactors (Figure 1.4) can be also used for other industrial processes like the production of food or pharmaceuticals (Perez-Garcia, et al., 2011).

Heterotrophic cultivation runs in axenic mode, being a highly productive system since it can sustain exponential growth for longer periods and reach higher biomass densities, lipid content, and specific growth rates, while reducing the time of scale-up and associated costs (Mohamed, et al., 2011; Barros, et al., 2019). Despite the reproducibility of the high cell density obtained (50 - 170 g L^{-1}), it has some disadvantages: it is known that only a few strains grow in this regime, the release of CO_2 is difficult, single contamination can lead to culture failure and less light-induced metabolites content (Perez-Garcia, et al., 2011; Barros, et al., 2019; Khan, et al., 2016). Heterotrophic cultivation can be followed by autotrophic cultivation, in a two-stage production process, to overcome some of those advantages (Barros, et al., 2019).

Figure 1.4 Allmicroalgae's industrial fermenters of 5000 L

1.5.4. HARVESTING

The harvesting process, together with dewatering, represents 3 to 15% of the microalgae biomass production costs (Fasaei, et al., 2018). Economic and environmental sustainability is highly dependent on the method chosen for biomass harvest (Singh & Patidar, 2018). Therefore, the recovery of biomass from very diluted cultures in PBR's is very expensive and closely related to labor and energy demands (Fasaei, et al., 2018). Microalgae harvesting from PBR's consists of concentrating the culture solids from 0.05% to 1-5% (dry basis), while dewatering further concentrates them in a slurry containing 15-25% of dry matter, or in a dry product, that follows the usual biomass processing (Muylaert, et al., 2017; Fasaei, et al., 2018). To obtain such culture concentrations at a large scale is often necessary to combine multiple operations (Fasaei, et al., 2018). A defined standard method for separating biomass from the liquid in all plant types of microalgae production has not yet been found, however, a range of available technologies can be selected according to microalgae specificities and their final application (Uduman, et al., 2010). Usually, there is a combination of these two types of methods: one density-based (sedimentation, coagulation, flotation, and centrifugation),

with one based on particles size (filtration or screens). A flocculation method can be combined with each previous method to facilitate the separation of the suspended microalgae (Singh & Patidar, 2018; Liu, et al., 2013) and to reduce energy requirements. After the harvesting process, the remaining water/culture medium can still be reused. Nevertheless, it should be noticed that the harvesting method used can strongly influence the final quality of the water/culture medium and consequently the microalgae growth (Farooq, et al., 2015).

1.5.1. DISSOLVED ORGANIC MATTER

During regular growth, microalgae release part of the organic matter (OM) fixed during photosynthesis to water (Bjornssen, 1988; Hulatt & Thomas, 2010). Microalgae use two ways to release extracellular organic matter (EOM): by exudation, in which most large polymers are actively transported through the cell membrane; and by leakage, a passive diffusion, where small, uncharged molecules easily cross the cell membrane (Bjornssen, 1988; Thornton, 2014). EOM contributes to the increase of dissolved organic matter (DOM) present in the culture medium.

The DOM composition is mostly composed of low molecular weight compounds, such as proteins, and poly-, oligo- and mono-saccharides (carbohydrates), lipids, and organic acids (Pivokonsky, et al., 2006; Zhang, et al., 2013). As a microalgae culture grows, the concentration of polysaccharides in DOM may decrease, probably consumed by bacteria (Hadj-Romdhane, et al., 2013), while the protein concentration increases (Pivokonsky, et al., 2006). The diversity of these proteins is the same until the death phase is reached, when the release of intracellular OM occurs and new proteins come out due to autolysis (Pivokonsky, et al., 2006). DOM can be assimilated and depleted by bacteria or reabsorbed by microalgae, stimulating microalgae growth (Bjornssen, 1988; Farooq, et al., 2015). However, the production of some released DOM can be induced in an attempt to eliminate microalgae competitors or predators, but it might lead to own growth inhibition (Grobbelaar, 2004; Zhang, et al., 2013; Loftus & Johnson, 2017), or to form complex compounds with minerals *e.g.* iron, making it to precipitate and, therefore unavailable for absorption by microalgae (Pivokonsky, et al., 2006).

The production of growth inhibitors is not a very common phenomenon within the genera *Chlorella* despite reports from Pratt in 1940 relating chlorellin compound as being

autoinhibitory (Scutt, 1964). However, colony (or cell aggregates) formation in *C. vulgaris* can be induced by the use of a recycled medium, or by the presence of predators and their exoproducts in the culture medium, *e.g.* the presence of the protist *Tetrahymena thermophila*, or the predatory cyanobacteria *Vampirovibrio chlorellavorus* (Fisher, et al., 2016; Ganuza, et al., 2016).

1.5.2. Water footprint and microalgae production sustainability

The environmental and economic sustainability of food systems is required in a time when water scarcity is being aggravated by climate change and population growth (FAO, 2018). Besides energy efficiency, efficient water use is essential to achieve sustainable industrial production of microalgae. Unlike to what happens with soybean or corn crops, the water used in the cultivation of microalgae is not lost to the soil, instead, can be reused mainly in closed systems, in which there is no evaporation (Lee & White, 2019).

The use of PBRs imparts a total water footprint in the range of 2.4-6.8 m^3 of water needed to produce 1 kg of dry biomass, 60% of which is associated with PBR operation – electricity and nutrients production (Martins, et al., 2018). Water also has significant energy requirements, from collection to pumping (Murphy & Allen, 2011).

After biomass harvesting, the reuse of the growth medium contributes to the reduction of the water footprint and the total use of the remaining nutrients in the water, contributing to economic sustainability (Yang, et al., 2011). However, it is important to understand the underlying implications, since autoinhibitory OM may be present, which will represent even lower biomass quality or productivity (Hadj-Romdhane, et al., 2013). DOM composition highly depends on taxa, so each cultivated strain reacts differently to its recycled growth medium (Loftus & Johnson, 2017; Loftus & Johnson, 2019). Nevertheless, different water treatments have already been described to remove the remaining DOM (Zhang, et al., 2016; Farooq, et al., 2015). Although DOM and water management can be challenging (Lee & White, 2019), it is important to overcome the problems of the culture medium recycling process to reduce the water footprint associated with microalgae cultivation.

1.6. MICROALGAE PRODUCTION AT ALLMICROALGAE

At Allmicroalgae´s facilities, strict autotrophic microalgae are initially cultivated indoors in a well-controlled room to further serve as inoculum for flat panels in the greenhouse, which in turn are used to inoculate tubular flow-through photobioreactors (PBR) outdoors. The scale-up process for *Chlorella vulgaris* is slightly different since the inoculum is produced heterotrophically before inoculation of autotrophic outdoor bioreactors, as described by Barros et al. (2019). At Allmicroalgae (Figure 1.5) there are 19 tubular PBR with different scale sizes, small (10 m^3), medium (35 m^3), large (90 m^3), and extra-large (180 m^3), allowing for large scale biomass production. Biomass harvesting is carried out by an ultrafiltration system for further processing. After biomass pasteurization, spray drying is applied to obtain the dry biomass. However, biomass can also be sold as a frozen wet paste, being processed by centrifugation instead of spray drying. The production of *Chlorella vulgaris* biomass at Allmicroalgae is certified for food safety by ISO 22000.

Figure 1.5. Left Partial view of Allmicroalgae industrial facilities. **Right** Tablets of *C. vulgaris*.

Currently, five species are regularly cultivated by Allmicroalgae to be marketed for food or feed applications: *Phaeodactylum tricornutum*, *Nannochloropsis* sp., *Scenedesmus obliquus*, *Chlorella vulgaris,* and *Tetraselmis chui.* However, only the last two are approved for human nutrition, being sold in powder, tablets (Figure 1.6), and capsules under the brand ALLMA. Some partnerships allowed the development of food products based on microalgae, such as crunches, cookies, pasta, etc.

1.7. AIMS OF THE THESIS

The main goal of this thesis was to optimize the culture medium recycling process of *Chlorella vulgaris* in order to reduce process costs and the water footprint associated with its industrial cultivation. Besides, the influence of the use of recycled medium on the composition of biomass and its functional properties for later application in food was also studied. To achieve this, the following specific objectives were considered:

- Assess the growth of *Chlorella vulgaris* under three different proportions of recycled medium, in several reuses;
- Evaluate the effect of culture medium recycling on the physicochemical and technofunctional properties of biomass.

2. MATERIALS AND METHODS

All the microalgal growth trials were performed at Allmicroalgae's facilities from January to November 2020, including daily growth assessment and pigment analysis. The quantification of protein and ash content of the produced biomass was carried out on the first week of January 2021 at the Center of Marine Sciences of the University of Algarve. All the other analyses were performed between November and December 2020 at the premises of the VALOREN Laboratory, at the School of Agriculture, Polytechnic of Coimbra.

2.1. RECYCLED CULTURE MEDIUM

After *C. vulgaris* industrial cultivation in tubular PBR's, algal biomass industrial harvesting and concentration (up to 5%) was performed using an industrial tangential flow filtration system (TFF) (Pall with wusp 6443 membranes in PVDF with 0.2 µm pore size) from where the permeate was collected into an intermediate bulk container (IBC). This permeate was prepared and used to be applied as a recycled culture medium, both in laboratory and pilot scale microalgae cultivation.

For laboratory-scale cultivation in BC, the permeate was filtered through a 0.7 µm glass fiber filter (VWR™, Leuven, Belgium), followed by filter sterilization (0.2 µm filter upper cups, VWR™, Leuven, Belgium) before its first reuse (R1). For further reuse (R2), the permeate, consisting of water and unused nutrients, underwent pH adjustment and re-supplementation with nutritive organic medium up to 2 mM L^{-1} of ammonium.

In the case of pilot-scale cultivation in FP, the permeate from FTT was used directly as recycled culture medium (R1).

2.2. CULTIVATION IN RECYCLED CULTURE MEDIUM

Recycled culture medium (RCM) was reused for 3 consecutive times (R1, R2, and R3) to grow *C. vulgaris* at three different ratios: 100% (P100), 70% (P70), and 50% (P50) being P100 composed exclusively of recycled medium and P70 and P50, recycled medium diluted with fresh culture medium (FCM) (Table 2.1). Cultivation with fresh culture

medium (FCM) was used as a control. 3 biological replicates were used for each condition.

Table 2.1 Composition of the culture medium per condition and trial. FCM – fresh culture medium, TFF - tangential flow filtration, RCM – recycled culture medium.

