UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

Dipartimento di Agraria

Dottorato di ricerca in Sustainable agricultural and forestry systems and food security

PhD dissertation

Bioconversion of food industry by-products and waste streams into omega-3 fatty acids using aquatic protists

Promotor

Ch.mo Prof. Raffaele Sacchi

PhD student

Russo Giovanni Luca

Co-promotors

Ch.mo Prof. Paolo Masi

Dr. Antonio Luca Langellotti

Coordinator

Prof. Albino Maggio

XXXIV° ciclo

Thesis committee

Promotor

Prof. Dr Raffaele Sacchi

Professor of food science and technology

University of Naples Federico II

Co-promotors

Prof. Dr Paolo Masi

Professor of food science and technology

University of Naples Federico II

Dr Antonio Luca Langellotti

CAISIAL center

University of Naples Federico II

CONTENTS

CHAPTER 1 4

General introduction

Aim and thesis outline

CHAPTER 2 12

Sustainable production of food grade omega-3 oil using aquatic protists: reliability and future horizons

CHAPTER 3 37

Valorization of second cheese whey through cultivation of extremophile microalga *Galdieria sulphuraria.*

CHAPTER 4 56

Production of Omega-3 Oil by *Aurantiochytrium mangrovei* Using Spent Osmotic Solution from Candied Fruit Industry as Sole Organic Carbon Source

CHAPTER 5 77

Formulation of New Media From Dairy and Brewery Wastes For a Sustainable Production of DHA-rich oil By *Aurantiochytrium mangrovei*

CHAPTER 6 102

Techno-economic assessment of alternative food waste medium for the production of heterotrophic biomass rich in omega-3 oil

CHAPTER 7 123

Conclusion and future perspectives.

Chapter 1

General introduction

It has been amply reported that about a third of the food produced in the world for human consumption becomes a waste or is lost (FAO, 2013). In numerical terms, circa 1.3 billion tons of food wastes are produced every year, causing approximately US$ 750 billion in economic losses worldwide. According with FAO data, most of the food wastes are generated from household, representing the 53% of the total production of food waste (Figure 1). However household wastes are a difficult substrate to treat and their collection would be not an energy-efficient solution (Anal 2017). On the contrary, the wastes generated from agri-food industries have a stable chemical composition and are well segregated. Only from the production section, there are more than 700 million tons of food waste and side-stream that are generated from the agro-food industry annually. In many cases, these streams should not be considered a waste, but more as by-products, or specifically, as food by products (FBW).

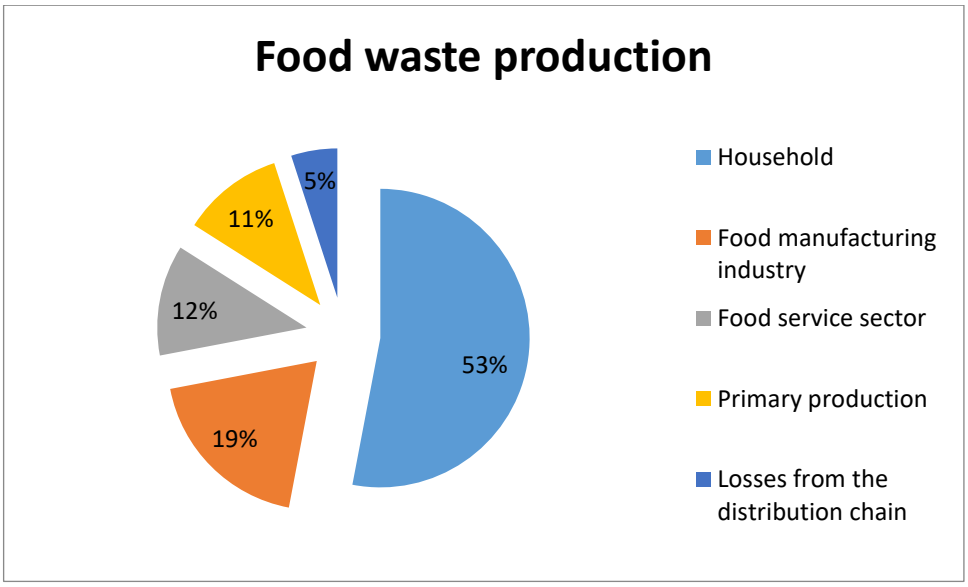

Figure 1. Principal sources of food waste generation worldwide. Source Anal et al., 2017

The FBWs produced around the world differs from country to country on the basis of main market developed. For example, in India about 65%–70% of the organic pollutants are produced from food and agro-product industries, such as distilleries, sugar factories, dairies, fruit canning, meat processing, pulp, and paper mills (Rajagopal 2008). In south east Asiatic

countries instead, the main market is represented by the palm oil industry. In fact Malaysia presently accounts for 28% of world palm oil production and 33% of world exports (MPOC 2018), producing a huge amount of polluted palm oil mill effluent (POME). Fia et al. (2012) showed that the industrial wastewater released in coffee-producing regions (e.g. Brazil, Vietnam and Colombia) has become a large environmental problem, creating the need for low cost treatment technologies (Fia et al. 2012).

Food wastewaters represent not only a problem for the producers, but most of all an environmental problem. It has been estimating that for each ton of FW produced there is an emission of about 2 tons of CO_2 (European Commission, 2010). In fact, the resources used to produce food that is eventually lost or wasted, account for approximately 4.4 gigatonnes of greenhouse gas emissions (CO_2 equivalent) annually, making food loss and waste the world's third largest emitter, after only China and the United States (Blakeney 2019). Modern industries tend to consider organic waste from food industry as a new important resource due to possibility of recycling and energy recovery, trying to adapt and to modify their processes so that any by-product can be recovered or recycled. In fact, many large companies no longer consider food by-products as a waste but more as an opportunity to start new processes (Mussatto, Dragone, and Roberto 2006; Russo et al. 2021). In European Union, every year are produced more than 250 mil MT of FBWs resulting from a variety of food manufacturing activities (Fava et al. 2015). The most used methods currently adopted for disposal of wastes include landfilling, having as the main disadvantage the high cost of transport; incineration, not convenient because of the low calorific value and high water content; feeding for animals, having the limit that not every waste is suitable for animal feeding, and biological treatment. Biological treatment is performed by anaerobic digestion that is based on different types of feedstock, such as urban organic waste, food waste from the food processing industry and manure. This lead to two outputs: one is biogas, which can be used in transport as a replacement for fossil fuels. The other output is bio-digest, which can be used as a replacement for artificial fertilizer (Lantz et al. 2007). In Mediterranean countries instead the dairy and brewery production are one of the main food industries, and it has also reached importance in other parts of the world (Australia, Chile, the United States, South Africa and China) (Ganesh et al. 2010; Stasinakis, Charalambous, and Vyrides 2022).

Dairy industry is one of the main food industries in Italy and Europe, with tons of cheese produced every year. In Italy the main dairy product is the mozzarella cheese, with a production of more than 250,000 tons every year (Castrica et al. 2020). This production leads

to different type of by-products and side-streams. For the obtainment of mozzarella cheese two main side-streams are generated: the cheese whey (CW) and the mozzarella stretching water (MSW). For the CW there are already many valorization processes through the obtainment of whey protein powder and other biotechnological processes (Barba 2021). The other categories of dairy wastewater, on the other hand, do not have valid alternatives to their reuse.

As a possible alternative, processes for adding value to FBWs should be developed. The alternative could be the upgrading concept: adding value to wastes by production of a product with desired properties, economic and ecological advantages (Laufenberg, Kunz, and Nystroem 2003; Russo et al. 2021). The FBWs can generate a number of natural bioactive molecules (vitamins, dietary fibers, pigments, organic acids, amino acids, etc.) of special interest for the food industry and the modern pharmaceutical and cosmetic industry. The market of these bio-based products is constantly increasing. At worldwide level, it increased from 77 to 92 billion from 2005 to 2010 and it's going to increase up to 228 and 515 billion in 2015 and 2020, respectively (without considering biofuels and pharmaceuticals) (Fava et al. 2015).

In order to enhance these by-products and wastewater, the aquatic protists (commonly known as microalgae) can play a primary role in converting waste to products with high added value. Many studies investigated the potential value of dual applications of microalgae for wastewater treatment and biomass production in the last years (Abreu et al. 2012; Anal 2017; Cheirsilp, Suwannarat, and Niyomdecha 2011; Girard et al. 2017; Nasir et al. 2015; Zimermann et al. 2020) proving the flexibility of these organisms to use the nutrients present in the FBWs. Most industrial wastewaters contain many nutritive substances, such as organic carbon, nitrogen, phosphorus, and other compounds. This composition makes wastewater suitable for the growth of biomass and aquatic protists (Perez-Garcia et al. 2015).

Aim and thesis outline

The aim of this research was to valorize the food by-products and wastes using aquatic protists producers of high added value molecules. In particular we studied the side-streams from dairy, brewery and sugar industry as new medium for aquatic protists cultivation in order to obtain commodities and omega-3 oil. This research aim was explored in a sequence of separate studies published or submitted to scientific journals.

The first chapter is a general introduction followed by 5 works reported as scientific papers that are published or submitted to scientific journals.

The **Chapter 2** shows a review in which we investigated sustainable processes based on FBWs for obtaining omega-3 rich oil through the cultivation of aquatic protists.

In the **Chapter 3** we evaluated the growth of an extremophile red alga (*Galdieria sulphuraria*) using a medium made from second cheese whey. This dairy by-product was successfully metabolized by the microalga without the utilization of any particular pre-treatment. Moreover the cultivation medium was optimized using a response surface methodology in order to enhance the biomass productivity, obtaining similar performance of the control with standard media.

However, in order to obtain a biomass rich in omega-3 oil, we explored new alternative medium made from candied fruit industry waste for the cultivation of the DHA producer *Aurantiochytrium mangrovei*. In **Chapter 4** was studied the utilization of spent osmotic solution from candied fruit production as source of organic carbon for *A. mangrovei*. We found that this food waste alone is capable to satisfy the carbon demand of the alga for its growth, obtaining a DHA content similar to the control. Nevertheless the supplementation of a nitrogen source was necessary to grow *A. mangrovei* because SOS has a very low content of nitrogen. For that reason, we explored other FBWs for the cultivation of this aquatic protists. In **Chapter 5** we studied a blend of dairy and brewery wastes for the development of a medium that can satisfy the whole nutrient demand of *A. mangrovei*. In that study we evaluated the utilization of a dairy effluent (mozzarella stretching water) coupled with spent yeast from brewery industry. For these FBWs some pre-treatments were necessary in order to increase the nutrient bio-availability. In the specific, an enzymatic hydrolysis of dairy waste was conducted, obtaining high biomass productivity.

In **Chapter 6** the insights of this thesis were combined in a techno-economic model for the evaluation of the cost-impact of FBWs as new nutrient source for heterotrophic cultivation of

A. mangrovei. In this model we compared the standard cultivation with the new elaborated food waste-based medium, showing that the operative cost can be significantly reduced used FBWs instead of bulk materials.

Finally, in **Chapter 7** the conclusions and future perspectives extrapolated from previous studies are reported and summarized.

References

Abreu, Ana P., Bruno Fernandes, António A. Vicente, José Teixeira, and Giuliano Dragone. 2012. "Mixotrophic Cultivation of Chlorella Vulgaris Using Industrial Dairy Waste as Organic Carbon Source." *Bioresource Technology* 118:61–66.

Anal, Anil Kumar. 2017. "Food Processing By-Products and Their Utilization: Introduction." in *Food Processing By-Products and their Utilization*.

Barba, Francisco J. 2021. "An Integrated Approach for the Valorization of Cheese Whey." *Foods* 10(3).

Blakeney, Michael. 2019. *Food Loss and Food Waste*.

Castrica, Marta, Vera Ventura, Sara Panseri, Giovanni Ferrazzi, Doriana Tedesco, and Claudia Maria Balzaretti. 2020. "The Sustainability of Urban Food Systems: The Case of Mozzarella Production in the City of Milan." *Sustainability (Switzerland).*

Cheirsilp, Benjamas, Warangkana Suwannarat, and Rujira Niyomdecha. 2011. "Mixed Culture of Oleaginous Yeast Rhodotorula Glutinis and Microalga Chlorella Vulgaris for Lipid Production from Industrial Wastes and Its Use as Biodiesel Feedstock." *New Biotechnology*.

Fava, Fabio, Grazia Totaro, Ludo Diels, Maria Reis, Jose Duarte, Osvaldo Beserra Carioca, Héctor M. Poggi-Varaldo, and Bruno Sommer Ferreira. 2015. "Biowaste Biorefinery in Europe: Opportunities and Research & Development Needs." *New Biotechnology*.

Fia, Fátima R. L., Antonio T. Matos, Alisson C. Borges, Ronaldo Fia, and Paulo R. Cecon. 2012. "Treatment of Wastewater from Coffee Bean Processing in Anaerobic Fixed Bed Reactors with Different Support Materials: Performance and Kinetic Modeling." *Journal of Environmental Management*.

Ganesh, Rangaraj, Rajagopal Rajinikanth, Joseph V. Thanikal, Ramamoorty Alwar Ramanujam, and Michel Torrijos. 2010. "Anaerobic Treatment of Winery Wastewater in Fixed Bed Reactors." *Bioprocess and Biosystems Engineering*.

Girard, Jean Michel, Réjean Tremblay, Nathalie Faucheux, Michèle Heitz, and Jean Sébastien Deschênes. 2017. "Phycoremediation of Cheese Whey Permeate Using Directed Commensalism between Scenedesmus Obliquus and Chlorella Protothecoides." *Algal Research*.