Condition	R1	R2	R3
FM	100% FCM	100% FCM	100% FCM
P100	100% permeate from TFF	100% RCM from R1	100% RCM from R2
P70	70% permeate from TFF 30% FCM	70% RCM from R1 30% FCM	70% RCM from R2 30% FCM
P50	50% permeate from TFF 50% FCM	50% RCM from R1 50% FCM	50% RCM from R2 50% FCM

Due to the small-scale cultivation volumes at the laboratory (1 L) and pilot-scale (50-80 L), after the first reuse (R1), the RCM was separated from biomass using different methods according to the production scale.

At the laboratory-scale, the RCM was collected after biomass harvesting through centrifugation for 8 min at 3500 rpm. The supernatant was filtered through a 0.7 µm glass fiber filter followed by a second filtration (sterile 0.2 µm filter). The pH and ammonium content were adjusted at the beginning of each trial.

At the pilot-scale, the RCM for R2 and R3 trials was obtained through a biomass flocculation process as described by Maji et al. (2018), with the CO_2 injection to lower the pH to 4 as quickly as possible. Each portion of supernatant separated from the biomass, from the same condition, was mixed, recovered and homogenized in an intermediate bulk container. 25 ppm of chlorine was added to avoid nutrients consumption or deterioration by bacteria or other microorganisms, until reuse.

2.3. MICROALGAL CULTIVATION

Axenic *Chlorella vulgaris* strain 0002 CA was obtained from Allmicroalgae's culture collection. In Allmicroalgae, this strain is first cultivated heterotrophically to later be used as inoculum for cultivation on several photobioreactors operating in organic autotrophic

mode. Heterotrophic and autotrophic modes applied by the company are described by Barros et al. (2019).

For the cultivations performed in this study, the inoculum was collected from industrial fermenters (heterotrophy) (Figure 1.4) to be cultured autotrophically, by a fed-batch process, in an industrial nutritive organic medium (confidential composition), with an ammonium concentration from 0.5 to 2 mM.L^{-1} as a nitrogen source, and supplemented with iron and micronutrients (0.22%, v/v). The pH of the culture was maintained at around 6.5 through manual addition of NaOH (10 M) (Fisher Scientific, UK) or injection of pure CO_2 (Linde, Portugal). Both culture pH and temperature were measured using a pH meter combo pH&EC (Hanna instruments).

At laboratory-scale, *C. vulgaris* was cultivated autotrophically in 1 L bubble column (BC) bioreactors (Figure 2.1A and B) at room temperature (24 ºC). The filtered air (0.2 µm glass fiber filter) provided constant aeration at 400 mLmin^{-1} and the use of white LEDs provided constant irradiance (750 µmol photons m^{-2} s^{-1}) in the 24:00 hour photoperiod. The chlorine present in the water used for the culture media was neutralized with sodium thiosulfate and then autoclaved for 40 min at 120 ºC before use.

Figure 2.1 Laboratory-scale - bubble column reactors (1 L) with *Chlorella vulgaris* cultivation after inoculation (A) and a few days later (B). Pilot-scale - flat-panel reactors before inoculation (C) and after inoculation (D).

At pilot-scale, *C. vulgaris* was cultivated autotrophically in flat panel (FP) bioreactors (Figure 2.1C and D), aerated continuously with filtered compressed air (0.2 µm glass fiber filter). The temperature was kept below 30 ºC by an irrigation system.

According to the time of the year, the photoperiod of each trial was adjusted to different light:dark periods according to Table 2.1. The trial with the first medium reuse (R1) occurred in a 12:12 h photoperiod, while the second (R2) had one hour less of light and the third (R3) two hours less. Throughout the pilot-scale trial, the working volume of the bioreactors used in each medium reuse decreased as shown in Table 2.2. This was due to the successive losses of culture medium during the harvest of the biomass between the reuses.

Table 2.2 Pilot-scale trials: photoperiod according to the time of year and the working volume of the photobioreactor for each medium reuse.

Trials	Photoperiod (light:dark)	Time of the year	Photobioreactor working volume
R1	12:12 h	September 25th to October 10th	80 L
R2	11:13 h	October 16th to October 25th	55 L
R3	10:14 h	November 4th to November 13th	50 L

2.4. GROWTH ASSESSMENT

Microalgae were observed daily by optical microscopy. Cell growth was determined by measuring the optical density (OD) of the culture at 750 nm, using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, Massachusetts, USA). Microalgae dry weight (DW) was determined by filtering 10 mL of algae suspension through a pre-weighed 0.7 µm glass fiber filter, washed with the same volume of distilled water and finally dried in a moisture analyzer MA 50.R (Radwag, Poland) to constant weight. The DW was calculated as the difference between the weight of the dried filter after and before filtration, per filtered volume. A linear correlation (Figure A.1, Appendix A) was established to obtain DW by OD measurement (equation 2.1).

$$\mathrm{DW}\ (\mathrm{g\ L^{-1}}) = 0.445\ \mathrm{OD}_{750} + 0.059 \quad (2.1)$$

Specific growth rate (µ) was calculated through equation 2.2, where X_1 and X_2 represent the biomass concentration at the beginning and end of the exponential phase, t_1 and t_2 are the corresponding times (in days, d) for those concentrations.

$$\mu\,(d^{-1}) = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1} \tag{2.2}$$

Volumetric biomass productivity (*P*) represented in equation 2.3 is the biomass produced at an interval time (days) per the initial working volume (V_t, L) of the reactor. Due to evaporation, the working volume in the reactor was not stable, and so, the total biomass (X) in the reactor each day was calculated by multiplying the obtained biomass concentration (DW, g L^{-1}) by the real working volume (V_r, L) at the time of sampling, as shown by equation 2.4.

$$P\,(gL^{-1}d^{-1}) = \frac{X_2 - X_1}{(t_2 - t_1) \times V_t} \tag{2.3}$$

$$X\,(g) = DW \times V_r \tag{2.4}$$

Areal biomass productivity (P_a) was calculated through equation 2.5, where volumetric productivity (equation 2.3) was multiplied by the initial working volume of the bioreactor (V_t) divided by the ground area occupied by the bioreactor (A, m^2) under cultivation.

$$P_a\,(g\,m^{-2}\,d^{-1}) = \frac{P\,V_t}{A} \tag{2.5}$$

2.5. DETERMINATION OF AMMONIUM CONTENT

Ammonium (NH_4^+) content was determined using the test kit NH_4/NH_3 from Sera (Sera, Heinsberg, Germany), according to the manufacturer´s instructions. The result of the reaction was read at 697 nm (Genesys™ 10SUV-VIS spectrophotometer, Thermo Fisher Scientific™, USA) and compared to a calibration curve of ammonium nitrate (Figure A.2, Appendix A).

2.6. BIOMASS HARVESTING

Once the cultures from pilot-scale cultivation reached the stationary phase, algal biomass was harvested by centrifugation at 3500 rpm for 8-10 min at 4 ºC (Hermle Z400K centrifuge, Labortechnik GmbH, Wehingen – Germany), while the supernatant was reused as RCM. The harvested cells were transferred to falcon tubes and frozen at -20 ºC

for at least 24 hours and freeze-dried for 3 to 4 days (UNICRIO MC-4L -60ºC freeze dryer). Freeze-dried samples were stored at -20 ºC until further biomass analysis.

2.7. Biomass Analysis

The biomass resultant from the pilot-scale trials was analyzed regarding its biochemical composition, (protein, ash, and pigments content) and functional properties (water and oil old capacity, foaming and emulsification properties).

2.7.1. Pigments content

For pigments extraction, 10 mg of dry biomass were weighed into a glass tube, 6 mL of acetone were added with zirconia beads (2.5% (v/v) of total volume) and homogenized by vortex (Velp Scientifica Classic Advanced) for 10 min at 1200 rpm. The sample was centrifuged at 3500 rpm for 10 min at 4 ºC (Hermle Z400K centrifuge, Labortechnik GmbH, Wehingen – Germany). These extraction steps were repeated until total loss of the pellet color.

The pigments extracted and recovered in the supernatant were analyzed by Genesys 10SUV-VIS spectrophotometer (Thermo Fisher Scientific™, USA) in a scanning spectrum from 380 to 700 nm for the determination of chlorophylls (a and b), pheophytins (a and b), and carotenoids content. The spectrum corresponding to each pigment was decomposed from all data and the corresponding content calculated using a mathematic model developed by Allmicroalgae for that purpose.

2.7.1. Ash content

The total ash content was determined using a gravimetric method (equation 2.6). Samples (approximately 50 mg of freeze-dried biomass) were placed in a pre-weigh porcelain crucible and weighed before and after being burn in a muffle furnace (J. P. Selecta, Sel horn R9-L) for 8 hours at 550 ºC.

$$Ash\ content\ (\%) = \frac{m_{ash}}{m_{biomass}} \times 100 \qquad (2.6)$$

where: m_{ash} is the mass of the ash, that remains after sample burn, and $m_{biomass}$ is the initial mass of the sample.

2.7.2. Protein content

Approximately 1 mg of freeze-dried biomass was weighted in aluminum capsules. The capsules were inserted in Vario el III (Vario EL, Element Analyser System, GmbH, Hanau, Germany) in which the CHN composition was determined. The total protein content was determined by multiplying the percentage of nitrogen (N) by a factor of 6.25 (Molazadeh, et al., 2019) as shown by equation 2.7.

$$Protein\ content\ (\%) = N \times 6.25 \quad (2.7)$$

2.7.3. Water-holding capacity and oil holding capacity

The water holding capacity (WHC) and oil holding capacity (OHC) were performed according to the method described by Kunarayakul et al. (2018) and Waghmare et al. (2016). In pre-weighed Eppendorf tubes, 0.05 g of freeze-dried biomass were suspended in 0.5 mL of distilled water or dispersed in 0.5 mL of sunflower oil, for 30 seconds in a vortex. The samples were left for 30 min at room temperature and then centrifuged at 7000 rpm (Ibx – MC 7000 series) for 10 min.

For WHC the supernatant was drained off, the excess water was removed at 50 ºC for 25 min, and the samples weighed again.

For OHC, the supernatant oil was removed by pipetting, the sample was centrifuged a second time for 2 min and the remaining oil was drained off before being weighed again. Analyses were made in duplicates and equations 2.8 and 2.9 were used to calculate WHC and OHC, respectively.

$$WHC\ (g\ g^{-1}) = \frac{m_{wet\ biomass} - m_{dry\ biomass}}{m_{dry\ biomass}} \quad (2.8)$$

$$OHC\ (g\ g^{-1}) = \frac{m_{oiled\ biomass} - m_{dry\ biomass}}{m_{dry\ biomass}} \quad (2.9)$$

where: $m_{wet\ biomass}$ is the mass of the sample with the water that remains after treatment; $m_{oiled\ biomass}$ is the mass of the sample with the oil that remains after treatment; and $m_{dry\ biomass}$ is the mass of the dry sample.

2.7.4. FOAMING PROPERTIES

Biomass foaming capacity (FC) and foaming stability (FS) were determined by the method described by Kunarayakul et al. (2018) with slight modifications. 0.225 g of freeze-dried biomass were suspended in 45 mL of 0.1 M phosphate buffer (pH 7) for 30 s of stirring followed by 15 s of homogenization at 3500 rpm (IKA ® T25 digital ULTRA-TURRAX ®). Triplicates from the previous suspension (15 mL each, V_i) were homogenized again for 1 min at 18 000 rpm in a 400 mL beaker, to form a foam. After homogenization, the liquid and the foam were immediately transferred to a measuring cylinder, and the total volume was registered (V_f). Foam volume was measured right after homogenization (V_{Fo}) and after 30, 40, and 60 min (V_{Ft}) to observe foam stability at room temperature. FC and FS, presented as percentage, were evaluated by equations 2.10 and 2.11, respectively.

$$\mathrm{FC}\ (\%) = \frac{V_f - V_i}{V_i} \times 100 \quad (2.10)$$

$$\mathrm{FS}\ (\%) = \frac{\mathrm{V_{Ft}}}{\mathrm{V_{F0}}} \times 100 \quad (2.11)$$

2.7.5. EMULSIFICATION PROPERTIES

Emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined according to the method of Pearce and Kinsella (1978) and Barac et al. (2010), and Kunarayakul et al. (2018) with adaptations. 0.080 g of freeze-dried biomass were suspended in 8 mL of distilled water for 45 s of homogenization at 3500 rpm (IKA ® T25 digital ULTRA-TURRAX ®). 1.25 mL of organic sunflower oil was added to each duplicate of 3.75 mL from the previous biomass suspension for further homogenization in a 50 mL falcon tube, for 1 min at 22 000 rpm. 25 µL of the emulsion was taken from the bottom of the tube after homogenization (A_0) and after 10 min (A_{10}), and each time immediately mixed with 4.75 mL of 0.1% SDS (sodium dodecyl sulfate) solution. The absorbance of these solutions was measured at 500 nm. EAI e ESI were calculated according to Pearce and Kinsella (1978) as follows:

$$EAI(m^2 g^{-1}) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \varphi \times 10000} \quad (2.12)$$

$$ESI\ (\text{min}) = A_0 \times \frac{\Delta t}{A_0 - A_{10}} \qquad (2.13)$$

where, DF = dilution factor (200); c = biomass concentration (g mL^{-1}) in the aqueous phase, before emulsion formation (≈ 10000 g mL^{-1}); φ = the oil fraction used for the emulsion (0.25); Δt = 10 min; and the absorbance at 0 (A_0) and 10 min (A_{10}).

2.8. Statistical analysis

Statistical analysis was performed using SigmaPlot v14.5 software - trial version from Systat Systems, San Jose, USA. A one-way analysis of variance (ANOVA), followed by the Bonferroni test, was used for data comparison and evaluation of statistically significant differences between groups. A p-value < 0.05 was considered statistically significant. Data are expressed as the means of 3 independent biological replicates ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. GROWTH ASSESSMENT UNDER RECYCLED MEDIA

3.1.1. LABORATORY-SCALE TRIALS

Permeate, from industrial TFF, was used as recycled culture medium to evaluate the growth performance of microalgae at different RCM concentrations (100% - P100, 70% - P70, and 50% - P50). Each recycled medium was reused three consecutive times R1, R2, and R3.

The first approach to recycling the medium consisted of the laboratory-scale experiments in 1L bubble column photobioreactors under axenic conditions. The cultures were therefore grown under controlled conditions of light, temperature, nutrients, and aeration as described in the material and methods section. The cultures to which only the fresh medium was applied were used as the control group. The cultures with recycled media were assigned as P100, P70, and P50.

After 13 days of cultivation, microalgae growing in all tested conditions reached the stationary phase, as shown in Figure 3.1. The DW achieved in the fresh medium was 1.77 ± 0.10 g L^{-1} and 1.68 ± 0.25 g L^{-1} in P50. DW achieved was highest when using 100 and 70% permeate, 1.92 ± 0.21 g L^{-1} and 1.80 ± 0.13 g L^{-1}, respectively, however, no significant difference ($p > 0.05$) was found between the fresh and recycled medium experiments on final DW, productivities, and specific growth rate (0.145 – 0.160 d^{-1}) at the first reuse (R1). The global productivities obtained were around 0.109 – 0.126 g L^{-1} d^{-1}, achieving maximum productivity values of 0.219 – 0.324 g L^{-1} d^{-1} (Figure 3.1). Therefore, at the laboratory-scale and under the tested conditions, *C. vulgaris* growth was not affected by the first reuse of the RCM.

The obtained results are in line with successful medium reuses of *Chlorella* sp. by Hadj-Romdhane et al. (2012; 2013) and Zhu et al. (2013). Higher productivity, than the obtained in the present work, was reported for *C. vulgaris* for continuous cultivation mode during 63 days, 0.56 ± 0.07 g L^{-1} d^{-1}. A reason for such difference can be attributed to the continuous cultivation mode, maintaining cell growth on exponential phase, and to the lower maximum cell densities achieved, DW= 1.5 g L^{-1}, avoiding the shadow effect, with

comparison to the obtained in this study (Figure 3.1), and contributing to higher photosynthetic efficiency. A second possible reason might be the permanent feeding used in Hadj-Romdhane et al. (2012; 2013), while in this study some essential nutrients might have become growth limiting. Lower productivity than the obtained in the present work was reported by Lam & Lee (2014) for *C. vulgaris* growth under fresh medium cultivation at laboratory-scale, 0.032 g L^{-1} d^{-1}, showing that the values obtained are within the range reported in the literature.

The obtained specific growth rates (0.145 – 0.160 d^{-1}) in this work are above the reported by Machado, et al (2020), 0.122 d^{-1}, for the organic growth of *C. vulgaris* at a laboratory scale (2 L).

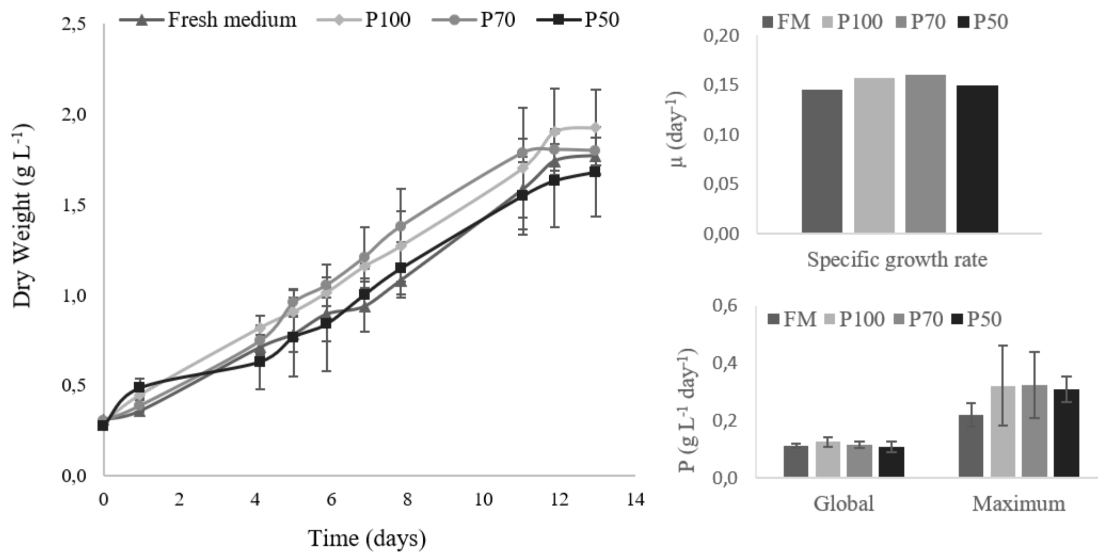

Figure 3.1 Cultivation of *C. vulgaris* in bubble column photobioreactors (1 L) at the first culture medium reuse (R1). FM - fresh medium (control); P100 - 100% permeate, P70 - 70% permeate; P50 - 50% permeate. **Left Graph** Growth curves. **Right Graph** (**upper**) Specific growth rate. **Right Graph** (**lower**) global and maximum volumetric productivity. Statistically significant differences were not found ($p \geq 0.05$) in any of the analyzed parameters.

After the first reuse (R1), the RCM was handled as described in sub-chapter 2.2, to be reused for a second time (R2). On day 4, culture contamination with a filamentous fungus was detected (Figure 3.2, A and H), on what was the first attempt for R2. The

second attempt of *C. vulgaris* cultivation took place, however, contamination by fungi appeared consistently after a few days of cultivation (Figure 3.2, B-H). *Chlorella vulgaris* was interacting with fungi leading to the formation of "green balls" (Figure 3.2, E-G) larger than 1 cm in diameter. This interaction limited the assessment of microalgae growth. This type of interactions of microalgae and fungi have been reported and studied as a possibility for bio-flocculation as a new strategy for biomass harvesting (Alam, et al., 2016; Demir, et al., 2020; Chen, et al., 2018). According to the literature, the morphology of fungal pellets indicates that the filamentous fungus found in this study belongs to the *Aspergillus* genera (Toscano P., et al., 2018; Li, et al., 2019; Wrebe, et al., 2014).

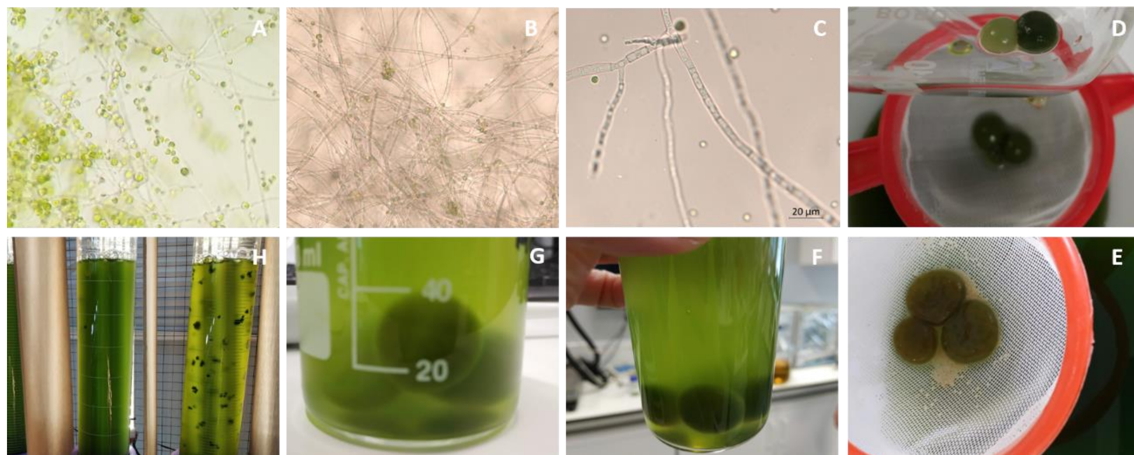

Figure 3.2 Microscopic observation (400X amplification) of fungi hyphae and microalgae (A-C) and fungi interaction with microalgae (D–H). A and H were captured on the 1st attempt to R2, B, C, D, G, F, and E were from a 2nd attempt.