Lantz, Mikael, Mattias Svensson, Lovisa Björnsson, and Pål Börjesson. 2007. “The Prospects for an Expansion of Biogas Systems in Sweden - Incentives, Barriers and Potentials.” *Energy Policy*.

Laufenberg, Günther, Benno Kunz, and Marianne Nystroem. 2003. “Transformation of Vegetable Waste into Value Added Products: (A) the Upgrading Concept; (B) Practical Implementations.” *Bioresource Technology*.

MPOC. 2018. “Malaysian Palm Oil Council.” Retrieved October 13, 2019 (http://mpoc.org.my/malaysian-palm-oil-industry/).

Mussatto, S. I., G. Dragone, and I. C. Roberto. 2006. “Brewers’ Spent Grain: Generation, Characteristics and Potential Applications.” *Journal of Cereal Science.*

Nasir, Nurfarahana Mohd, Nur Syuhada Abu Bakar, Fathurrahman Lananan, Siti Hajar Abdul Hamid, Su Shiung Lam, and Ahmad Jusoh. 2015. “Treatment of African Catfish, Clarias Gariepinus Wastewater Utilizing Phytoremediation of Microalgae, Chlorella Sp. with Aspergillus Niger Bio-Harvesting.” *Bioresource Technology*.

Perez-Garcia, Octavio, Yoav Bashan, Yoav Bashan, and Yoav Bashan. 2015. “Microalgal Heterotrophic and Mixotrophic Culturing for Bio-Refining: From Metabolic Routes to Techno-Economics.” in *Algal Biorefineries: Volume 2: Products and Refinery Design*.

Rajagopal, Deepak. 2008. “Implications of India’s Biofuel Policies for Food, Water and the Poor.” *Water Policy*.

Russo, Giovanni L., Antonio L. Langellotti, Maria Oliviero, Raffaele Sacchi, and Paolo Masi. 2021. “Sustainable Production of Food Grade Omega-3 Oil Using Aquatic Protists: Reliability and Future Horizons.” *New Biotechnology* 62(January):32–39.

Stasinakis, Athanasios S., Panagiotis Charalambous, and Ioannis Vyrides. 2022. “Dairy Wastewater Management in EU: Produced Amounts, Existing Legislation, Applied Treatment Processes and Future Challenges.” *Journal of Environmental Management*.

Zimermann, Jéssika D. ar. Fernandes, Eduardo Bittencourt Sydney, Maria Luísa Cerri, Isabella Kuroki de Carvalho, Kathlyn Schafranski, Alessandra Cristine Novak Sydney, Luciano Vitali, Samantha Gonçalves, Gustavo Amadeu Micke, Carlos Ricardo Soccol, and Ivo Motin Demiate. 2020. “Growth Kinetics, Phenolic Compounds Profile and Pigments Analysis of Galdieria Sulphuraria Cultivated in Whey Permeate in Shake-Flasks and Stirred-Tank Bioreactor.” *Journal of Water Process Engineering* 38:101598.

Chapter 2

Sustainable production of food grade omega-3 oil using aquatic protists: reliability and future horizons

This chapter has been published as:

Russo, G. L., Langellotti, A. L., Oliviero, M., Sacchi, R., & Masi, P. (2021). Sustainable production of food grade omega-3 oil using aquatic protists: reliability and future horizons. *New Biotechnology*.

Abstract

Biotechnological production of omega-3 polyunsaturated fatty acids (PUFAs) has become a commercial alternative to fish oil in the past twenty years. Compared to PUFA production by fatty fishes that from microorganisms has increased due to its promising sustainability and high product safety and also to the increasing awareness in the expanding vegan market. Although autotrophic production by microalgae seems to be more sustainable in the long term, to date most of the microbial production of omega-3 is carried out under heterotrophic conditions using conventional fermentation technologies. The present review critically analyzes the main reasons for this discrepancy and reports on the recent advances and the most promising approaches for its future development in the context of sustainability and circular economy.

Keywords: Polyunsaturated fatty acids, microalgae, fermentation, sustainability, biorefinery, food waste

Introduction

Omega-3 polyunsaturated fatty acids (n3-PUFAs) are recognised as fundamental elements in the human diet, with a series of health effects and benefits in the treatment of several pathologies. The low ratio between omega-6 and omega-3 series in the modern diet involves an increase in the risks of cardiovascular diseases, type 2 diabetes and some types of cancer in genetically predisposed individuals [1]. Alpha-linolenic acid (ALA, C18:3 n-3) is an omega-3 PUFA found in some biomass such as walnuts, flax, soybean, chia, hemp and *Echium plantagineum L*. [2]. ALA is the precursor for the biosynthesis of omega-3 very long chain polyunsaturated fatty acids (VLC-PUFAs) such as stearidonic acid (SDA, C18:4), eicosapentaenoic acid (EPA, C20:5), docosapentaenoic acid (DPA, C22:5) and docosahexaenoic acid (DHA, C22:6).

For most animals, VLC-PUFAs are essential components of cell membranes in neural and muscle tissues and are precursors of signaling molecules (bioactive lipid mediators) [3]. Moreover, DPA is the second most frequent constituent of the human brain and is important in pregnancy and foetal neural development [4]. Nevertheless, de novo synthesis of EPA and DHA is only performed efficiently by some taxa of aquatic protists, generally described as microalgae, representing the main source of these fatty acids in the biosphere [5]. VLC-PUFAs are transferred through trophic chains to invertebrates and fish, and then to terrestrial

consumers, including humans. Terrestrial plants do not produce VLC-PUFAs and most vertebrates, including humans, cannot synthesise the conversion of ALA to DHA efficiently due to the lack or poor expression of the required enzymes [5]. Thus, EPA, DPA and DHA are considered essential fatty acids because they must be obtained from food or supplementary sources [1].

Although humans have evolved to genetically adapt to a ratio of omega-6/omega-3 fatty acids of about 4 to 1, to date the worldwide availability of VLC-PUFAs seems insufficient to meet the demand [6]. The main source of VLC-PUFAs in the human diet is fish oil. Aquaculture of fatty fish rich in omega-3 depends on the fish forage that provides fish meal and fish oil, the key fish feed ingredients. Coupled with fisheries for direct human consumption, this affects the fish stocks that are predicted to be irreversibly damaged in the near future [7]. Therefore, in order to fight the rising cost of fish oil, the content of vegetable biomass in fish diets is progressively increasing, resulting in a lower VLC-PUFA content in fish muscle [8].

Bivalve mollusc culture seems to be a promising approach to meet both the VLC-PUFA and protein future demands. However, it comes with the barriers of food allergies and biotoxin hazard risks [9]. Other alternatives could be to harvest zooplankton, such as krill and copepods, but that has potential consequences on the marine environment if performed on a large scale [10]. A further promising approach could be the transfer of VLC-PUFA cluster genes from microorganisms to the crops commonly used for vegetable oil production [2]. However, this could be hindered by legal restrictions on the cultivation of genetically modified organisms, such as in Europe.

With the above-listed assumptions, the scientific community has focused on research into sustainable biomass production for VLC-PUFAs. Cultivation of VLC-PUFA rich microorganisms, such as marine protists (e.g. microalgae), is the most promising and viable solution to meet the current gap between VLC-PUFA supply and demand. This review thus critically discusses the availability and promises of the aquatic protists to be used in this application. Strategies to enhance sustainability and reduce the cost of the production process are also discussed. Moreover, a special focus on nutrient recycling from industrial food by-products and wastes using fermentation technologies is included, coupled with a biorefinery model to recover all high-value chemical compounds from biomass to achieve more sustainable production in the context of the circular economy.

Exploitation of metabolic biodiversity of aquatic protists for VLC-PUFA production

The term 'protist' generally refers to all unicellular eukaryotes, ranging from algae to heterotrophic flagellates, which are placed into a single kingdom of Protista [11]. However, 'microalgae' refers to a polyphyletic group of photosynthetic organisms, such as prokaryotic cyanobacteria and unicellular eukaryotes. Therefore, because the term "microalgae" does not recognise that many protists can also grow heterotrophically or mixotrophically, currently protist is used to describe single-celled eukaryotes in general [12]. Many genera are obligate photoautotroph, but some species can grow also mixotrophically such as *Brachiomonas, Chlorella, Chlorococcum, Cyclotella, Euglena, Haematococcus, Nannochloropsis, Navicula, Nitzschia, Ochromonas, Phaeodactylum, Rhodomonas* and *Scenedesmus* [13].

Many of them are also facultative heterotrophs, belonging to genera *Amphora, Ankistrodesmus, Chlamydomonas, Chlorella, Chlorococcum, Cyclotella, Dunaliella, Euglena, Nannochloropsis, Nitzschia, Ochromonas* and *Tetraselmis* [13]. Moreover, some protists are obligate heterotrophs such as *Crypthecodinium* and thraustochytrids, but also many dinoflagellates such as *Oxyrrhis* and *Gyrodinium*.

Protists are important VLC-PUFA producers and, therefore, are considered possible candidates for industrial production of EPA and DHA. Those producing VLC-PUFAs belong mainly to marine phytoplankton [14]. However, some freshwater protists, such as *Monodus subterraneus* and *Trachydiscus minutus* are considered potential EPA-producers [15,16]. Generally, protists grown in heterotrophy and mixotrophy have increased VLC-PUFA content [17]. VLC-PUFA content and cultivation strategies of some genera of marine and freshwater protists are summarised in Table 1.

Table 1. Reported cultivation strategies and average content of EPA, DHA and DPA as % of total fatty acids (TFA) from some genera of marine and freshwater protists.

Phylum	Genus	Cultivation type	n3-LCPUFAs (% of TFA)	Ref.
Bacillariophyta	*Phaeodactylum*	P, M	18.6 EPA. 1.3 DHA	[16]
	Nitzschia	P, M, H	13.8 EPA 1.1 DHA	[76]
	Skeletonema	P	10.9 EPA, 1.4 DHA	[76]
	Thalassiosira	P	15.1 EPA, 3.9 DHA	[14]
	Odontella	P	19.8 EPA, 2.9 DHA	[76]
	Cyclotella	P, M, H	15.4 EPA, 1.2 DHA	[76]
Ochrophyta	*Nannochloropsis*	P, M, H	21.0 EPA	[16]
	Chloridella	P	28.7 EPA	[16]
	Monodus	P, M, H	12.0 EPA, 2.3 DHA	[16]
	Trachydiscus	P	38.7 EPA	[15]
Rhodophyta	*Porphyridium*	P, M, H	16.7 EPA	[16]
Cryptophyta	*Rhodomonas*	P	10.7 EPA, 6.9 DHA	[76]
	Chroomonas	P	13.4 EPA, 4,7 DPA	[76]
Chlorophyta	*Tetraselmis*	P, M, H	9.5 EPA	[16]
	Koliella	P	5.2 EPA	[16]
Heterokonta - Bygira	*Aurantochytrium*	H	39 DHA	[77]
	Schizochytrium	H	43.1 DHA	[36]
	Thraustochytrium	H	69 DHA 13 DPA	[77]
	Ulkenia	H	13.7 DHA 2.4 DPA	[77]
Haptophyta	*Emiliania huxleyi*	P	19.7 DHA	[14]
	Isochrysis	P, M, H	1.9 EPA, 6.6 DPA, 14.3 DHA	[16]
	Pavlova	P, M	27.8 EPA, 6,6 DPA, 6,6 DHA	[76]
Miozoa	*Amphidinium*	P, M	7.6 EPA, 2.6 DPA, 10.4 DHA	[16]
	Crypthecodinium	H	28.8 DHA	[19]
	Pyrocystis	P, M, H	24.3 EPA, 41.1 DPA	[16]
	Prorocentrum	P, M	24.1 EPA, 20.6 DHA	[16]
	Oxyrrhis marina	H	1.4 EPA, 15.3 DHA	[18]

Legend: P= photoautrophic, M= mixotrophic, H= heterotrophic cultivation

Among the obligate heterotrophic protists, it is reported that the phagotrophs *Ochromonas marina* and *Gyrodimium dominans* produce more EPA and DHA when fed on dried yeast [18], while thraustochytrids (*Aurantochytrium spp., Thraustochytrium spp.* and *Schizochytrium spp*.) and dinoflagellate *Crypthecodinium cohnii* are considered mainly DHA producers [19,20]. In particular, for thraustochytrids, a DHA content of more than one third of the total fatty acids is usually reported [20,21]. Moreover, *Schizochytrium sp*. is also used for the industrial production of DPA [22]. Mixotrophic growth has been reported to improve lipid productivity of many protists. In particular, *Nannochloropsis gaditana, N. oculata, Dunaliella*

salina and *Chlorella sorokiniana* produce a higher amount of lipids in mixotrophy, compared with photoautotrophy [23,24].

Besides the regular metabolism, it is possible to enhance the lipid productivity of protists by appropriate strain selection and by inducing mutagenesis and/or genetic engineering [13,25]. A study has reported that mutants of *Nannochloropsis oculata* increased the levels of EPA after N-methyl-N-nitrosourea-induced mutagenesis [25]. In *Pavlova lutheri*, instead, after mutation by UV-light, the EPA and DHA content were 32.8% and 32.9% (as % dry biomass) respectively, higher than those of native strain [26]. Recently, an improvement in DHA and EPA content of *Schizochytrium sp*. by 81.5% and 172.5% respectively was reported, that could be of interest to apply at an industrial scale [27].