Previous preliminary attempts for the R1 trial also resulted in contaminations, either with microflagellates or bacterial proliferation (data not shown), suggesting the importance of the microbiome in recycling the medium. According to what is reported in the literature, bacteria, and microalgae establish a mutualistic relationship: while consuming the organic matter released by microalgae to the RCM, bacteria release vitamin B and remineralizes sulfur, nitrogen, and phosphorous that support microalgae growth. The presence of bacteria has a direct effect on dissolved organic matter (DOM) release (Grossart & Simon, 2007) (Muhlenbruch, et al., 2018), however, the lack of asepsis during laboratory experiments was limiting the performance of the culture. The

obtained results, also supported by the literature, showed the challenges on a laboratory-scale (small-scale) for the reuse of industrial permeate. Therefore, only trials for R1 and R2 were performed at the laboratory-scale, and trials were continued on a pilot-scale.

3.1.2. PILOT-SCALE TRIALS

Three consecutive cultivations (R1, R2, and R3) were performed with recycled medium, on pilot-scale flat panel photobioreactors. Permeate, from the TFF membranes, was used directly in R1. At the end of R1, as well as in the case of R2 (second reuse), the RCM was recovered after harvesting the biomass by a flocculation/sedimentation process induced by reducing pH. The sedimentation efficiency of the biomass in the R1 and R2 trials was 90 ± 5%, allowing the supernatant to be collected and reused as a recycled culture medium for the third reuse (R3), with only a residual amount of biomass. At R1, the culture reached the stationary phase after 9 days of cultivation and, thus, the following cultivations were also carried out during this period. The growth curves during R1, R2, and R3 are shown in Figures 3.3, 3.4, and 3.5, respectively, in which the solar irradiance is also recorded. Growth curves are describing the total algal biomass (Equation 2.4) as a function of time. The temperature of the culture medium at the time of each sampling can be seen in Figure B.3 (Appendix B).

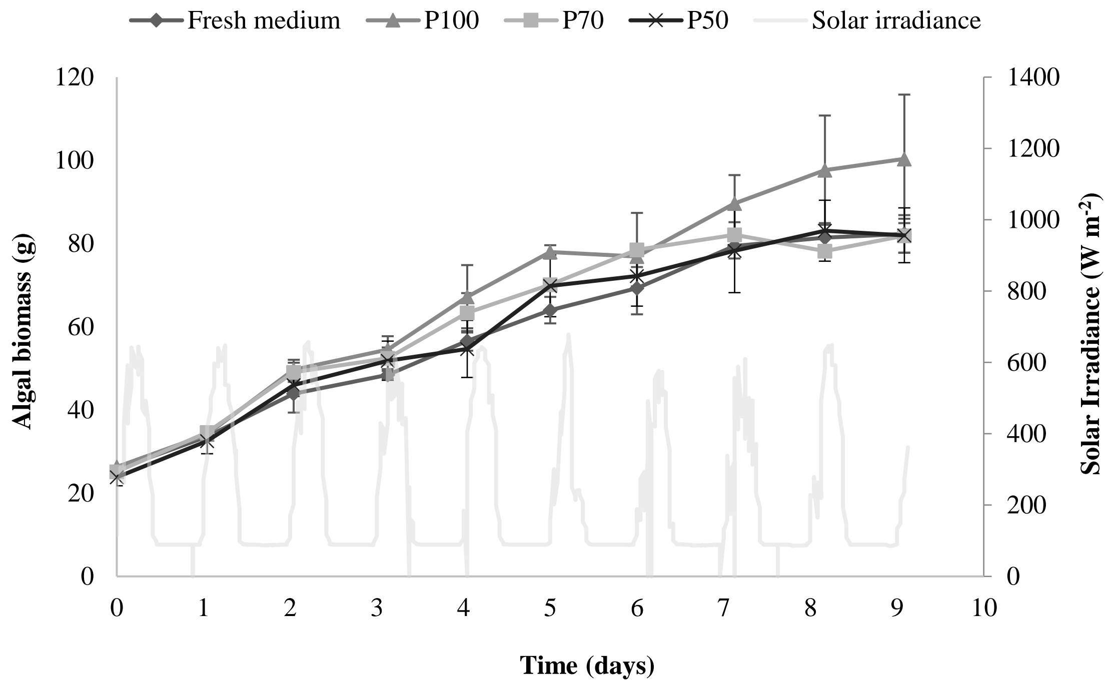

Figure 3.3 Growth curves of *C. vulgaris* cultivated in pilot-scale flat panel photobioreactors (80 L) at the first culture medium reuse (R1). FM - fresh medium (control); P100 - 100% permeate, P70 - 70% permeate; P50 - 50% permeate. 12:12 hours photoperiod. Peak solar irradiance (SI) of 678.5 W m^{-2}. Results are the mean values and standard deviation of three biological replicates. No statistically significant differences between the final biomass were found ($p \geq 0.05$).

After the first reuse of the medium, the use of 100% of permeate (P100) allowed to reach 100.4 ± 15.4 g of total biomass, the highest value achieved, compared to the FM (82.3 ± 4.5 g), P70 (81.9 ± 4.1 g), and P50 (81.9 ± 6.6 g) (Figure 3.3). However, no statistically significant differences were found between the tested conditions, as shown by the specific growth rates and productivities (volumetric and areal) presented in Table 3.1.

During R1, the growth of *Chlorella vulgaris* was not inhibited by the reuse of the permeate from previous industrial cultivation, regardless of the ratio applied. Complete permeate reuse (P100) reached the highest DW value even when compared to the control with fresh medium, though not statistically significant, similarly to what happened at the laboratory-scale on R1.

Table 3.1 Biomass productivities and specific growth rate (μ) of *C. vulgaris* in pilot-scale flat panel photobioreactors (80 L) in the first culture medium reuse (R1). FM - fresh medium (control); P100 - 100% permeate, P70 - 70% permeate; P50 - 50% permeate.

	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)		Areal productivity ($g\ m^{-2}\ d^{-1}$)		μ (d^{-1})
	Global	Maximum	Global	Maximum	
FM	0.077 ± 0.007	0.154 ± 0.023	52.1 ± 3.0	102.4 ± 15.7	0.130 ± 0.009
P100	0.100 ± 0.020	0.238 ± 0.025	67.2 ± 13.8	157.7 ± 12.3	0.149 ± 0.017
P70	0.077 ± 0.005	0.193 ± 0.029	51.5 ± 3.3	128.3 ± 20.9	0.133 ± 0.005
P50	0.077 ± 0.009	0.208 ± 0.068	52.2 ± 6.1	141.2 ± 46.0	0.138 ± 0.004

12:12 hours photoperiod. Peak solar irradiance of 678.5 W m^{-2}. Results are the mean values ± standard deviation of three biological replicates. Statistically significant differences at ($p < 0.05$).

No statistically significant differences ($p \geq 0.05$) were found in growth rates or productivities between tested conditions during the second reuse of permeate (R2), as shown in Table 3.2. Again, higher ratios of RCM resulted in the highest total biomass obtained, P100 = 33.4 ± 10.0 g and P70 = 33.1 ± 4.3 g, while the control group achieved 28.4 ± 2.3 g, and P50 = 28.1 ± 4.1 g (Figure 3.4). Besides no statistical differences, the specific growth rate, global and maximum productivities were higher for P100 (Table 3.2). Productivity obtained at R2 is higher than reported by Lam & Lee (2014) for *Chlorella vulgaris* cultivation under FM, 0.016 g L^{-1} d^{-1}, probably due to the differences between the photobioreactors type, despite the same size.

Despite no statistically significant differences found, both R1 and R2 showed a tendency for a higher amount of biomass when using a higher proportion of recycled medium, suggesting that, at the tested conditions, the organic matter dissolved in the RCM was stimulating *C. vulgaris* growth. As the methods used for the recovery of the culture media are not the same used for the laboratory and pilot-scale, it was interesting to note that same similarity on growth. The microalgae growth at the pilot-scale was not affected by the excessive DOM and the yellow color of the RCM, as previously reported by Loftus & Johnson (2017). Similar results to the present study were reported by Beevi, et al. (2016) with the green microalgae *Chlorococcum* sp, on which, by increasing the pH of the medium to 12, cells flocculated up to 94%, and the liquid was recycled for further cultivation without significant interferences in culture performance.

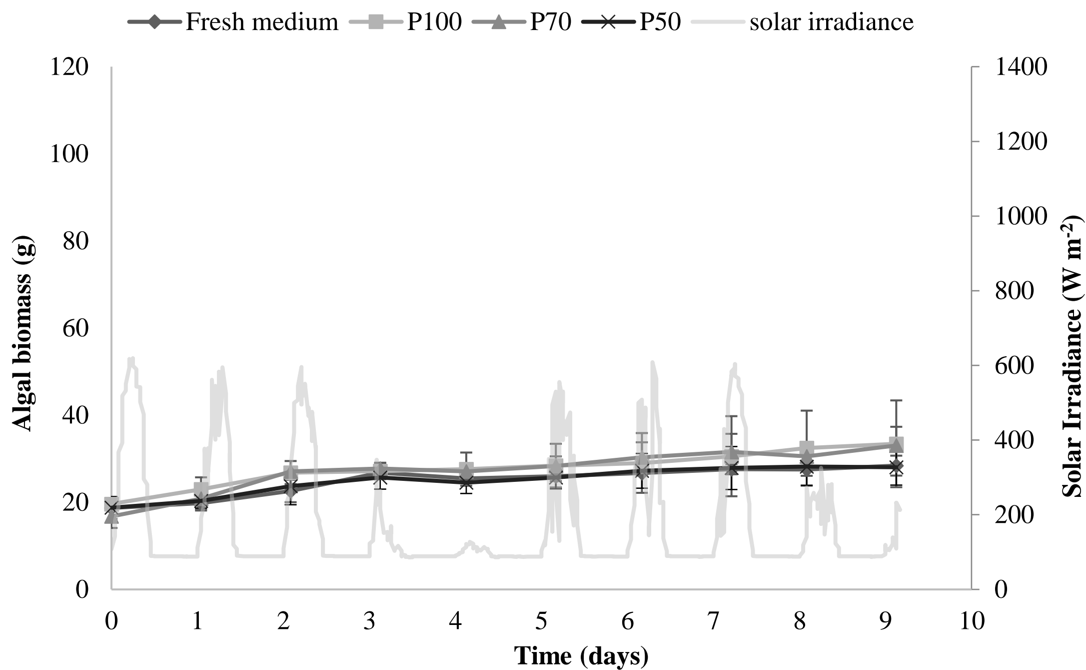

Figure 3.4 Growth curves of *Chlorella vulgaris* cultivated in pilot-scale flat panel photobioreactors (55 L) at the second culture medium reuse (R2). Fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. 11:13 (light:dark) hours photoperiod. Peak solar irradiance (SI) of 619 W m^{-2}. Results are the mean and the standard deviation of three biological replicates. Statistically significant differences between the obtained final biomass were not found ($p \geq 0.05$).