State of production technologies

The cultivation technology for aquatic protists represents a key point for VLC-PUFA production and improvement of lipid yields. Cultivation technologies are based largely on the metabolism of the species. Autotrophic cultivation is the oldest method to cultivate microalgae, and the main industrial technology used in autotrophy is the open pond system [28]. Open ponds are built in a raceway or circular configuration; in the former, biomass surface is exposed to sunlight as much as possible and its movement is guaranteed by paddle wheels that provide regular mixing and recirculation, preventing biomass sedimentation; in the second configuration instead, the tank has a cylindrical shape and biomass is continuously stirred by a pivoted rotating agitator. However, due to several limitations, this configuration has been set aside and not used for industrial cultivation [29].

In order to prevent contamination and to control critical parameters such as CO2 utilisation, light intensity and temperature, photobioreactors (PBRs) and indoor ponds are developed [30]. Nevertheless, the development of increasingly sophisticated PBRs with lower investment costs has been one of the main targets of recent years [28]. Closed PBRs allow control over all the growth parameters and avoid wasting CO2. However, the high investment cost for these plants remains the main problem [30]. The classic PBR designs are the tubular (vertical and horizontal) and flat-panel systems. The tubular design is made with transparent tubing where the culture flows with a certain speed [31], while the flat panel reactors consist of two parallel panels (usually made in PVC) between which there is a layer where biomass grow [32]. Nevertheless, these designs have some disadvantages such as high investment

costs, difficulty in light absorption and biomass harvesting and the absence of possibility to scale up in large-scale production [32,33]. To obtain a more uniform light distribution in tubular PBRs, an innovative design where the tubes are immersed in a suspension of light-scattering silica nanoparticles were designed [33]. This reactor was tested to grow *Chlamydomonas reinhardtii*, a protist rich in VLC-PUFAs.

Unlike autotrophy, heterotrophic conditions require the addition of an organic carbon source but not light. Thus, heterotrophic growth can be performed in conventional microbial bioreactors, reducing the initial investment costs. Recent studies have shown that the use of "closed" biofermenters for the production of VLC-PUFAs, is the best methods to produce these fatty acids [34]. Some engineering strategies have been established in the context of VLC-PUFA production from many protists, such as fed-batch fermentations. In fed-batch strategies, the amount of organic carbon is not supplied to the culture all at once but is spread out over time, depending on the metabolic rate of the species [35]. In fed-batch cultivation of *Schizochytrium sp*., enhancement of DHA production and doubling of lipid productivity, compared to batch cultivation methods, have been demonstrated [36]. However, fed-batch has the limitation of low volumetric productivity [35]. For that reason, a continuous cultivation mode (where the volume of bioreactors is constant) was also developed for PUFA-producer species. Different strategies to increase the lipid productivity were developed; the most common strategy relies on nitrogen starvation, which induces lipid accumulation. However, that causes a drop in biomass growth rate [37]. To overcome this limitation, an innovative multi-stage continuous cultivation was developed to obtain a good compromise between the growth rate and relative amount of the target molecules such as VLC-PUFA and/or secondary metabolites [37,38]. In a recent study, a three stage approach has been developed for *Schizochytrium sp*. cultivation, obtaining an increase of lipids, DHA content and DHA productivity by 47.6%, 64.3% and 97.1% respectively, in comparison with the two-stage fermentation process [38].

Mixotrophic cultivation technologies are similar to those used for autotrophy, with minor modifications. One of the main technological challenges for mixotrophy, is to design a cost-effective system ensuring axenicity (requires steam-sterilisation) and, at the same time, also providing natural or artificial light. In a recent study, flat-panel PBRs were used to test an industrial scale-up for *Chlorella vulgaris* in mixotrophic conditions, concluding that there is ample room for engineering improvements [39]. An interesting technological variant was proposed in another study for mixotrophic cultivation. In this work, the lipid production of a mutant *Scenedesmus sp*. Z-4 was enhanced with an ultrasonic treatment, which led to an

improvement of enzyme activity, cell membrane permeability and substrate transportation [40]. The amount and type of organic carbon to be used in mixotrophic cultivation requires further studies to establish the correct combinations for each species and strain [23]. There are currently a large number of studies related to mixotrophic cultivation for *Chlorella sp*., *Nannochloropsis sp*. and *P. tricornutum*, but very few for other species [13,17,23,39,41]. This aspect is a limiting factor for industrial scale-up, as the mixotrophy is still limited to a few species, and often to those that do not produce a good amount of VLC-PUFAs.

Biorefinery concept to enhance sustainability and lower production costs

Aquatic protists are an important source of high-value compounds, including those producing VLC-PUFA: chlorophyll, canthaxanthin, lutein and beta-carotene from *Nannochloropsis sp*. [42], fucoxanthin from *P. tricornutum* [43], exopolysaccharides and phycobiliproteins from *Porphyridium cruentum* [34,44], β-carotene and violaxanthin from *N. gaditana* [23], carotenoids from thraustochytrids [45] and astaxanthin from *Aurantiochytrium sp*. [46]. Therefore, one of the strategies to improve the sustainability of VLC-PUFA production by aquatic protists, is the exploitation of all the possible high-value co-products from the whole biomass and the residual spent medium for cultivation [47]. One of the possible downstream biorefinery approaches is shown in Figure 1.

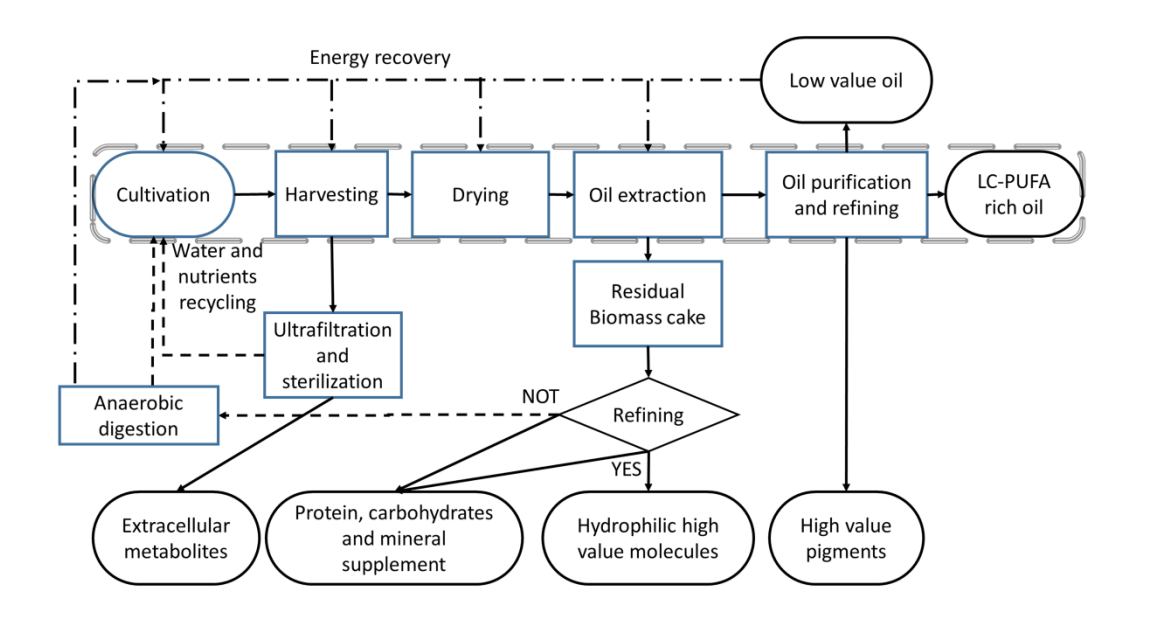

Figure 1. Multiproduct biorefinery model for the obtainment of algae oil using as a platform a VLC-PUFA rich protist

Many molecules such as extracellular polymeric substances (EPS) are released during the cultivation process. Moreover, residual spent media after harvesting is rich in residual nutrients that could be recycled into the cultivation process, which is a common practice especially in autotrophic cultivation to lower production costs [48].

After lipid extraction, the residual defatted biomass (cake) can also be used to recover high-value compounds (i.e. phycobiliproteins) and/or used as protein-, carbohydrates- and mineral-rich biomass for feed supplementation [49]. The lowest value application of the residual cake could be anaerobic digestion for the recovery of energy and mineral nutrients in the production process. In a recent study, the defatted biomass was used as feedstock for the production of bio-hydrogen through anaerobic digestion and also to recover reducing sugars that were reused in the cultivation process [50]. Moreover, the process for lipid purification and omega-3 concentration requires the removal of high value pigments (i.e. carotenoids) and short chain fatty acids suitable for energy production into the biorefinery. On-site conversion for energy production often requires additional equipment, increasing capital costs; also, valorisation of co-products in other markets may have better economic sustainability [47].

Some omega-3 rich protists show a high content of light-harvesting pigments such as phycobiliprotein (PBP) that are widely used as natural dyes for food and cosmetics. *P. purpureum* has a total PBP content of 4.8% on a dry basis consisting of 70% phycoerythrin, 20% C-phycocyanin and 10% allo-phycocyanin, all pigments with high economic value [44]. In fact, a study estimated the total cost for highly purified PE production in *P. cruentum* at USD 1.17 mg^{-1}, while the commercial price of standard PE is higher than USD 30 mg^{-1} [51].

From the omega-3 crude oil, it is also possible to recover some high-value carotenoids. Fucoxanthin (FX) represents a major carotenoid in diatoms and presents several health benefits thanks to its claimed antioxidant, anti-inflammatory, anticancer, and antihypertensive activities [52]. Recently the FX production of 13 diatoms in photoautotrophy were studied, reporting the highest value in *Odontella aurita* (>0.20 mg $L^{-1}d^{-1}$) [53]. Another study obtained 5.97 mg L^{-1} of FX using *P. tricornutum* with a supplementation of spent yeast (versus 1.82 mg L^{-1} from the control) [43].

Thraustochytrids are reported to be a source of xanthophyll carotenoid astaxanthin. Astaxanthin productivity of 9.48 mg $L^{-1}day^{-1}$ was reported through the cultivation of *Aurantiochytrium sp*. mutant, with a yield of 40 mg L^{-1} [46]. Another study reported an astaxanthin yield of 162.14 μg g^{-1} from *Thraustochytrium sp*. S7 optimized with response

surface methodologies [54]. Astaxanthin and β-carotene represent almost half of the global carotenoid market, which was estimated to be $1.2 billion in 2016 and is expected to increase to over $1.5 billion by 2021 [55]. Others postulated a biorefinery model for biofuel production that can also be implemented in the cultivation of PUFA-rich protists. The authors concluded that without an integrated approach, microalgal biodiesel could never be produced economically [56]. From the reported data, it is clear that a proper biorefinery design with a proper fractionation system as suggested in Figure 1, can also be of environmental and economic profit for VLC-PUFA production.

Use of recycled nutrients for food-grade PUFA production

Despite the ability that many protists have to grow on wastewater and polluting substances, here we will evaluate only the use of food grade elements for the development of the biomass. From the perspective of a more economical cultivation process, heterotrophy has a great advantage; without the light requirement, heterotrophic protists can also grow in a dark colored media or in the presence of suspended solids that make the passage of light difficult [57]. This advantage could be exploited also for mixotrophic cultivation.

Nutrients required during the cultivation of PUFA-rich microorganisms contribute significantly to the overall costs and carbon footprint of the final product. To overcome this limitation, the recycling of nutrients from agro-industrial flue gas, side-streams, waste and by-products seems to be one of the best approaches for VLC-PUFAs production from aquatic protists [58]. Although there are different sources of agro-industrial waste, those ones coming from the food industry can be reused in fermentation technologies to produce food-grade high value products. Food industry by-products and waste (FBW) are characterised by high amounts of organic carbon, proteins and mineral salts, which could be usefully recovered for biomass cultivation [59,60]. Moreover, these FBW are easily obtained, being produced in large quantities, particularly those from the agro-industry, for which an increase is expected in coming years [61]. To date, some FBW have been successfully used for the cultivation of microorganisms. Among them, the cheapest are sugar molasses, corn steep liquor (CSL), whey permeate (WP) and glycerol. These by-products are successfully used in the cultivation of aquatic protists rich in VLC-PUFAs; other available FBW such as brewery by-product and food waste showed promising results (Table 2).

Table 2. Aquatic protists rich in n-3 LC-PUFAs cultivated using agro-industrial by-products.

Food by-products	Species	Total lipids (% of biomass)	LC-PUFA content (% of TFA)	Biomass concentration (g L^{-1} DW)	Ref.
Corn steep powder + glycerol	*Aurantiochytrium sp. n. AF0043*	31.14 %	DHA 29.7% DPA 6.0%	29.78	[78]
Cheese whey + corn steep liquor	*Crypthecodinium cohnii* CCMP 316	28.7%	DHA 8.5-17 %	2.0 g L^{-1} day^{-1}	[79]
Tofu whey	*Schizochytrium sp.* S31	56.8 %	DHA 22.5% DPA 3.9% EPA 1.4 %	13.3	[75]
Carob pulp	*Crypthecodinium cohnii* CCMP 316	9.2 %	DHA 48% DPA 2.1%	42.0	[80]
Potato processing water	Thraustochytriidae sp. AS4-A1	38%	DHA 28.9%	9.0	[81]
Liquid from brewery by-product	Thraustochytriidae sp. AS4-A1	31%	DHA 21.5% DPA+ EPA 21.5%	9.01	[81]
Liquid from brewery by-product + yeast extract + monosodium glutamate	Thraustochytriidae sp. AS4-A1	50%	DHA 13.3%	15.2	[81]
Saline water from demineralization of cheese whey + glycerol	*Schizochytrium limacinum*	35 %	DHA 48.46 %	28.40	[82]
Saline water from demineralization of cheese whey + yeast extract + glycerol	*Japonochytrium marinum*	51.47 %	DHA 48.98 %	24.72	[82]
Cane molasses	*Schizochytrium sp. CCTCC M209059*	41.22 %	DHA 37.9% DPA 12.08% EPA 1.16%	21.94	[83]
Cane molasses + algae-residue	*Schizochytrium sp.*	32.8%	DHA 45.26%	55.54	[84]
High-fructose corn syrup	*Aurantiochytrium sp.* YLH70	64.9%	DHA 39.41%	78.5	[85]
Brewery spent yeast	*Aurantiochytrium sp.* KRS101	38.1%	DHA 34.2%	31.8	[86]
Brewery spent yeast	*Phaeodactylum tricornutum*	N.r.	EPA 16%	0.8	[43]
Food waste + glycerol + antioxidant	*P. tricornutum* strain E70	35%	ARA 5.6% DHA 3.3% EPA 25.9%	N.r.	[87]

N.r.= Not reported; Values expressed as g $L^{-1}d^{-1}$ refers to the biomass productivity

FBW usually needs pre-treatment before use as nutrients. Complex organic solids such as carbohydrates (starch, cellulose, lactose etc.), lipids and proteins must be hydrolysed to release basic components (i.e. monosaccharides and amino acids) that are easily usable by microorganisms [58]. The pre-treatments aim to a) remove particulates and reduce colouring effect for mixotrophic cultivation (avoiding light-shading effects); b) increase the bioavailability of organic compounds (i.e. particle size reduction, protein and carbohydrate hydrolysis); c) remove or reduce the number of toxic compounds; and d) increase stability of FBW and related carbon loss during transport and storage before use [62]. However, it is not well known if the pre-treatment of FBW could affect fatty acid production by aquatic protists, but the utilization of FBW in the culture media could induce changes in the biomass biochemical composition [60]. Different approaches to treatments for microalgal cultivation have been evaluated in the last few years, one such approach being enzymatic hydrolysis. Commercial amylolytic and proteolytic enzymes were used in submerged fermentations to treat food waste for *Chlorella pyrenoidosa,* obtaining a hydrolysate rich in glucose and free amino nitrogen (FAN) [63]. Fungal hydrolysis was reported also as an effective pre-treatment of food waste for heterotrophic cultivation of *Schizochytrium mangrovei* and *C. pyrenoidosa* [64]. Anaerobic digestion (AD) of agro-industrial wastes, wastewater and by-products coupled with microalgae cultivation is reported as a strategy to couple bioenergy production and nutrient recovery from liquid digestate rich in ammonia, phosphate and organic acids [65]. Liquid digestate from agro-industrial waste has also been used to cultivate EPA-rich diatom *P. tricornutum* [59].

All these studies suggest that FBWs are interesting sources of organic carbon, mineral salts and nitrogen. However, more in-depth and extensive research is required for sustainable FBW pre-treatments, selection of suitable strains and optimization of culture conditions. In fact, it would be interesting to combine the concept of biorefinery explained above with the possibility of reusing low-cost nutritional sources to make VLC-PUFA production process economically and environmentally more sustainable.

Economy and sustainability

The so-called “omega-3 algae oils” are considered niche products in the market [13]. Very few companies have the production platform in place for it and mainly use thraustochytrids in closed bioreactors, and only a handful of companies sell food-grade omega-3 oil made using a photosynthetic technology (Table 3).

Table 3. Main companies producing n-3 LC-PUFA oils from protists. Information provided by direct mail interviews with some of the companies and company websites.

Company – registered trademark	Product	Strains used	Cultivation technology	Carbon source	Production area	Ref.
DSM - VERAMARIS®	Omega 3 algae oil	*Schizochytrium sp.*	Biofermenters	Dextrose from corn	Netherlands	[88]
DSM – Martek Biosciences	Omega 3 algae oil	*Schizochytrium sp.*	Biofermenters	N.r.	N.r.	[89]
ALGORIGIN®	Omega 3 capsules	*Schizochytrium sp.*	Biofermenters	N.r.	England	INW – [90]
Goerlich-Pharma - BIOPLUS	Algae oil – Capsules - Algal oil Powder	*Schizochytrium sp*.	Biofermenters	N.r.	Germany	[91]
CELLANA	Omega 3 for feed and nutraceutical	Various marine strains (unknown)	Phototrophic open pond - PBR combination	Carbon dioxide	USA	[92]
LYXIA®	Algae oil bulk Algae oil powder	*Nannochloropsis salina Schizochytrium sp.*	Phototrophic open raceway pond - Biofermenters	Carbon dioxide	China	[93]
Qualitas Health - IWI LIFE®	Omega 3 capsules	*Nannochlorpsis sp.*	Phototrophic open raceway pond	Carbon dioxide	USA - Mexico	[94]
Source-Omega	Algae oil	*Schizochytrium sp.*	Biofermenters	Sugars from corn industry	USA	[95]
Corbion - AlgaPrime™ DHA	Omega 3 for aquaculture, pet food and livestock	*Schizochytrium sp.*	Biofermenters	Sugar from sugar cane	Brazil	INW – [96]
FERMENTALG	Omega 3 for nutraceuticals DHA oil	Various strains (*Ulkenia sp.*, S*chizochytrium sp*.)	Biofermenters	N.r.	France	[97]

Chambio – ALGAMEG-3	Algal oil powder	*Schizochytrium* AlgaMEG-3TM	Biofermenters	N.r.	Taiwan	[98]
Algarithm	Algal oil – oil powder	*Schizochytrium sp.*	Biofermenters	N.r.	Canada	[99]
Algaenutra	Algal oil – oil powder	*Schizochytrium sp.*?	Biofermenters	N.r.	China	[100]
Arizona Algae Products – EPA15+	EPA omega 3 algae oil	*Nannochloropsis WPRO30+*	Closed Photobioreactors and covered raceway	Carbon dioxide	Arizona, USA	INW – [101]
Mara Renewables Corporation	DHA omega 3 algae oil	*Schizochytrium T18*	Biofermenters	Glucose	UK	INW – [102

N.r. = Not reported; INW = interview to producers; Numbers refer to web page of producers

Most of the photosynthetic production plants for omega-3 rich microalgae have the niche specialties for aquaculture as a core market. However, the VLC-PUFA market price is predicted to grow at an average annual rate of 13.5% worldwide, reaching a value of $5 billion in 2020 [66]. The market supply is ensured by fish oil and a fluctuating value of about 1 million metric tons of fish oil per annum is reported from whole fish and fishery by-products from 2015 to 2018, with a mean price of $1,600 ton^{-1} [67]. The main destination for fish oil is the aquaculture sector; other markets include terrestrial animal feed, direct human consumption and other special uses. The Global Organization for EPA and DHA reported a total share of 111,210 metric tons of EPA and DHA ingredients in 2018, of which about 2,000 tons were algae oil [68]. In terms of volume, dietary supplements are the market leader (63.8%) followed by pet food supplementation (24.8%), infant formulas (4%) and the remaining are fortified foods and pharma products [67].

For a view of the final market of omega-3 from supplements and other foods, an interesting study reports an evaluation of the unit price of EPA-DHA in some products available in supermarkets [69]. The lowest economic value was observed for cheap fish oil with an EPA-DHA price of $60 kg^{-1}. Instead, a price of $180 was reported for 1 kg of EPA-DHA from frozen sardine, while prenatal DHA and nutraceutical omega-3 supplements showed a cost range of $870 to 2500 kg-1.

The literature is scarce regarding the analysis of production costs and life cycle assessment of aquatic protists cultivation for VLC-PUFA production, but many authors have reported data on biodiesel production. For phototrophic cultivation, one study lists the main factors for lowering production costs as: the biological productivity of the microalgal strain, the photosynthetic efficiency of the cultivation system and geographical location which influences solar irradiation and temperature, and access to cooling water for PBRs [70]. It was concluded that using microalgae with 6% of their biomass consisting of EPA and DHA, cultivated in flat panel PBRs in Spain, have the lowest production cost ($39 kg-1 of EPA/DHA equivalents) with respect to the use of tubular systems and open pond raceways.

Another study reported on *Tetraselmis suecica* cultivated in PBRs, a biomass cost of $14 kg^{-1} at 1-ha scale, modelling a cost of $5.7 kg^{-1} for 100-ha. However, it was concluded that locating the plant in more favorable climatic conditions (e.g. in Tunisia), the final cost of the biomass could be reduced by up to $3.6 kg−1 at the 100-ha scale [71]. Others have reported, in a techno-economic analysis of heterotrophic biofuel production using *C. protothecoides*, that for a plant producing 10.126 ton yr^{-1} biodiesel, the production cost was $1.224 ton^{-1}. It

was stated that the investment was not profitable for biofuel alone but it should improve if the biomass were sold at a high price and a technology that is less energy intensive used to harvest, break the cell wall and to extract the oil [72]. This could be the case in a factory dedicated to the omega-3 oils.

Generally, 20-30% of the total cost of biomass production is represented by biomass harvesting, while the equipment cost for the extraction/esterification of oil from biomass is 6% of the total equipment cost [73]. The conventional method for lipid extraction involves the use of organic solvents, but first, a suitable cell disruption must be conducted to extract lipids. In order to increase extraction yields, novel techniques have been developed to aid cell wall disruption. These techniques are principally ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE) and supercritical fluid extraction (SCF), which are also used on an industrial scale. [72].

Another important cost in mixotrophic and heterotrophic cultivation is linked to the use of organic substrates. Using pure chemicals as a carbon source is not feasible for large scale operation if the aim is to compete with the reference market of the final product (omega-3 from fish in the present case). It is estimated that the glucose represents about 80% of the total medium cost, so that using by-products can cut down the costs [74]. The organic carbon and nitrogen substrates should be supplied from by-products of other processes to overcome this limitation [72].

A recent study reported a production cost for DHA produced by *Schizochytrium sp*. S31 using standard media in the range of \$52.2-157.2 kg^{-1}, while a further improvement of the process using a sustainable medium reduced it to \$15.4 kg^{-1} [75]. Another report using laboratory results based on oil and high-value pigments produced by *Nannochloropsis sp*. in indoor polyethylene bag PBRs, found that 82% of the costs were associated with light, 13% with water, 4% with nutrient consumption and an unusual 1% with harvesting [42]. Data from these later works suffer from not considering labor, equipment, land investments and indirect costs.

Conclusion

Aquatic protists can be used effectively for the industrial production of long chain omega-3 for human consumption. Quality, safety and ethical issues related to this oil generate consumer motivation to pay more than they would do for fish oil. However, for protists to

emerge from the niche market of vegan supplements and establish in the massive food market, some steps in research and development are required to meet economic and environmental sustainability standards. First, screening and selection of wild-type and mutant strains are required to identify the species with the highest EPA and DHA productivity. Secondly, optimisation of cultivation protocols and technologies, utilisation of agro-food by-products as low-cost nutrients for media formulation and recovery of high-value co-products from the residual biomass in a biorefinery concept must all be explored to improve sustainability and meet the promise of protist cultivation as an alternative source of VLC-PUFAs.

References

[1] Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol 2018;9:345–81. https://doi.org/10.1146/annurev-food-111317-095850.

[2] Ruiz-Lopez N, Haslam RP, Usher S, Napier JA, Sayanova O. An alternative pathway for the effective production of the omega-3 long-chain polyunsaturates EPA and ETA in transgenic oilseeds. Plant Biotechnol J 2015;13:1264–75. https://doi.org/10.1111/pbi.12328.

[3] Calder PC. Very long-chain n -3 fatty acids and human health: fact, fiction and the future. Proc Nutr Soc 2018;77:52–72. https://doi.org/10.1017/S0029665117003950.

[4] Li J, Yin H, Bibus DM, Byelashov OA. The role of Omega-3 docosapentaenoic acid in pregnancy and early development. Eur J Lipid Sci Technol 2016;118:1692–701. https://doi.org/10.1002/ejlt.201600076.

[5] Gladyshev MI, Arts MT, Sushchik NN. Preliminary estimates of the export of omega-3 highly unsaturated fatty acids (EPA+DHA) from aquatic to terrestrial ecosystems. Lipids Aquat. Ecosyst., New York, NY: Springer New York; 2009, p. 179–210. https://doi.org/10.1007/978-0-387-89366-2_8.

[6] Tocher DR. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 2015;449:94–107. https://doi.org/10.1016/j.aquaculture.2015.01.010.

[7] Teh LS, Cheung WW, Christensen V, Sumaila U. Can we meet the Target? Status and future trends for fisheries sustainability. Curr Opin Environ Sustain 2017;29:118–30. https://doi.org/10.1016/j.cosust.2018.02.006.

[8] Gladyshev MI, Glushchenko LA, Makhutova ON, Rudchenko AE, Shulepina SP, Dubovskaya OP, et al. Comparative Analysis of Content of Omega-3 Polyunsaturated Fatty Acids in Food and Muscle Tissue of Fish from Aquaculture and Natural Habitats. Contemp Probl Ecol 2018. https://doi.org/10.1134/S199542551803006X.

[9] Tan K, Ma H, Li S, Zheng H. Bivalves as future source of sustainable natural omega-3 polyunsaturated fatty acids. Food Chem 2020;311:125907. https://doi.org/10.1016/j.foodchem.2019.125907.

[10] Olsen RE, Waagbø R, Melle W, Ringø E, Lall SP. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. CRC Press; 2010. https://doi.org/10.1201/9781439808634.

[11] Whittaker RH. New Concepts of Kingdoms of Organisms. Science (80-) 1969;163:150–60. https://doi.org/10.1126/science.163.3863.150.

[12] Caron DA, Worden AZ, Countway PD, Demir E, Heidelberg KB. Protists are microbes too: a perspective. ISME J 2009;3:4–12. https://doi.org/10.1038/ismej.2008.101.