Table 3.2 Biomass productivities and specific growth rate (μ) of *C. vulgaris* at pilot-scale flat panel photobioreactors (55 L) at the second culture medium reuse (R2). FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM.

	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)		Areal productivity ($g\ m^{-2}\ d^{-1}$)		μ (d^{-1})
	Global	Maximum	Global	Maximum	
FM	0.021 ± 0.008	0.088 ± 0.032	10.1 ± 3.9	42.6 ± 15.4	0.046 ± 0.018
P100	0.026 ± 0.018	0.083 ± 0.003	14.6 ± 10.7	45.7 ± 0.4	0.056 ± 0.032
P70	0.034 ± 0.011	0.124 ± 0.027	16.7 ± 5.7	61.4 ± 11.4	0.075 ± 0.026
P50	0.020 ± 0.009	0.080 ± 0.037	9.6 ± 4.5	39.6 ± 18.4	0.044 ± 0.019

11:13 (light:dark) hours photoperiod. Peak solar irradiance of 619 W m^{-2}. Results are the mean values and standard deviation of three biological replicates. Statistically significant differences at ($p < 0.05$).

In the R3 trial, a dark yellow color of the RCM in the P100 group was noticed even before inoculation. However, the color didn't significantly affect the measured absorbance (750 nm) of the RCM. The same phenomenon of color change for dark yellow along the recycling was reported by Hadj-Romdhane (2013). On the first days of the R3 trial, continuous biomass loss was registered (Figure 3.5) in all tested groups, including the control, FM, which was probably a result of the cloudy and cold days (T_{max} = 20ºC and T_{min} = 9ºC) recorded along the 9 days of the assay. The initial total algal biomass on each group was around 18 g, and, at the end, the total biomass obtained was very similar: FM = 21.2 ± 2.6 g; P100 = 17.5 ± 0.6 g; P70 = 19.9 ± 1.6 g; P50 = 20.7 ± 0.5 g. Contrarily to R1 and R2, in the R3 trial the P100 was the group reaching the lowest, and even negative, values of specific growth rate, μ = -0.005 ± 0.001 d^{-1} (Table 3.3) and global productivities: P = -0.002 ± 0.000 g L^{-1} d^{-1} and Pa = -0.9 ± 0.1 g m^{-2} d^{-1} (Table 3.3). The global productivities of P100 were statistically significantly different from the control group: P = 0.008 ± 0.005 g L^{-1} d^{-1} and Pa = 3.9 ± 2.3 g m^{-2} d^{-1}, but not from P70 and P50 (Table 3.3). The most likely reason for such low productivities, when compared with R1 and R2, were the adverse growth conditions registered. According to Wu & Zhuang (2016), adverse light intensity, temperature, or extreme pH are associated with higher microalgae DOM release to the medium. The low levels of solar irradiance observed along the trial might have affected photosynthesis, inhibiting microalgae growth (Olaizola & Grewe, 2019) and contributing to a possible increase in DOM, which has hindered P100 from recovery until the end of the trial. Other cultivation parameters like culture medium temperature (Figure B.3, Appendix B), pH, or ammonia concentrations were not significantly different from R1 and R2.

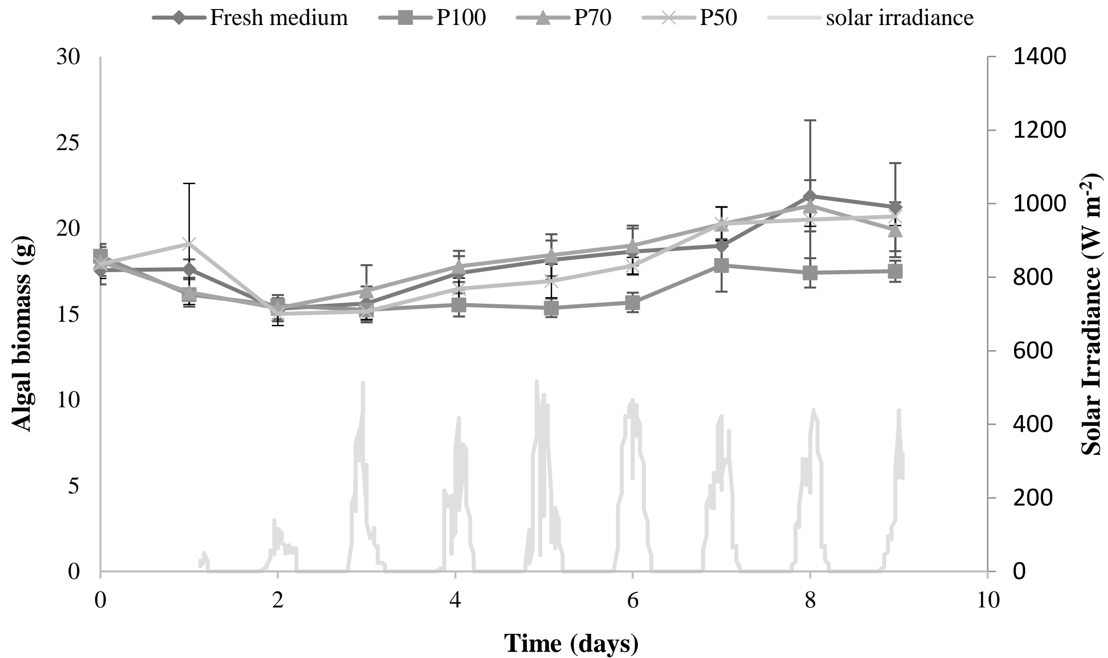

Figure 3.5 Growth curves of *Chlorella vulgaris* cultivated in pilot-scale flat panel photobioreactors (50 L) at the third culture medium reuse (R3). Fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. 10:14 (light:dark) hours photoperiod. Peak solar irradiance (SI) of 517 Wm^{-2}. Results are the mean and the standard deviation of three biological replicates. Statistically significant differences between the obtained final biomass were not found ($p \geq 0.05$). The absence of solar irradiance data on the 1st day of the trial is owed to an equipment failure.

Besides, according to Loftus & Johnson (2017), the DOM released and accumulated over the trials can affect the physical and chemical properties of the culture medium, having an impact on the uptake and availability of nutrients, gas exchanges, cell aggregation, and shading. That shading was observed by the dark yellow color detected in P100. Further analytical studies to analyze the RCM composition would be needed to clarify the reasons behind this result.

The existence of an auto-inhibitory growth substance, chlorellin, produced by *Chlorella vulgaris,* was reported in 1940 (Pratt & Fong), as affecting *C. vulgaris* when more concentrated and aged RCM. Contrarily, Hadj-Romdhane (2013) didn't notice any growth inhibition after growing *C. vulgaris* for 63 days in a recycled culture medium, however, biomass was harvested before reaching the stationary phase which, accordingly to Pivokonsky, et al. (2006), moderates the amount of DOM present in the medium.

During the current work, the stationary phase was reached in every trial to promote the release of organic matter to the RCM and test it in the worst-case scenario. Although, during *Chlorella vulgaris* autotrophic industrial cultivation at Allmicroalgae, the culture is harvested during the exponential phase as in the study conducted by Hadj-Romdhane (2013), not allowing it to reach the stationary phase.

Table 3.3 Biomass productivities and specific growth rate (μ) of *C. vulgaris* at pilot-scale flat panel photobioreactors (50 L) at the third culture medium reuse (R3). FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Kd – the death rate constant (loss of viable cell).

	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)		Areal productivity ($g\ m^{-2}\ d^{-1}$)		μ (d^{-1})
	Global	Maximum	Global	Maximum	
FM	0.008 ± 0.005^{b}	0.085 ± 0.083^{b}	3.9 ± 2.3^{b}	39.6 ± 38.4^{a}	0.021 ± 0.012^{a}
P100	0.000^{a}	0.046 ± 0.026^{a}	0.0^{a}	20.8 ± 11.7^{a}	$K_d = 0.005 \pm 0.001$
P70	$0.004 \pm 0.004^{a,b}$	0.040 ± 0.005^{a}	$1.9 \pm 1.9^{a,b}$	18.2 ± 2.4^{a}	0.011 ± 0.010^{a}
P50	$0.006 \pm 0.002^{a,b}$	0.056 ± 0.034^{a}	$2.9 \pm 1.0^{a,b}$	25.1 ± 14.9^{a}	0.016 ± 0.006^{a}

10:14 (light:dark) hours photoperiod. Peak solar irradiance of 517 Wm^{-2}. Results are the mean values ± standard deviation of three biological replicates. Statistically significant differences at ($p < 0.05$).

In the three medium recycling trials, no significant differences were found in specific growth rates or productivities between the use of fresh medium and 70% and 50% RCM. The same was verified using 100% RCM in the two first reuses, however, at R3, differences were found between that and fresh medium, once cellular death has occurred at P100.

The present work showed that the permeate can be reused at least 3 times up to a concentration of 70%, not affecting microalgae growth at the pilot-scale when compared to the control. However, productivity and growth have continually decreased from R1 to R3, likely due to adverse abiotic conditions. Further experiments, at the industrial scale, would be necessary to adapt the procedure.

Farooq et al (2015) tested, at a laboratory-scale, the growth of *Chlorella vulgaris* in RCM, supplied with NO_3 as the nitrogen source, after different harvesting methods (centrifugation and flocculation). They found that biomass productivities were improved

by the RCM in comparison with a fresh medium, for three consecutive reuses. Using ammonium to supply the RCM of a marine *Chlorella* sp., Loftus & Johnson (2017) reported no differences between cultivation under RCM and fresh medium for 4 consecutive reuses. Further studies on how harvesting affects the cultivation would be necessary. Accordingly to Depraetere et al (2015), medium recycling has been associated with a size change of the population due to the small cells that can pass through the harvesting process.