[13] Perez-Garcia O, Bashan Y. Microalgal Heterotrophic and Mixotrophic Culturing for Bio-refining: From Metabolic Routes to Techno-economics. Algal Biorefineries, Cham: Springer International Publishing; 2015, p. 61–131. https://doi.org/10.1007/978-3-319-20200-6_3.

[14] Jónasdóttir S. Fatty Acid Profiles and Production in Marine Phytoplankton. Mar Drugs 2019;17:151. https://doi.org/10.3390/md17030151.

[15] Cepák V, Přibyl P, Kohoutková J, Kaštánek P. Optimization of cultivation conditions for fatty acid composition and EPA production in the eustigmatophycean microalga Trachydiscus minutus. J Appl Phycol 2014;26:181–90. https://doi.org/10.1007/s10811-013-0119-z.

[16] Lang I, Hodac L, Friedl T, Feussner I. Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biol 2011;11:124. https://doi.org/10.1186/1471-2229-11-124.

[17] D'Imporzano G, Silvia S, Davide V, Barbara S, Fabrizio A. Microalgae Mixotrophic Growth: Opportunity for Stream Depuration and Carbon Recovery. Prospect. Challenges Algal Biotechnol., Singapore: Springer Singapore; 2017, p. 141–77. https://doi.org/10.1007/978-981-10-1950-0_5.

[18] Yoon EY, Park J, Jeong HJ, Rho J-R. Fatty acid composition and docosahexaenoic acid (DHA) content of the heterotrophic dinoflagellate Oxyrrhis marina fed on dried yeast: compared with algal prey. ALGAE 2017;32:67–74. https://doi.org/10.4490/algae.2017.32.3.5.

[19] Chalima A, Taxeidis G, Topakas E. Optimization of the production of docosahexaenoic fatty acid by the heterotrophic microalga Crypthecodinium cohnii utilizing a dark fermentation effluent. Renew Energy 2020;152:102–9. https://doi.org/10.1016/j.renene.2020.01.041.

[20] Burja AM, Radianingtyas H, Windust A, Barrow CJ. Isolation and characterization of polyunsaturated fatty acid producing Thraustochytrium species: screening of strains and optimization of omega-3 production. Appl Microbiol Biotechnol 2006;72:1161–9. https://doi.org/10.1007/s00253-006-0419-1.

[21] Zhu L, Zhang X, Ji L, Song X, Kuang C. Changes of lipid content and fatty acid composition of Schizochytrium limacinum in response to different temperatures and salinities. Process Biochem 2007;42:210–4. https://doi.org/10.1016/j.procbio.2006.08.002.

[22] Drouin G, Rioux V, Legrand P. The n-3 docosapentaenoic acid (DPA): A new player in the n-3 long chain polyunsaturated fatty acid family. Biochimie 2019;159:36–48. https://doi.org/10.1016/j.biochi.2019.01.022.

[23] Menegol T, Romero-Villegas GI, López-Rodríguez M, Navarro-López E, López-Rosales L, Chisti Y, et al. Mixotrophic production of polyunsaturated fatty acids and carotenoids by the microalga Nannochloropsis gaditana. J Appl Phycol 2019;31:2823–32. https://doi.org/10.1007/s10811-019-01828-3.

[24] Wang J, Yang H, Wang F. Mixotrophic Cultivation of Microalgae for Biodiesel Production: Status and Prospects. Appl Biochem Biotechnol 2014;172:3307–29. https://doi.org/10.1007/s12010-014-0729-1.

[25] Chaturvedi R, Uppalapati SR, Alamsjah MA, Fujita Y. Isolation of quizalofop-resistant mutants of Nannochloropsis oculata (Eustigmatophyceae) with high eicosapentaenoic acid following N-methyl-N-nitrosourea-induced random mutagenesis. J Appl Phycol 2004;16:135–44. https://doi.org/10.1023/B:JAPH.0000044826.70360.8e.

[26] Meireles LA, Guedes AC, Malcata FX. Increase of the yields of eicosapentaenoic and docosahexaenoic acids by the microalgaPavlova lutheri following random mutagenesis. Biotechnol Bioeng 2003;81:50–5. https://doi.org/10.1002/bit.10451.

[27] Li Z, Meng T, Ling X, Li J, Zheng C, Shi Y, et al. Overexpression of Malonyl-CoA: ACP Transacylase in Schizochytrium sp. to Improve Polyunsaturated Fatty Acid Production. J Agric Food Chem 2018;66:5382–91. https://doi.org/10.1021/acs.jafc.8b01026.

[28] Suparmaniam U, Lam MK, Uemura Y, Lim JW, Lee KT, Shuit SH. Insights into the microalgae cultivation technology and harvesting process for biofuel production: A review. Renew Sustain Energy Rev 2019;115:109361. https://doi.org/10.1016/j.rser.2019.109361.

[29] Borowitzka MA. Culturing Microalgae in Outdoor Ponds. Algal Cult. Tech., Elsevier; 2005, p. 205–18. https://doi.org/10.1016/B978-012088426-1/50015-9.

[30] Faried M, Samer M, Abdelsalam E, Yousef RS, Attia YA, Ali AS. Biodiesel production from microalgae: Processes, technologies and recent advancements. Renew Sustain Energy Rev 2017;79:893–913. https://doi.org/10.1016/j.rser.2017.05.199.

[31] Nwoba EG, Ayre JM, Moheimani NR, Ubi BE, Ogbonna JC. Growth comparison of microalgae in tubular photobioreactor and open pond for treating anaerobic digestion piggery effluent. Algal Res 2016;17:268–76. https://doi.org/10.1016/j.algal.2016.05.022.

[32] Acién FG, Molina E, Reis A, Torzillo G, Zittelli GC, Sepúlveda C, et al. Photobioreactors for the production of microalgae. Microalgae-Based Biofuels Bioprod., Elsevier; 2017, p. 1–44. https://doi.org/10.1016/B978-0-08-101023-5.00001-7.

[33] Torzillo G, Chini Zittelli G. Tubular Photobioreactors. Algal Biorefineries, Cham: Springer International Publishing; 2015, p. 187–212. https://doi.org/10.1007/978-3-319-20200-6_5.

[34] Li S, Ji L, Shi Q, Wu H, Fan J. Advances in the production of bioactive substances from marine unicellular microalgae Porphyridium spp. Bioresour Technol 2019;292:122048. https://doi.org/10.1016/j.biortech.2019.122048.

[35] Xie D, Miller E, Sharpe P, Jackson E, Zhu Q. Omega-3 production by fermentation of Yarrowia lipolytica : From fed-batch to continuous. Biotechnol Bioeng 2017;114:798–812. https://doi.org/10.1002/bit.26216.

[36] Zhang M, Wu W, Guo X, Weichen Y, Qi F, Jiang X, et al. Mathematical modeling of fed-batch fermentation of Schizochytrium sp. FJU-512 growth and DHA production using a shift control strategy. 3 Biotech 2018;8:162. https://doi.org/10.1007/s13205-018-1187-1.

[37] Sung MG, Lee B, Kim CW, Nam K, Chang YK. Enhancement of lipid productivity by adopting multi-stage continuous cultivation strategy in Nannochloropsis gaditana. Bioresour Technol 2017. https://doi.org/10.1016/j.biortech.2016.12.100.

[38] Guo D-S, Ji X-J, Ren L-J, Yin F-W, Sun X-M, Huang H, et al. Development of a multi-stage continuous fermentation strategy for docosahexaenoic acid production by Schizochytrium sp. Bioresour Technol 2018;269:32–9. https://doi.org/10.1016/j.biortech.2018.08.066.

[39] Barros A, Guerra LT, Simões M, Santos E, Fonseca D, Silva J, et al. Mass balance analysis of carbon and nitrogen in industrial scale mixotrophic microalgae cultures. Algal Res 2017;21:35–41. https://doi.org/10.1016/j.algal.2016.10.014.

[40] Ren H-Y, Xiao R-N, Kong F, Zhao L, Xing D, Ma J, et al. Enhanced biomass and lipid accumulation of mixotrophic microalgae by using low-strength ultrasonic stimulation. Bioresour Technol 2019;272:606–10. https://doi.org/10.1016/j.biortech.2018.10.058.

[41] Cerón García MC, García Camacho F, Sánchez Mirón A, Fernández Sevilla JM, Chisti Y, Molina Grima E. Mixotrophic production of marine microalga Phaeodactylum tricornutum on various carbon sources. J Microbiol Biotechnol 2006;16:689–94.

[42] Ferreira AF, Ribeiro LA, Batista AP, Marques PASS, Nobre BP, Palavra AMF, et al. A biorefinery from Nannochloropsis sp. microalga – Energy and CO2 emission and economic analyses. Bioresour Technol 2013;138:235–44. https://doi.org/10.1016/j.biortech.2013.03.168.

[43] Yuan X, Liang L, Liu K, Xie L, Huang L, He W, et al. Spent yeast as an efficient medium supplement for fucoxanthin and eicosapentaenoic acid (EPA) production by Phaeodactylum tricornutum. J Appl Phycol 2020;32:59–69. https://doi.org/10.1007/s10811-019-01909-3.

[44] Kathiresan S, Sarada R, Bhattacharya S, Ravishankar GA. Culture media optimization for growth and phycoerythrin production fromPorphyridium purpureum. Biotechnol Bioeng 2007;96:456–63. https://doi.org/10.1002/bit.21138.

[45] Park H, Kwak M, Seo J, Ju J, Heo S, Park S, et al. Enhanced production of carotenoids using a Thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. Bioprocess Biosyst Eng 2018;41:1355–70. https://doi.org/10.1007/s00449-018-1963-7.

[46] Watanabe K, Arafiles KH V., Higashi R, Okamura Y, Tajima T, Matsumura Y, et al. Isolation of High Carotenoid-producing Aurantiochytrium sp. Mutants and Improvement of Astaxanthin Productivity Using Metabolic Information. J Oleo Sci 2018;67:571–8. https://doi.org/10.5650/jos.ess17230.

[47] Parsons S, Allen MJ, Chuck CJ. Coproducts of algae and yeast-derived single cell oils: A critical review of their role in improving biorefinery sustainability. Bioresour Technol 2020;303:122862. https://doi.org/10.1016/j.biortech.2020.122862.

[48] Fret J, Roef L, Blust R, Diels L, Tavernier S, Vyverman W, et al. Reuse of rejuvenated media during laboratory and pilot scale cultivation of Nannochloropsis sp. Algal Res 2017;27:265–73. https://doi.org/10.1016/j.algal.2017.09.018.

[49] Vidyashankar S, VenuGopal KS, Chauhan VS, Muthukumar SP, Sarada R. Characterisation of defatted Scenedesmus dimorphus algal biomass as animal feed. J Appl Phycol 2015;27:1871–9. https://doi.org/10.1007/s10811-014-0498-9.

[50] Naresh Kumar A, Min B, Venkata Mohan S. Defatted algal biomass as feedstock for short chain carboxylic acids and biohydrogen production in the biorefinery format. Bioresour Technol 2018;269:408–16. https://doi.org/10.1016/j.biortech.2018.08.059.

[51] Ruiz-Ruiz F, Benavides J, Rito-Palomares M. Scaling-up of a B-phycoerythrin production and purification bioprocess involving aqueous two-phase systems: Practical experiences. Process Biochem 2013;48:738–45. https://doi.org/10.1016/j.procbio.2013.02.010.

[52] Peng J, Yuan J-P, Wu C-F, Wang J-H. Fucoxanthin, a Marine Carotenoid Present in Brown Seaweeds and Diatoms: Metabolism and Bioactivities Relevant to Human Health. Mar Drugs 2011;9:1806–28. https://doi.org/10.3390/md9101806.

[53] Guo B, Liu B, Yang B, Sun P, Lu X, Liu J, et al. Screening of Diatom Strains and Characterization of Cyclotella cryptica as A Potential Fucoxanthin Producer. Mar Drugs 2016;14:125. https://doi.org/10.3390/md14070125.

[54] Singh D, Gupta A, Wilkens SL, Mathur AS, Tuli DK, Barrow CJ, et al. Understanding response surface optimisation to the modeling of Astaxanthin extraction from a novel strain Thraustochytrium sp. S7. Algal Res 2015;11:113–20. https://doi.org/10.1016/j.algal.2015.06.005.

[55] Sathasivam R, Ki J-S. A Review of the Biological Activities of Microalgal Carotenoids and Their Potential Use in Healthcare and Cosmetic Industries. Mar Drugs 2018;16:26. https://doi.org/10.3390/md16010026.

[56] Wijffels RH, Barbosa MJ. An Outlook on Microalgal Biofuels. Science (80-) 2010;329:796–9. https://doi.org/10.1126/science.1189003.

[57] Christenson L, Sims R. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol Adv 2011;29:686–702. https://doi.org/10.1016/j.biotechadv.2011.05.015.

[58] Ende SSW, Noke A. Heterotrophic microalgae production on food waste and by-products. J Appl Phycol 2019;31:1565–71. https://doi.org/10.1007/s10811-018-1697-6.

[59] Massa M, Buono S, Langellotti AL, Castaldo L, Martello A, Paduano A, et al. Evaluation of anaerobic digestates from different feedstocks as growth media for Tetradesmus obliquus, Botryococcus braunii, Phaeodactylum tricornutum and Arthrospira maxima. N Biotechnol 2017;36:8–16. https://doi.org/10.1016/j.nbt.2016.12.007.

[60] Massa M, Buono S, Langellotti AL, Martello A, Russo GL, Troise DA, et al. Biochemical composition and in vitro digestibility of Galdieria sulphuraria grown on spent cherry-brine liquid. N Biotechnol 2019;53:9–15. https://doi.org/10.1016/j.nbt.2019.06.003.