The obtained results are very promising for the sustainability of *Chlorella vulgaris* cultivation. When recycling the permeate, the nutrients are recovered from the culture media, and the freshwater need is reduced, contributing to reduce the associated costs and increase the environmental and economic sustainability of the entire microalgae cultivation process.

3.2. EFFECT OF RECYCLED CULTURE MEDIUM ON MICROALGAE BIOMASS PROPERTIES

Studies on the reuse of the culture medium focus mainly on the evaluation of cell growth, based on biomass productivity and growth rates, or on the analysis of DOM constituents released by microalgae under recycling conditions. However, *Chlorella vulgaris* is produced mainly for the food market and, for that, the analysis of the biochemical composition of the biomass was of extreme interest, allowing to understand if the use of the recycled medium had an impact on the nutritional properties of the resulting biomass.

Regarding biochemical composition, the pigment, protein, and ash content of the freeze-dried biomass from pilot-scale trials were evaluated.

It can be observed in Figure 3.6, that the carotenoid content was less in R3, yet not affected in each recycling trial (R1, R2, R3), being similar between the fresh medium and RCM. The chlorophyll content decreased along with the 3 trials with no statistically significant differences observed at R1 and R2 between FM and RCM (Figure 3.6). However, the recycling of the culture medium affected the chlorophyll content after the

third reuse when 100% permeate was used. At this stage, the chlorophyll content of *C. vulgaris* when grown using 100% RCM (17.6 ± 1.2 mg g^{-1}) does not differ from the fresh medium (21.6 ± 2.9 mg g^{-1}), however, it was statistically lower than P70 (23.2 ± 1.4 mg g^{-1}) and P50 (23.7 ± 1.2 mg g^{-1}). Higher cell densities are related to higher contents of chlorophyll (Oo, et al., 2017), which may explain the lower chlorophyll content in trials in which the biomass DW was lower.

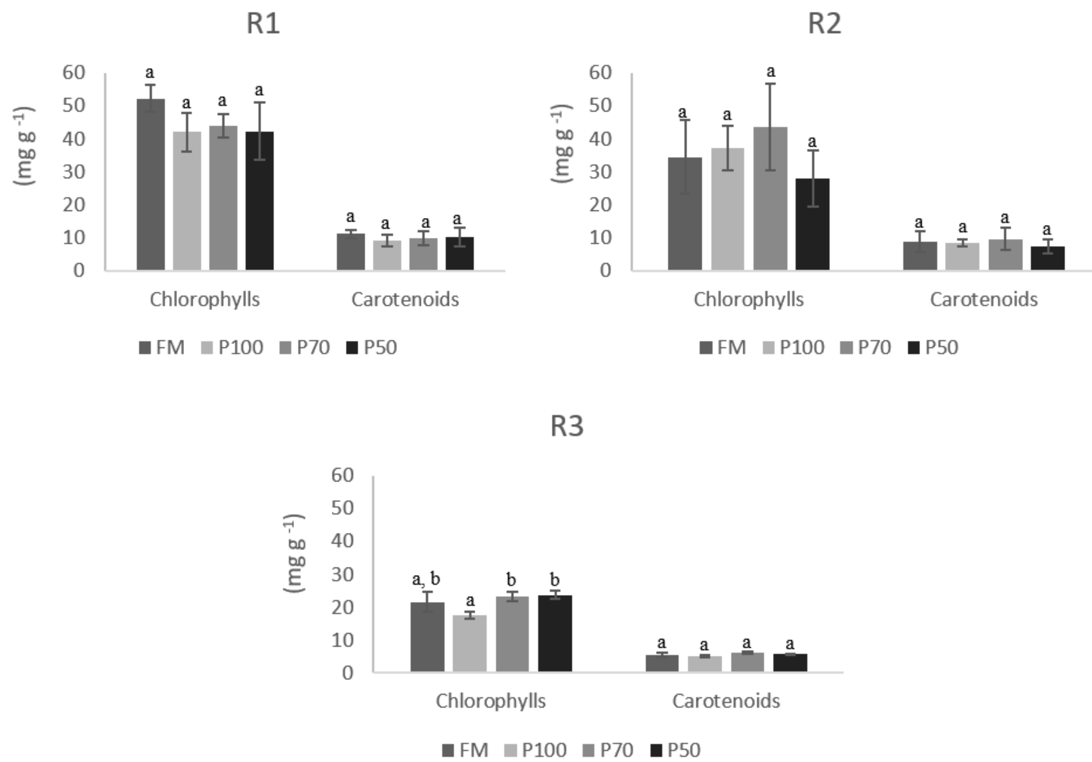

Figure 3.6 Total chlorophyll and total carotenoid contents from flat panels at the first (R1), second (R2), and third (R3) reuse of the culture medium. FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Results are the mean values and standard deviation of three biological replicates. Different letters within each group represent statistically significant differences ($p < 0.05$).

The spray-dried biomass of *C. vulgaris* produced at Allmicroalgae (in tubular PBR) is registered to have a chlorophyll content of 25.3 mg g^{-1} (certificate of analysis of *Chlorella vulgaris*, company's internal document - confidential), which is above the average chlorophyll contents in R3 trial. However, in R1 and R2 the chlorophyll content is significantly higher, which may indicate higher cell density achieved in the flat-panel

bioreactor than in a tubular photobioreactor, which presents higher light penetration. Hadj-Romdhane et al. (2013) reported no changes and no negative effect on chlorophyll content over 63 days of cultivation of *C. vulgaris* under RCM conditions.

In each recycling trial the mineral content in the microalgae biomass, represented by the ash content in Figure 3.7, was not statistically affected using RCM (P100, P70, and P50) when compared to fresh medium (FM). However, the range of total ash content was smaller in R3 (from 8.5% to 9.7%) compared to R2 and R1 (from 10.8% to 13.7%).

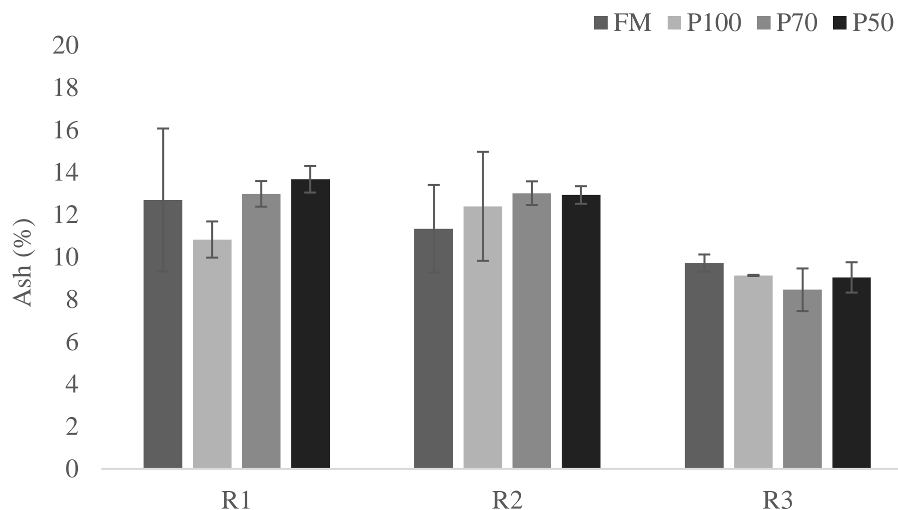

Figure 3.7 Ash content of *C. vulgaris* from flat panels bioreactors at the first (R1), second (R2), and third R3 reuse of the culture medium. FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Results are the mean values and standard deviation of three biological replicates. No statistically significant differences within each reuse were found ($p \geq 0.05$).

Chorella vulgaris biomass produced at Allmicroalgae is reported to have 10,2% ash content (certificate of analysis of *C. vulgaris*, company's internal document - confidential). In R1 and R2, the obtained ash contents are above this value, but the results for R3 were slightly lower. *C. vulgaris* is reported to have an ash content of 9.5% (Shalaby & Yasin, 2013), 9.0 % (Bach, et al., 2017), and 6.3 % (Tokusoglu & Unal, 2013), which falls within the ash values obtained at R3. In this study it was observed that the use of recycled medium seems to decrease the mineral content for *C. vulgaris*. Similarly, *Tetraselmis* sp., grown in RCM in open ponds systems, had a lower ash content after 3

reuses than in fresh medium cultivation, representing less minerals and consequently more organic matter in the cells grown in RCM (Fon Sing, 2014).

Regarding protein content, no statistical differences were detected between groups in any of the medium recycling trials, as shown in Figure 3.8. The protein values obtained during the 3 trials (R1 - 53.5-57.3%; R2 - 52.7-54.0% and R3 - 48.8-54.8%) meet the range of protein values reported for *Chlorella vulgaris*, 38% to 58% (Gouveia, et al., 2008; Barros, et al., 2019).

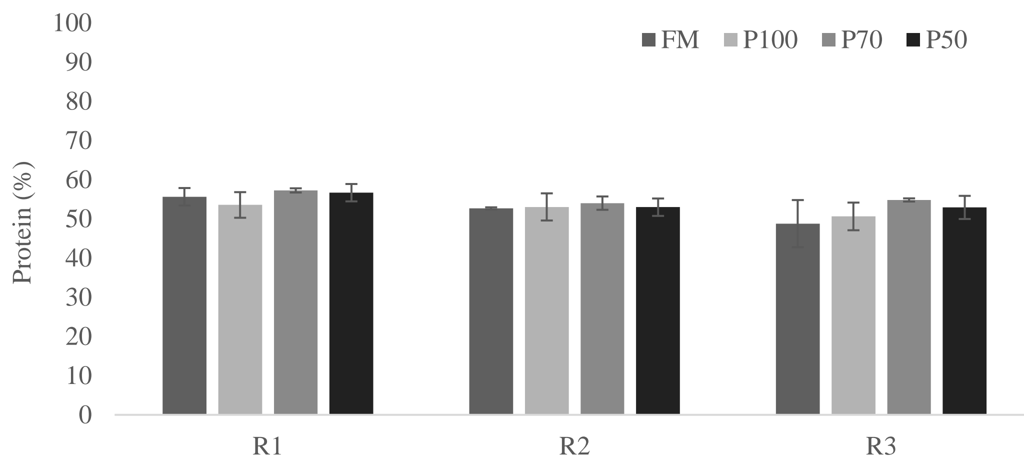

Figure 3.8 Protein content of *C. vulgaris* from flat panels bioreactors at the first (R1), second (R2), and third (R3) reuse of the culture medium. FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Results are the mean values and standard deviation of three biological replicates. No statistically significant differences within each reuse were found ($p \geq 0.05$).