[61] Pimentel-Moral S, Cádiz-Gurrea M de la L, Rodríguez-Pérez C, Segura-Carretero A. Recent advances in extraction technologies of phytochemicals applied for the revaluation of agri-food by-products. Funct. Preserv. Prop. Phytochem., Elsevier; 2020, p. 209–39. https://doi.org/10.1016/B978-0-12-818593-3.00007-5.

[62] Parthiba Karthikeyan O, Trably E, Mehariya S, Bernet N, Wong JWC, Carrere H. Pretreatment of food waste for methane and hydrogen recovery: A review. Bioresour Technol 2018;249:1025–39. https://doi.org/10.1016/j.biortech.2017.09.105.

[63] Pleissner D, Lau KY, Ki Lin CS. Utilization of food waste in continuous flow cultures of the heterotrophic microalga Chlorella pyrenoidosa for saturated and unsaturated fatty acids production. J Clean Prod 2017;142:1417–24. https://doi.org/10.1016/j.jclepro.2016.11.165.

[64] Pleissner D, Lam WC, Sun Z, Lin CSK. Food waste as nutrient source in heterotrophic microalgae cultivation. Bioresour Technol 2013;137:139–46. https://doi.org/10.1016/j.biortech.2013.03.088.

[65] Markou G, Wang L, Ye J, Unc A. Cultivation of Microalgae on Anaerobically Digested Agro-industrial Wastes and By-Products. Appl. Microalgae Wastewater Treat., Cham: Springer International Publishing; 2019, p. 147–72. https://doi.org/10.1007/978-3-030-13909-4_7.

[66] Rahman KM. Food and High Value Products from Microalgae: Market Opportunities and Challenges. In: Alam MA, Xu J-L, Wang Z, editors. Microalgae Biotechnol. Food, Heal. High Value Prod., Singapore: Springer Singapore; 2020, p. 3–27. https://doi.org/10.1007/978-981-15-0169-2_1.

[67] GLOBEFISH. GLOBEFISH Highlights January 2020 ISSUE, with Jan. – Sep. 2019 Statistics. FAO; 2020. https://doi.org/10.4060/ca7968en.

[68] GOED. The Global Organization for EPA and DHA Omega-3s. (2019). 2017-2018 Global EPA and DHA Ingredient Market Report. Salt Lake City, UT. 2019. https://goedomega3.com/purchase/ingredient-market-report (accessed April 23, 2020).

[69] A. Watters C, M. Edmonds C. A Cost Analysis of EPA and DHA in Fish, Supplements, and Foods. J Nutr Food Sci 2012;02. https://doi.org/10.4172/2155-9600.1000159.

[70] Chauton MS, Reitan KI, Norsker NH, Tveterås R, Kleivdal HT. A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: Research challenges and possibilities. Aquaculture 2015;436:95–103. https://doi.org/10.1016/j.aquaculture.2014.10.038.

[71] Tredici MR, Rodolfi L, Biondi N, Bassi N, Sampietro G. Techno-economic analysis of microalgal biomass production in a 1-ha Green Wall Panel (GWP®) plant. Algal Res 2016;19:253–63. https://doi.org/10.1016/j.algal.2016.09.005.

[72] Tabernero A, Martín del Valle EM, Galán MA. Evaluating the industrial potential of biodiesel from a microalgae heterotrophic culture: Scale-up and economics. Biochem Eng J 2012;63:104–15. https://doi.org/10.1016/j.bej.2011.11.006.

[73] Molina Grima E, Belarbi E-H, Acién Fernández F., Robles Medina A, Chisti Y. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv 2003;20:491–515. https://doi.org/10.1016/S0734-9750(02)00050-2.

[74] Li X, Xu H, Wu Q. Large-scale biodiesel production from microalgaChlorella protothecoides through heterotrophic cultivation in bioreactors. Biotechnol Bioeng 2007;98:764–71. https://doi.org/10.1002/bit.21489.

[75] Wang S-K, Wang X, Tian Y-T, Cui Y-H. Nutrient recovery from tofu whey wastewater for the economical production of docosahexaenoic acid by Schizochytrium sp. S31. Sci Total Environ 2020;710:136448. https://doi.org/10.1016/j.scitotenv.2019.136448.

[76] Mitani E, Nakayama F, Matsuwaki I, Ichi I, Kawabata A, Kawachi M, et al. Fatty acid composition profiles of 235 strains of three microalgal divions within the NIES Microbial Culture Collection. Microb Resour Syst 2017;33:19–29.

[77] Lee Chang KJ, Nichols CM, Blackburn SI, Dunstan GA, Koutoulis A, Nichols PD. Comparison of Thraustochytrids Aurantiochytrium sp., Schizochytrium sp., Thraustochytrium sp., and Ulkenia sp. for Production of Biodiesel, Long-Chain Omega-3 Oils, and Exopolysaccharide. Mar Biotechnol 2014;16:396–411. https://doi.org/10.1007/s10126-014-9560-5.

[78] Trovão M, Pereira H, Costa M, Machado A, Barros A, Soares M, et al. Lab-Scale Optimization of Aurantiochytrium sp. Culture Medium for Improved Growth and DHA Production. Appl Sci 2020;10:2500. https://doi.org/10.3390/app10072500.

[79] Isleten-Hosoglu M, Elibol M. Bioutilization of Cheese Whey and Corn Steep Liqour by Heterotrophic Microalgae Crypthecodinium cohnii for Biomass and Lipid Production. Akad Gıda 2017;15:233–41. https://doi.org/10.24323/akademik-gida.345256.

[80] Mendes A, Guerra P, Madeira V, Ruano F, Lopes da Silva T, Reis A. Study of docosahexaenoic acid production by the heterotrophic microalga Crypthecodinium cohnii

CCMP 316 using carob pulp as a promising carbon source. World J Microbiol Biotechnol 2007;23:1209–15. https://doi.org/10.1007/s11274-007-9349-z.

[81] Quilodrán B, Hinzpeter I, Hormazabal E, Quiroz A, Shene C. Docosahexaenoic acid (C22:6n−3, DHA) and astaxanthin production by Thraustochytriidae sp. AS4-A1 a native strain with high similitude to Ulkenia sp.: Evaluation of liquid residues from food industry as nutrient sources. Enzyme Microb Technol 2010;47:24–30. https://doi.org/10.1016/j.enzmictec.2010.04.002.

[82] Humhal T, Kastanek P, Jezkova Z, Cadkova A, Kohoutkova J, Branyik T. Use of saline waste water from demineralization of cheese whey for cultivation of Schizochytrium limacinum PA-968 and Japonochytrium marinum AN-4. Bioprocess Biosyst Eng 2017;40:395–402. https://doi.org/10.1007/s00449-016-1707-5.

[83] Ren L-J, Li J, Hu Y-W, Ji X-J, Huang H. Utilization of cane molasses for docosahexaenoic acid production by Schizochytrium sp. CCTCC M209059. Korean J Chem Eng 2013;30:787–9. https://doi.org/10.1007/s11814-013-0020-0.

[84] Yin F-W, Zhu S-Y, Guo D-S, Ren L-J, Ji X-J, Huang H, et al. Development of a strategy for the production of docosahexaenoic acid by Schizochytrium sp. from cane molasses and algae-residue. Bioresour Technol 2019;271:118–24. https://doi.org/10.1016/j.biortech.2018.09.114.

[85] Yu X-J, Yu Z-Q, Liu Y-L, Sun J, Zheng J-Y, Wang Z. Utilization of High-Fructose Corn Syrup for Biomass Production Containing High Levels of Docosahexaenoic Acid by a Newly Isolated Aurantiochytrium sp. YLH70. Appl Biochem Biotechnol 2015;177:1229–40. https://doi.org/10.1007/s12010-015-1809-6.

[86] Ryu B-G, Kim K, Kim J, Han J-I, Yang J-W. Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, Aurantiochytrium sp. KRS101. Bioresour Technol 2013;129:351–9. https://doi.org/10.1016/j.biortech.2012.11.049.

[87] Wang X, Balamurugan S, Liu S-F, Zhang M-M, Yang W-D, Liu J-S, et al. Enhanced polyunsaturated fatty acid production using food wastes and biofuels byproducts by an evolved strain of Phaeodactylum tricornutum. Bioresour Technol 2020;296:122351. https://doi.org/10.1016/j.biortech.2019.122351.

[88] Veramaris. Company info 2020. https://www.veramaris.com/what-we-do-detail.html#fermentative (accessed May 10, 2020).

[89] Martek. Martek Biosciences n.d. https://www.dsm.com/corporate/news/news-archive/2011/09-11-dsm-announces-receipt-of-final-competition-clearance-for-acquisition-of-martek.html (accessed October 6, 2020).

[90] ALGORIGIN. Algae cultivation 2020. https://algorigin.com/en/algae/schizochytrium/ (accessed April 15, 2020).

[91] BIOPLUS. Algae oil 2020. https://goerlich-pharma.com/en/omega-3/bioplus-algae-oil/ (accessed April 15, 2020).

[92] CELLANA. Renew omega-3 2020. http://cellana.com/products/renew-omega-3/ (accessed April 14, 2020).

[93] LYXIA. Our technology 2020. http://www.lyxia.com/our-technology/ (accessed April 16, 2020).

[94] Qualitas-health. Company info 2020. https://www.qualitas-health.com/our-company.

[95] Source-omega. Web page 2020. http://www.source-omega.com/ (accessed April 14, 2020).

[96] ALGAPRIME. Web page 2020. http://algaprime.com/ (accessed April 17, 2020).

[97] FERMENTALG. Algae strains 2020. https://www.fermentalg.com/science/strains/ (accessed April 20, 2020).

[98] Chambrio. Chambrio technology 2020. algameg-3.com/home/technology/ (accessed April 20, 2020).

[99] Algarithm. Web page 2020. https://www.algarithm.ca/ (accessed April 20, 2020).

[100] Algaenutra. Workshop 2020. http://www.algaenutra.com/en/About-Us/Workshop/ (accessed April 18, 2020).

[101] Azalgae. Web page 2020. https://www.azalgae.com/ (accessed April 15, 2020).

[102] Corporation MR. Company info n.d. https://www.maracorp.ca/about (accessed April 17, 2020).

Chapter 3

Valorization of second cheese whey through cultivation of extremophile microalga *Galdieria sulphuraria.*

This chapter has been published as:

Russo, G. L., Langellotti, A. L., Oliviero, M., Baselice, M., Sacchi, R., & Masi, P. (2021). Valorization of second cheese whey through cultivation of extremophile microalga *Galdieria sulphuraria*. *AIMS Environmental Science*, *8*(5), 435-448.

Abstract: Second cheese whey (SCW) or "*scotta*" in Italian, is a side-stream from the manufacturing of "*Ricotta*" cheese, obtained after thermal coagulation of whey proteins residue in the cheese whey. *Galdieria sulphuraria* is a thermophilic red algae well known for its metabolic capabilities to grow on wastewater and other saline effluents. In this work, the valorisation of SCW as nutrient source for the growth of *G. sulphuraria* has been investigated using different concentrations of SCW. The biochemical and fatty acids composition of the biomass obtained has been evaluated too. Small differences have been observed in terms of biomass obtained after 12 days of cultivation between the SCW media and the relative control with the same amount of reducing sugars. The fatty acids composition of *G. sulphuraria* grown in SCW showed a higher content of polyunsaturated fatty acids compared to the control. The biomass productivity using SCW media has also been optimized through response surface methodologies with supplementation of nitrogen source obtaining a biomass dry weight higher than 10 g L^{-1}.

Keywords: sustainability, PUFA, food waste, microalgae, dairy wastewater, bioconversion

3.1 Introduction

The dairy industry is one of the most important food industries in Europe. Cheese manufacturing produces different effluents including the second cheese whey (SCW) which, in particular, comes from "ricotta" cheese production. The SCW is the result of whey proteins thermal coagulation, which are separated to make ricotta cheese, while the liquid residue is destined to be an effluent. The SCW is an interesting by-product due to the presence of important nutrients like lactose, nitrogen, free aminoacids, mineral salts, phosphorous etc. [1]. However, SCW production causes huge environmental and economic problems for its disposal by producers [2]. Only in Italy, where it is known as "scotta", more than 1 million tons per year are produced [1,2].

In the last years, many efforts have been carried out to evaluate the biotechnological utilization of dairy wastewater or cheese whey (CW) [3]. Actually, there is not a real utilization of this by-product and it is destined to disposal by the producers. Usually, dairy effluents are treated with physicochemical or biological processes. A biological process that has been evaluated in the last years involves the use of microalgae [4].

These aquatic protists are microrganisms widely known for their sustainable bioremediation capacity. In fact, they provide great opportunities to recycle nutrients present inside food waste or effluents such as sludge or saline wastewater [5,6]. The most used microalgae are *Chlorella sp.* and *Scenedesmus sp*., for which the growth on pretreated dairy effluents has been widely studied [7,8]. However, there is another group of microalgae that provides great opportunities as biorefinery platforms: the extremophile algae [9]. Extremophile algae are capable to grow in harsh conditions such as high salinity concentration or very low pH. Among them, *Galdieria sulphuraria* is a promising heterotrophic red algae that has been cultivated for the production of pigments, antioxidants and for the removal of nitrogen, sugars and phosphorus from wastewaters [4,10,11]. In fact, *G. sulphuraria* is an important producer of C-phycocyanin which exhibits ability to remain stable at high temperatures up to 60 °C [12]. This property makes this pigment very useful in various fields of forensic sciences including biotechnology, molecular biology, and recombinant technology.

Thanks to its great metabolic flexibility, this microalga is an interesting biomass for the bioconversion of food by-products and waste in molecules with high added value. Moreover, *G. sulphuraria* is a thermo-acidophilic microalga capable to grow at pH lower than 2 and at temperature higher than 50 °C, which are important to prevent bacterial contamination that could affect the growth performance [11].

In scientific literature few works evaluated *G. sulphuraria* growth on food by-products. In particular, Massa et al., (2019) reported the biochemical composition for samples grown on spent cherry brine liquid [6]; while Zimermann et al., (2020) studied the growth kinetics of *G. sulphuraria* grown on whey permeate using, however, cell count as only growth parameter [4]. Scherhag and Ackermann (2020) instead, evaluated the sugar removal from fruit wastewater by *G. sulphuraria* (SAG 21.92) [10]. However, very few studies have been found on biomass optimization of these food wastes by using statistical methods such as response surface methodologies.

Therefore, for the first time, in this work SCW has been used as nutrient media for the cultivation of *G. sulphuraria*. The growth kinetics and the biochemical composition of the microalga have been determined. The biomass optimization of new SCW media has been established through response surface methodology (RSM).

3.2Materials and methods

Galdieria sulphuraria standard growth conditions

G. sulphuraria (SAG 107.79) was obtained from Culture Collection of Algae at the University of Göttingen (Germany). Regular sub-culturing of photoautotrophic algae were made every 4 weeks on liquid and agar slants of Cyanidium Medium [6]. To obtain the transition from autotrophy to heterotrophy, the Allen medium [13] was used as standard media (SM), with an addiction of 30 g L^{-1} of glucose as organic carbon source and 1.32 g L^{-1} of $(NH_4)_2SO_4$ as nitrogen source. The cultures were placed in a dark room at 27 °C ± 1 and the mixing was provided through an air bubbling system equipped with a filter of 0.22 µm in order to prevent any contamination and to provide oxygenation to the culture. The pH of culture was set at 1.5 with the addition of H_2SO_4 5 N.

Characterization and treatment of Second Cheese Whey

The SCW was gently provided by a dairy industry in the area of Salerno (Italy) that produces mozzarella and other fresh cheeses. The samples were taken from the accumulation tanks of the company and immediately stored at −18 °C for the transport in the laboratory. In these tanks only the SCW was present and not any other type of dairy wastewater.

Prior any analysis, the SCW was filtered through a 1 µm filter to remove coarse solids. The chemical analyses involved the measurement of dry matter (g L^{-1}), volatile solids (g L^{-1}), ash (g L^{-1}), pH (using a Mettler-Toledo pH-meter), reducing sugars using 3,5-Dinitrosalicylic acid (DNS) method [14], protein content following Bradford method [15], nitrates (cadmium reduction method), ammonium (N-NH_4) (salicylate method), phosphate content (P total) (acid digestion method) [16] and free amino nitrogen (FAN). FAN content was estimated with ninhidrin reaction method described by Lie (1973) [17]. All the analyses were carried out in triplicate.

For the treatments, prior the cultivation, the SCW was heated up at 75 °C x 10 min to promote the precipitation of residual casein and then centrifuged at 4695 x g for 15 min. at 10 °C. The clean surnatant was then collected and used for the analyses and for the cultivation trials.

***G. sulphuraria* growth test and optimization using SCW**

To evaluate the utilization of SCW as nutrient source, four different concentrations of this effluent have been investigated. The scotta was diluted to reach four different concentrations in reducing sugars (RS): 1.0%, 1.5%, 2.0% and 2.5%, corresponding to 22%, 34%, 45% and

57% of concentration (v/v) respectively. The dilution was made with distilled water. As control, *G. sulphuraria* was cultivated in Allen medium at 1.0%, 1.5%, 2.0% and 2.5% of glucose. For the samples with SCW, pH was adjusted to a final value of 1.5 using H_2SO_4 5 N as the SM.

For this test, *G. sulphuraria* cultivation was carried for 12 d in air-lift reactor of 3 L with a working volume of 2 L at 27±1 °C in a dark room. The inoculum for the SCW test was previously acclimatized with the by-product to improve the growth performances.

After this, in order to enhance the biomass concentration using the new formulated media, a response surface method (RSM) was also used. A three level full factorial central composite design (CCD) was used to determine the effect of added nitrogen and glucose to the new media. The optimization consisted of 14 runs conducted in two blocks with 4 cubic points (or factorial points), 4 axial points (or star points) and 3 center points for each block. The independent factors used were glucose and $(NH_4)_2SO_4$. The three levels (−1, 0 and +1) set for glucose were 0, 5 and 10 g L^{-1} supplemented to the SCW media, while for NH_4SO_4 was 0, 0.4 and 0.8 g L^{-1}.

The mathematical relationship of the response (Y) to the significant independent variables X1 and X2 is given by the following quadratic polynomial equation (1):

$$Y = \beta 0 + \Sigma i=1 \beta iXi + \Sigma i=1 \beta ii Xi2 + \Sigma i=1 \Sigma j=I+1 \beta ij XiXj \quad (1)$$

Where Y is the predicted response; Xi and Xj are the coded values; β0 the independent coefficient; βi,,j is the linear coefficient associated to each independent factor (Xi,j) and βij and βii are the coefficient for interaction and quadratic effects respectively [18]

The optimization test was conducted at 27±1 °C using 1 L Erlenmeyer flask with a working volume of 600 mL and the mixing provided by an air bubbling system equipped with a filter (as described above).

Growth parameters

The monitoring of the growth performances for control and treated samples were obtained through growth curves using standard gravimetric methods on daily aliquots of cultures. The

Dry cell weight (DCW, g L^{-1}) was obtained after centrifugation at 4695 x g for 15 min and the pellet was rinsed twice to remove any residual salt. The pellet was dried at 70 °C until the constant weight was reached. The residual surnatant was filtered at 0.45 μm and immediately stored at −18 °C for further analysis. For the definition of the growth kinetics, the specific growth rate (μ), maximum specific growth rate (μ_{max} day^{-1}), maximum biomass obtained (X_{max}), and tX_{max} were calculated.

The maximum specific growth rate was calculated at the exponential stage following the equation (2):

$$\mu max\ (\text{day}^{-1}) = \frac{(\ln DCW2 - \ln DCW1)}{t2 - t1} \quad (2)$$

Where DCW1,2 is the dry cell weight at time 1 and 2 respectively.

To evaluate the nutrient consumption by *G. sulphuraria*, the free amino nitrogen (FAN) in residual media was assessed. The FAN content was obtained using the ninhidrin assay and the residual reducing sugar by DNS method.

Biochemical composition and lipid analysis of biomass

After the period of cultivation (12 d), the biomass was harvested using a continuous centrifuge at 3005 x g and washed with distilled water. The wet biomass obtained was lyophilized for all the analysis. The carbohydrate determination was obtained with the Dubois method [19] using 1 gr of freeze-dried sample of *G.sulphuraria*. The ash content of the biomass was determined gravimetrically in a muffle furnace at 550 °C until achieving constant weight.

The lipid content of biomass was determined by the Bligh and Dyer method [20] using chloroform:methanol 2:1 (v/v). The lipids extracted were suspended in 1 mL of hexane and then converted to their relative methyl esters by adding 200 μL of KOH 2 N in methanol for 30 s at room temperature. The fatty acids profile was obtained in gas chromatography system (Shimadzu GC-17A) coupled with a flame ionization detector (GC/FID). The GC was equipped with a fused silica capillary column (SPTM-2560, 75m x 0,18 mm, i.d. 0,14 μm film thickness) and using helium as gas carrier. The identification of the fatty acids methyl esters

(FAME) was obtained after the injection of pure standards FAME from Larodan (Malmoe, Sweden) and comparing the relatives retention time. Acquisition software used for identification of FAME was the Class-VP chromatography data system, vers. 4.6 (Shimadzu Italia, Milan).

The protein content of the biomass was obtained with Kjeldahl method [21]. The cellular protein value was calculated using the conversion factor of 6.25.

Statistical analysis

The data were analyzed using SPSS software, version 23 (IBM Corp., Armonk, NY, USA). All the analyses were carried out in triplicate, and average values with standard deviation were reported. One-way analysis of variance (ANOVA) was applied using raw data to test for significant differences among the samples. $p < 0.05$ was considered statistically significant. Tukey's test was used for post-hoc analysis when there were significant differences among the samples. The optimization process was evaluated with RSM analysis, performed in 'R' (RStudio with 'R' version 3.0.2, RCore Team, Vienna/Austria).

Results and discussion

Characterization of SCW

The chemical and physical composition of SCW is reported in Table 1. The characteristics of this by-product are not very different from cheese whey because it is slightly acid (pH 5.9) and characterized by a good content of reducing sugars, mainly lactose (up to 44 g L^{-1}). The sugars and nitrates content of SCW were higher than those reported in literature [22]. The high RS content is very interesting for heterotrophic cultivation of *G. sulphuraria*, which was reported to be able to grow on many different organic carbon sources [23,24]. The analysis showed a residual content of protein, probably due to an inefficient process of flocculation during the "Ricotta" cheese production. However, the residual organic nitrogen could be very interesting for cultivation of *G. sulphuraria* which is able to use both inorganic and organic forms of nitrogen for its growth (i.e. aminoacids) [25]. The C:N ratio of the SCW was approximately 30, while the ratio of the SM is 43. That could affect the biochemical and lipid composition of the biomass obtained, as reported by other authors [26]. Total phosphorus (P total) of SCW was 96 mg L^{-1}, that is lower respect to the standard medium (110 mg L^{-1}), and

the resulting N:P ratio was 4.89 (higher than the SM). P is important for the synthesis of phospholipids and nucleic acids in microalgae [27].

Table 1. Chemical and physical characterization of second cheese whey (SCW) used for the growth of *G.sulphuraria.*

Parameters	Value
pH	5.9±0.2
Ash (g L^{-1})	5.5±0.5
Dry weight (g L^{-1})	58.4±0.6
Volatile solids (g L^{-1})	53.3±0.4
NH_4-N (mg L^{-1})	25±1.3
NO_3-N (mg L^{-1})	80±1.2
N total (g L^{-1})	0.59±0.1
Free amino nitrogen (mg L^{-1})	231.14±16.4
P total (mg L^{-1})	96.1±0.4
Reducing sugars (g L^{-1})	43.4±0.9
Protein content (g L^{-1})	3.1±0.6

Values expressed as mean ± SD (n=3)

The nutrient content of the SCW was not so far from the SM. Therefore, it was possible to test this dairy by-product for the cultivation of *G. sulphuraria.*

G. sulphuraria growth performances on SCW

The growth curves of *G. sulphuraria* grown using SCW are reported in Figure 1. The curves have been separated to better understand the growth performance on the various formulation of SCW medium with controls at the same amount of RS. No significant differences were observed for microalgae cultivated with scotta at 1% in RS respect to the relative control (fig. 1a). However the biomass obtained at 1% in RS is lower respect to the control.

For samples at 1.5% in RS, a difference can be observed (in terms of concentration) after 12 days of cultivation (5.1 g L−1 for the control and 4.2 g L−1 for the SCW samples), and the overall growth performance was lower respect to the control. However, these differences were not significant (fig. 1b). With SCW medium at 2% RS instead, *G. sulphuraria* showed a longer lag phase respect to the control, reaching only at 10th day a concentration similar to the relative control. At SCW 2.5% RS, microalgae showed worst growth performance compared to the samples at 2.0% RS, with a longer lag phase respect to the control, and reaching the

maximum concentration after 11 days of cultivation. The biomass obtained for SCW sample at 2.5% RS was significantly lower than the control (fig. 1d).

However, for SCW medium at 2.5% RS, the productivity is lower to the sample at 2.0%, this can be explained by an inhibition effect of higher concentration of "scotta". In fact, in the work of Zimermann et al., (2020) high concentrations of whey permeate resulted toxic for the growth of *G. sulphuraria*, and the author used concentration below the 40% (v/v) [4].

To further define the growth kinetics of *G. sulphuraria* in SCW media, the growth rate, Xmax and μ_{max} were calculated and reported in Table 2. After 4 days of cultivation, the growth rates of the controls are higher than the samples with SCW, except for the sample at SCW 1.5% RS. At 7th day, significant differences between the controls and the SCW samples are reported for the samples at 2 and 2.5% RS, denoting the longer lag phase reported for the culture with higher concentration of scotta (Fig. 1c, d). In particular for the samples at 2.5% RS the difference is more pronounced.

Table 2. Specific and maximum growth rate (μ_{max}), maximum concentration reached (X_{max}) and cultivation time for maximum concentration (tX_{max}) of *G.sulphuraria* growth on different concentrations of SCW diluted from 1.0 to 2.5% in reducing sugars (RS).