Studies that evaluate the content of dissolved protein in RCM are common. However, there is no information available about the effect of RCM on the biomass protein content (Loftus & Johnson, 2017). On the other hand, studies on lipid content are common in the biodiesel industry (Farooq, et al., 2015). The results obtained for *C. vulgaris* in this study are above the range of 38.9 to 41.3% protein content, reported in the literature for 4 different strains of *Chlorella* grown in fresh basal medium on pilot-scale Green-wall PBR (Guccione, et al., 2014), which shows that the biomass protein content is not affected by recycling of the culture medium.

The functionality of the biomass was also assessed based on water and oil holding capacity, foaming and emulsifying properties.

In terms of water holding capacity (WHC) and oil holding capacity (OHC), no statistical differences were detected between the groups in any of the recycling trials as shown in Figure 3.9, not even on R3, where biomass productivity (Table 3.3) differed significantly between P100 and the control group (FM). Despite no WHC differences between R1, R2, and R3, OHC values determined at R2 are lower than R1 and R2.

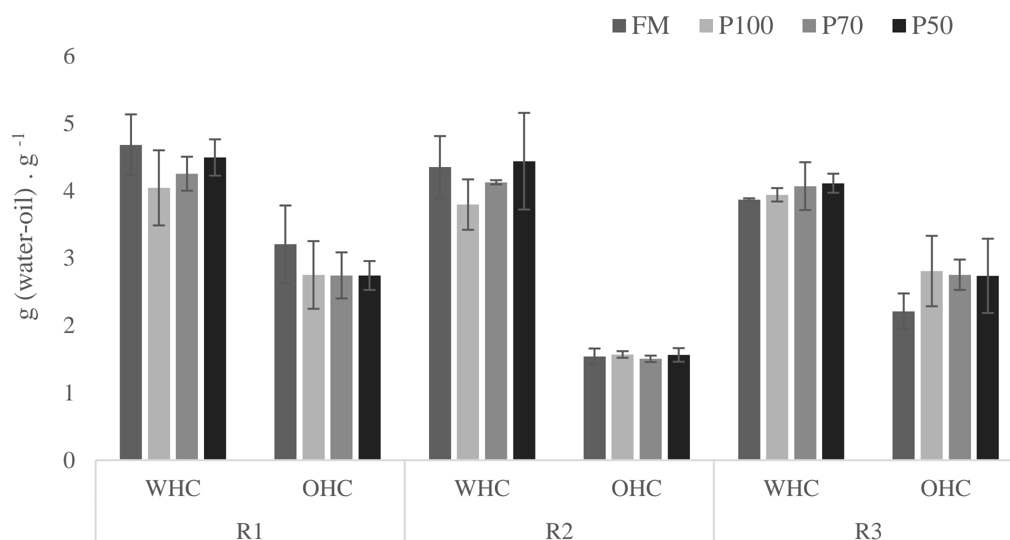

Figure 3.9 Water holding capacity (WHC) and oil holding capacity (OHC) of *C. vulgaris* biomass from flat panels bioreactors, at the first (R1), second (R2), and third (R3) reuse of the culture medium. FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Results are the mean values and standard deviation of three biological replicates. No statistically significant differences within groups were found ($p \geq 0.05$).

In this study, the WHC of the biomass was higher than that obtained by Waghmare (2016) for protein concentrates of *C. pyrenoidosa* (WHC = 3.09 ± 0.01 $g.g^{-1}$). These differences can be explained by the fact that other components of biomass, such as carbohydrates, also contribute to water retention increase. The OHC of *C. pyrenoidosa* was reported as 2.02 ± 0.04 $g.g^{-1}$ (Waghmare, et al., 2016), according to the results obtained (1.50 - 3.20 $g.g^{-1}$).

The water holding capacity (WHC) in microalgae has been related to the protein and carbohydrate content of biomass (Shalaby & Yasin, 2013). Spreadable processed cheese (Tohamy, et al., 2018), imitation processed cheese (Shalaby & Yasin, 2013), and butter cookies (Gouveia, et al., 2007) food properties were reported to be positively affected by *C. vulgaris* incorporation. The addition of microalgae biomass in food product formulations, even when added in a small proportion, allows the retention of the water responsible for changes in the food firmness.

The high OHC is desirable for food formulations such as mayonnaise (Waghmare, et al., 2016) or cookie dough (Gouveia, et al., 2007), being related to the emulsifying capacity. *C. vulgaris* mixed with acid casein curd, acts as an egg substitute and increases stability and viscosity of mayonnaise, positively affecting the sensory and nutritional characteristics of the final product (Abd El-Rasik & Mohamed, 2013). This product is already on the market as a vegan alternative to the traditional mayonnaise.

The foaming capacity (FC) of *Chlorella vulgaris* freeze-dried biomass was tested within groups, but no differences were found at any recycling stage between FM and RCM cultivations (Figure 3.10). The same behavior was found regarding foam stability at 30, 40, and 60 min (Figure 3.10). In general, R3 showed higher FC values, but lower FS than R1 and R2. It is reported that the electrostatic repulsion between protein molecules, which contributes to higher FC, is reduced by the presence of salts (Pereira, et al., 2018). And so, the lower ash content in R3 (Figure 3.7) can explain the higher FC in this case (Figure 3.10).

In accordance with the obtained results for FM at R3, 95% FC was reported for *C. pyrenoidosa* protein concentrate (Waghmare, et al., 2016). However, in all other conditions (FM, P100, P70, and P50) and reuses (R1 and R2), FC values are below 80%. In the same study, microalgae protein concentrate was reported with an FS after 180 min of 97% (Waghmare, et al., 2016). FS is a very important property during the whipping process. It is related to protein ability to stabilize air-water interfaces in foam-based foods (Dai, et al., 2020), or foam-like structures, such as bread dough. When incorporated in wheat flour dough, *C. vulgaris* had positively affected rheology and viscoelastic characteristic (Graça, et al., 2018).

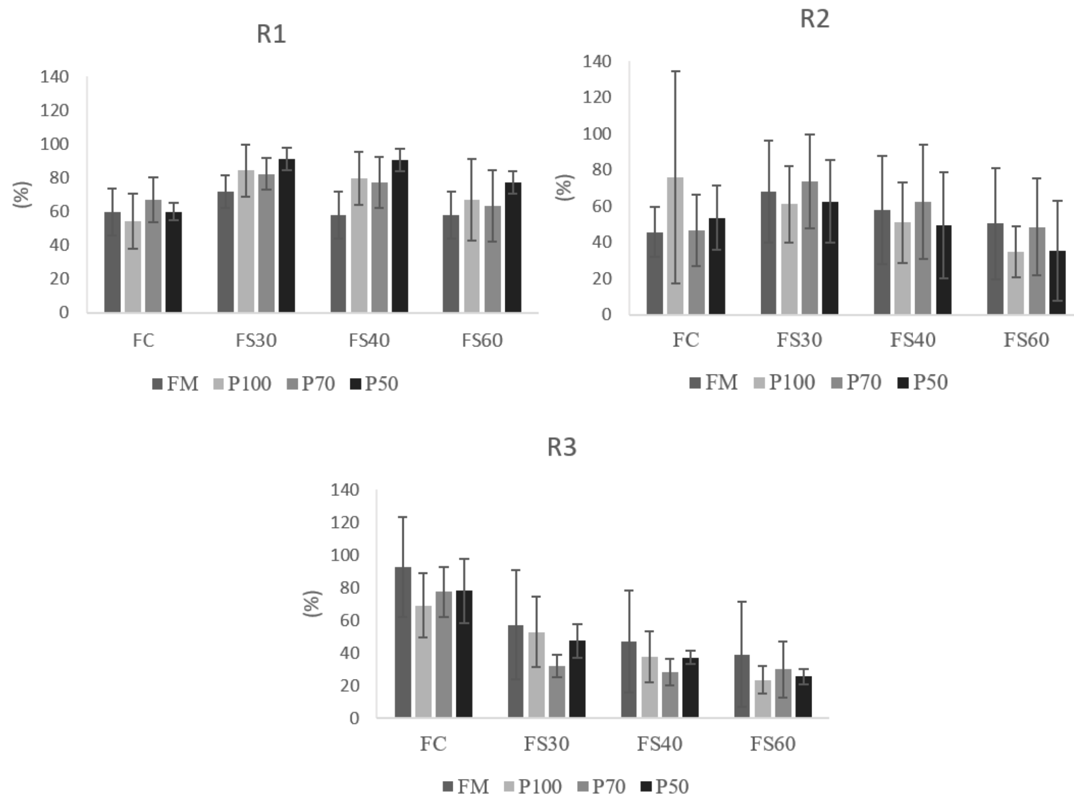

Figure 3.10 Foaming capacity (FC) and foaming stability after 30 (FS30), 40 (FS40), and 60 min (FS60) after foaming formation, of *C. vulgaris* from flat panels bioreactors, at the first (R1), second (R2) and third (R3) reuse of the culture medium. FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Results are the mean values and standard deviation of three biological replicates and three analytical replicates. No statistically significant differences within groups were found ($p \geq 0.05$).

The biomass produced in the present work presented an emulsifying activity index (EAI) and an emulsifying stability index (ESI) with no statistical differences between groups or between the three recycling trials (Figure 3.11). The suspension of freeze-dried biomass of *Chlorella vulgaris* in phosphate buffer, at pH 7, when emulsified with sunflower oil, didn´t present high EAI or ESI, resulting in an unstable emulsion. Raymundo, et al. (2005) studied the fat mimetic capacity of *Chlorella vulgaris* biomass and reported that, despite the high protein content, these microalgae cannot be used as the only emulsifier because the development of the emulsion structure does not occur. However, these authors also mentioned that when protein isolates (like pea protein) were added to the mixture, it was possible to obtain very stable emulsions, allowing a reduction in the amount of oil for the oil-in-water emulsion. Purified algae protein has been reported

as a competitive emulsifier when compared to commercial ones (Ursu, et al., 2014). Notwithstanding the end-use given to *C. vulgaris* biomass by the food industry or consumers, the emulsifying properties are not affected at any stage of the medium recycling procedure, not impacting the biomass EAI or ESI.

Overall, we can conclude that the *C. vulgaris* biomass functional properties were not affected by the use of recycled medium during cultivation, even when lower productivity was obtained.

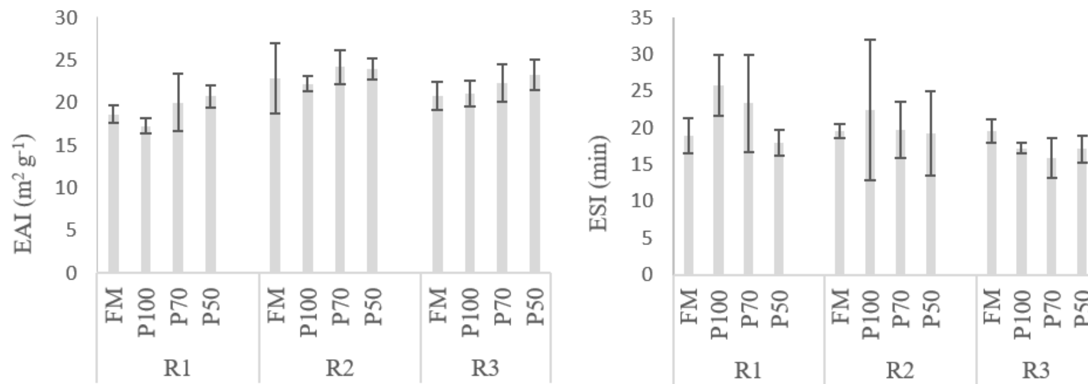

Figure 3.11 Emulsifying activity index (EAI) and emulsifying stability index (ESI) of *C. vulgaris* biomass from flat panels bioreactors, at the first (R1), second (R2), and third (R3) reuse of the culture medium. FM - fresh medium (control); P100 - 100% RCM, P70 - 70% RCM; P50 - 50% RCM. Results are the mean values and standard deviation of three biological replicates. No statistically significant differences within groups were found ($p \geq 0.05$).

4. CONCLUSIONS AND FUTURE PERSPECTIVES

Chlorella vulgaris was successfully grown at laboratory-scale bubble column photobioreactors and the growth parameters were not different between fresh and the first recycled culture medium (R1). Contamination with fungi appeared on the second reuse trial (R2) and resulted in the formation of fungal pellets with incorporated microalgae, after which no further laboratory-scale trials were performed.

The growth of *C. vulgaris* was successful when grown in pilot-scale flat panel photobioreactors under recycled culture medium for three consecutive reuses. A decrease in the productivities was noticed from R1 to R2 and R3 which was probably due to the influence of the abiotic factors. However, no significant differences were detected between the RCM and fresh medium at R1 and R2 trials. Only on the third (R3), and last, reuse trial the RCM at 100% has significantly affected the biomass global productivities of the microalgae when compared to the control. However, dilutions of the RCM at 70 and 50% did not significatively affect growth comparatively to fresh medium.

Biochemical analysis of the biomass obtained from pilot-scale trials showed that the carotenoids, ash, and protein contents were not affected either by the use of the recycled culture medium (P100, P70, and P50) neither by the number of reuses (R1, R2, and R3). However, chlorophyll content was negatively affected when RCM at 100% was applied at R3.

The foaming and emulsifying properties of *C. vulgaris* biomass were not affected by the use of recycled medium or the number of reuses.

The results obtained in this study are very promising for subsequent industrial upscaling. Its implementation will avoid the rejection of thousands of cubic meters of water per year, contributing both to the reduction of the water footprint associated with the cultivation of microalgae and to the use of the remaining nutrients still present in the culture medium.

In the future, the replicability of this work in photobioreactors on an industrial scale will be of great interest to Allmicroalgae, as well as the environmental and economic assessment associated with the impact of the recycling process. It will be also very

important to monitor the harvesting efficiencies throughout reuse, to ensure that they are not compromised by a possible selection of cells according to their size, which could affect the harvesting costs.

For future work, it would be interesting to repeat the R3 trial to assess growth in favorable conditions of light and temperature. The mineral content in P, Na, Ca, Mg, or Fe was not analyzed in this work, but it would be interesting to check if changes in its concentration occur for *Chlorella vulgaris* in the tested cultivation conditions. The access to the lipid content of biomass would also bring more insights since changes in the lipidome represent an immediate stress response.

The presence of other microorganisms and the knowledge of how their metabolism can contribute to the composition of dissolved organic matter, which varies with the culture aging, would allow a better correlation of cause and effect to the results obtained.

Finally, the applicability of the present work at Allmicroalgae will directly contribute to better water management and sustainability of the production process, reducing the water footprint and cultivation costs.

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Appendixes

A. Calibration curves

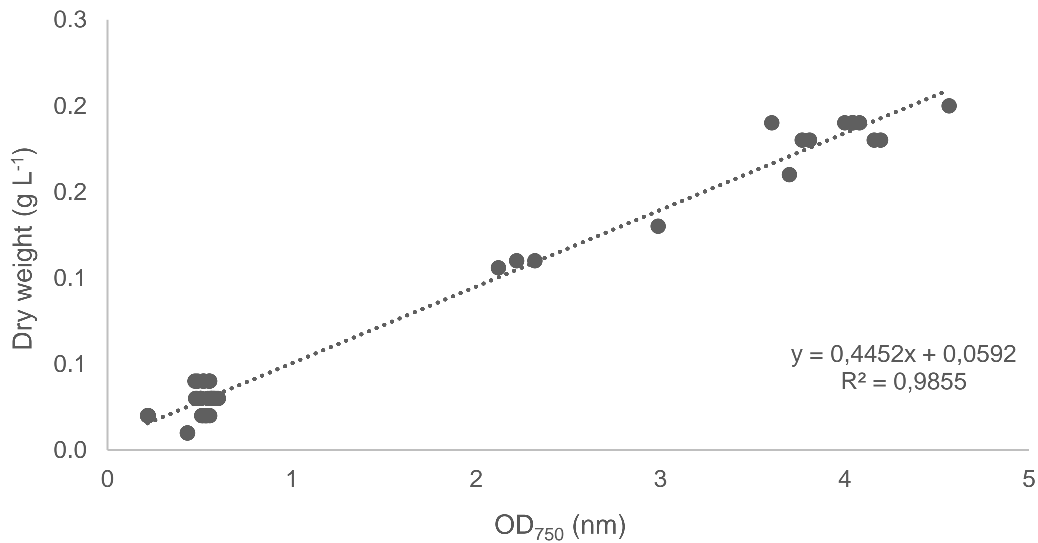

Figure A.1 Calibration curve of optical density (OD) at 750 nm and *Chlorella vulgaris* dry weight, n = 49.

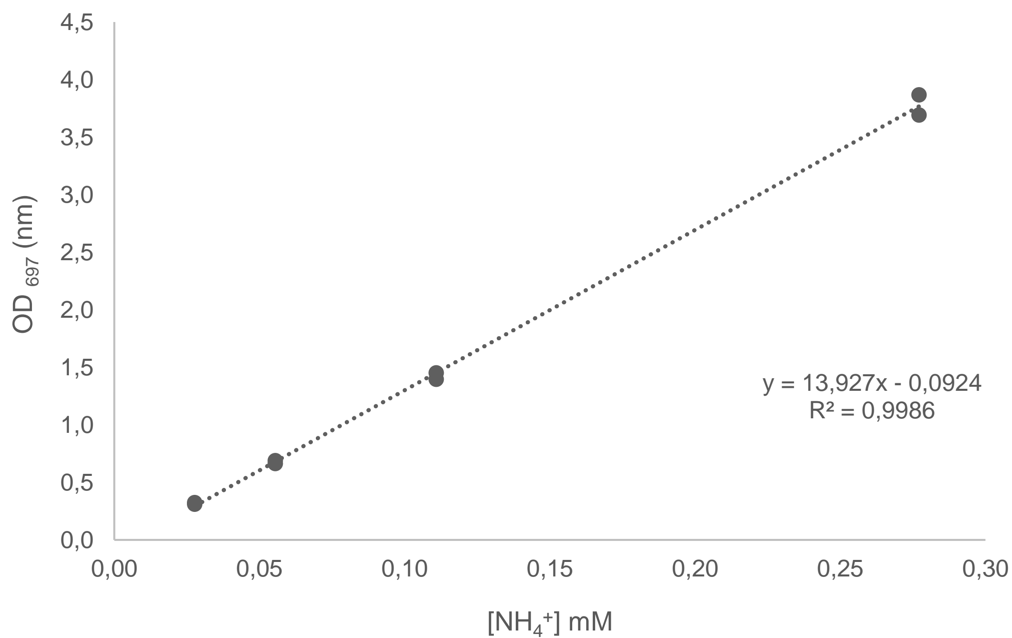

Figure A.2 Calibration curve of the optical density (OD) at 697 nm and the ammonium concentration, n = 8.

B. Pilot-scale assay related data

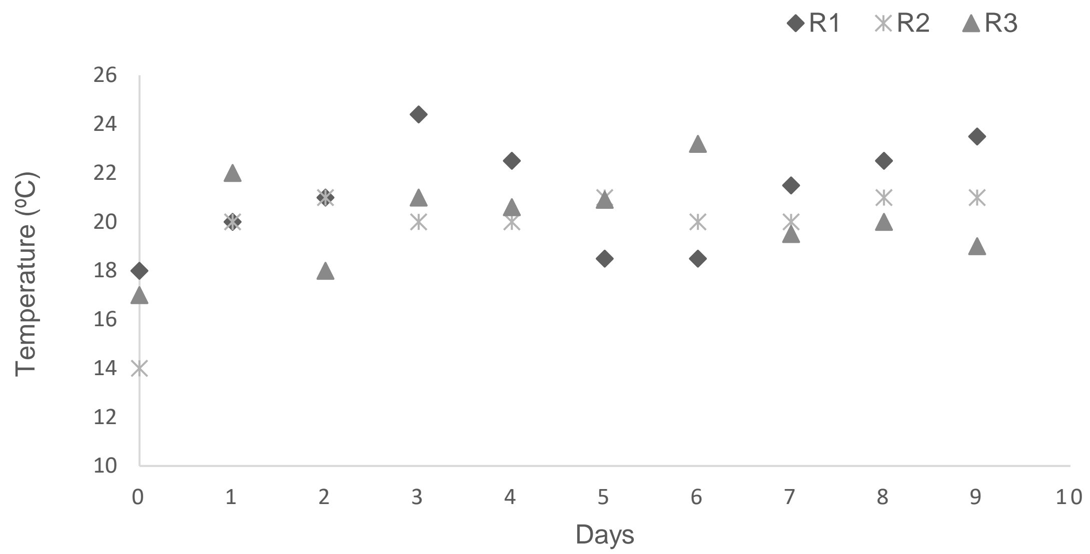

Figure B.3 Temperature of the culture medium at sampling time during the 9 days of first, second, and third medium reuse, R1, R2, and R3, respectively.