Sample	μ (4 d)	μ (7 d)	μ_{max}	X_{max} (g L^{-1})	tX_{max} (d)
Control 1.0% RS	0.305±0.03	0.202±0.01	0.174±0.02	4.09±0.21	8
SCW 1.0% RS	0.285±0.01	0.191±0.01	0.191±0.01	3.87±0.17	7
Control 1.5% RS	0.279±0.02	0.219±0.02	0.203±0.03	5.15±0.32	8
SCW 1.5% RS	0.287±0.02	0.203±0.01	0.186±0.02	4.35±0.19	9
Control 2.0% RS	0.325±0.03	0.256±0.02	0.199±0.03	6.10±0.29	8
SCW 2.0% RS	0.250±0.02	0.212±0.02	0.159±0.03	6.02±0.40	11
Control 2.5% RS	0.345±0.02	0.259±0.02	0.209±0.01	6.89±0.28	9
SCW 2.5% RS	0.259±0.01	0.165±0.01	0.147±0.02	5.33±0.21	11

Values expressed as mean ± SD (n=3)

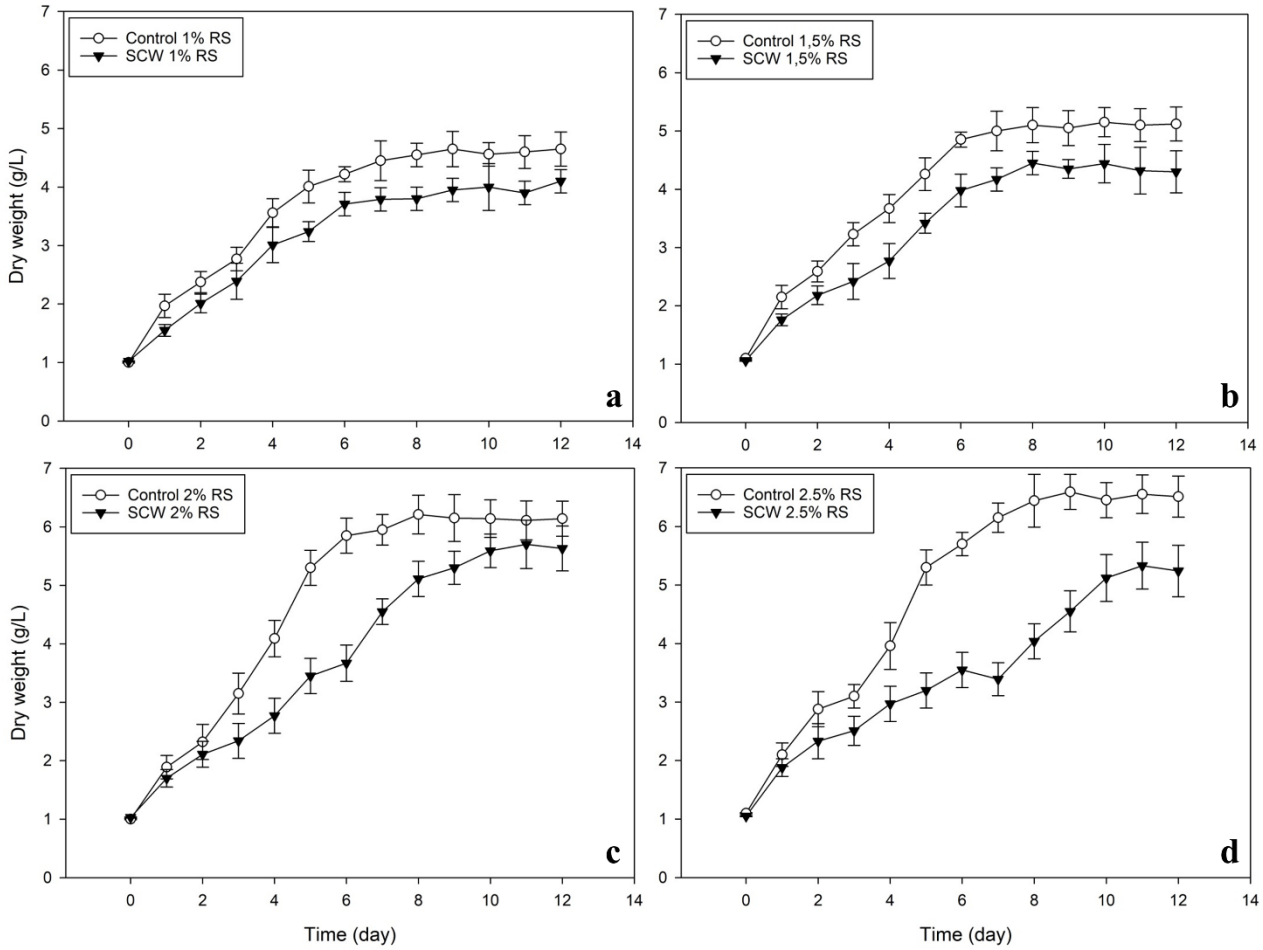

Figure 1. Growth curves (mean ± SD) of *Galdieria sulphuraria* growth with four formulations of second cheese whey medium diluted at 1.0% (a), 1.5% (b), 2.0% (c) and 2.5% (d) concentration in reducing sugars (RS). The controls refer to standard media with the same percentage of reducing sugars.

Both the samples at 2.0 and 2.5% RS reached the maximum biomass concentration after 11 days. This can be explained by a necessity of the microalga to adapt to the new media, which is something already known for this type of biomass [6]. About the uptake of lactose by *G. sulphuraria*, instead, some studies reported an utilization of this disaccharide by the alga [4,28], showing that lactose can be actually transported in to the cell by a low-affinity transport system. However it is actually not clear if lactose uptake is slower than the glucose uptake for *G. sulphuraria.*

The low free nitrogen present in SCW forced *G. sulphuraria* to assimilate organic nitrogen from peptides and aminoacids (I.e. FAN) residual after pre-treatments. In figure 2 is reported the FAN content on the supernatant collected after biomass harvesting. The FAN content is almost depleted in SCW at 1% RS after 12 days of cultivation. At 2.5% RS instead, the

residual content of FAN is still high after cultivation. At 2.0% the residual FAN concentration is the same of the sample at 1.5%, proving a better uptake of nitrogen in that condition.

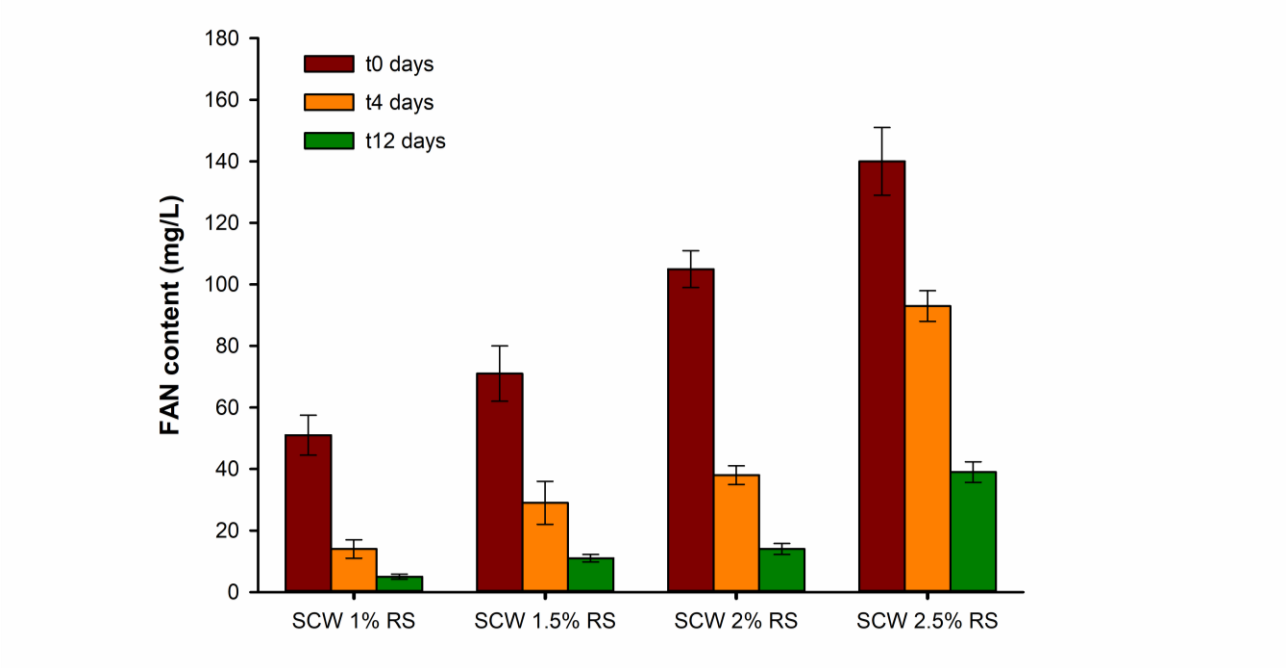

Figure 2. Free amino nitrogen content (mean ± SD) of residual water after centrifugation of biomass, sampled at beginning (t0), four days (t4) and twelve days (t12) of cultivation.

Other studies reported an assimilation of nitrogen from aminoacids and peptides present in growth medium by *Cyanidium caldarium* (formerly named *Galdieria sulphuraria*), but with a slower uptake [29]. This, combined to the presence of lactose as only carbon source, can explain the slower productivity compared to the controls. However, the SCW media formulation resulted interesting for the growth of *G. sulphuraria*, but an optimization of the medium is required to increase the biomass production.

Biochemical and fatty acids composition of biomass cultivated

The proximal composition of *G. sulphuraria* growth on various SCW formulations is reported in Figure 3. Carbohydrates results the principal biochemical component of the microalga, in agreement with other authors [6,30,31]. In general, the amount of carbohydrates increases with increasing concentration of SCW (in terms of RS); while the amount of proteins is lowered when the concentration of SCW increases. It is reported that heterotrophic cultures of *G. sulphuraria* accumulate carbohydrates (principally glycogen) when more glucose or

reducing sugars are added to the culture media [32], which explains the higher content of carbohydrates in the samples with an higher percentage of RS. The α-glucan (glycogen) is the primary form of carbohydrates accumulation in *G. sulphuraria*. Moreover, a natural glycerol glycoside (named "floridoside") is an interesting molecule found in this red alga due to its therapeutic properties (bone formation promotion and modulation of immune system) [33].

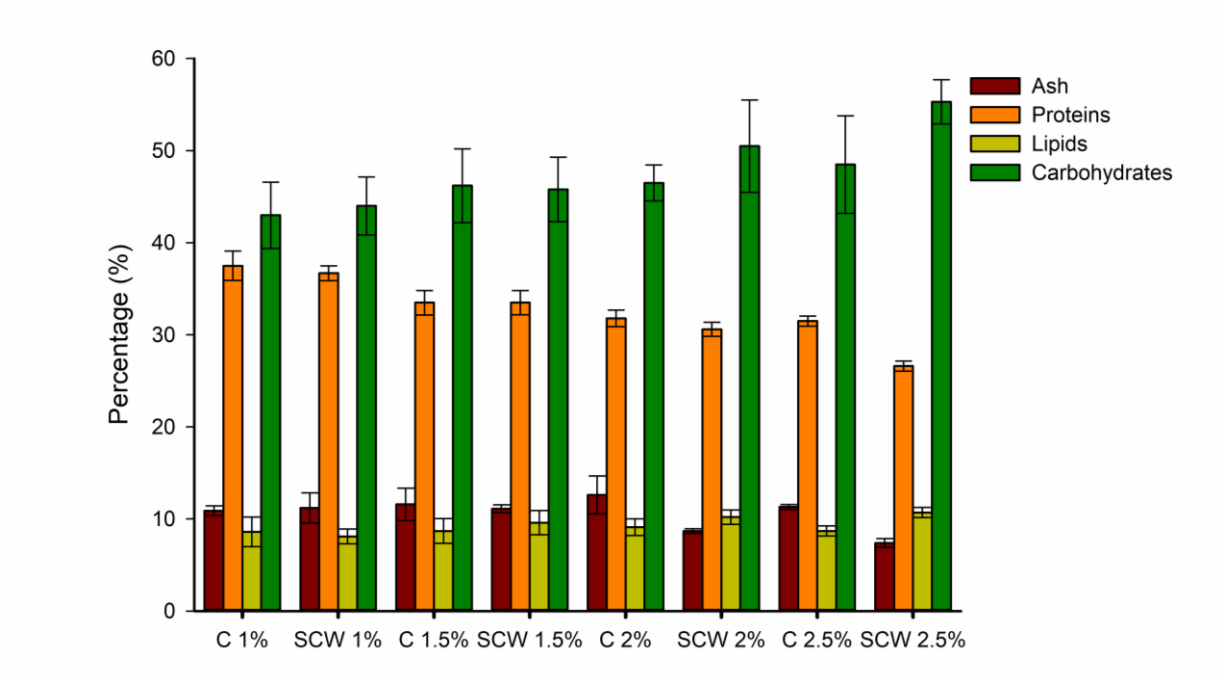

Figure 3. Chemical composition of *Galdieria sulphuraria* grown with SCW media diluted at four concentrations of reducing sugars (1-2.5%) respect to the standard medium (C) with the same percentage of reducing sugars (error bars refers to SD). Values are expressed as percentage of dry weight.

The protein content showed no significant differences between the SCW samples and the relative controls, but the SCW 2.5% in RS showed a protein content lower than the control (26% vs. 32%). The protein content of SCW samples was in line with another study [31] that used standard culture conditions.

The lipids content was about 10% of DW, without significant differences between the samples. This value is higher than others found in literature [6,30]. However, on the fatty acids profile (Table 3) it can be observed that there are significant differences between the samples growth on SCW media and the relative control in standard conditions. The control showed a greater concentration of oleic acid (up to 78% of Total fatty acids, TFA) respect to the SCW samples (30-60% of TFA) which showed instead a greater concentration in saturated fatty acids (SFA). The SFA content of SCW samples is significantly higher than the control,

in agreement with another study on *G. sulphuraria* growth in heterotrophic conditions [32]. The amount of oleic acid obtained in the control is higher than another reported in literature for *G. sulphuraria* [30], while the amount of linoleic acid (C18:2) is lower.

Table 3. Fatty acids profile (g/100g) on the lipids extract from *G.sulphuraria* grown on SCW diluted at different reducing sugars (RS) concentrations (1-2.5%).

Fatty acids	Control	SCW 1.0%	SCW 1.5%	SCW 2.0%	SCW2.5%
C14:0	0.21 ± 0.07^{a}	4.54 ± 1.10^{d}	2.29 ± 0.37^{c}	1.6 ± 0.21^{b}	1.76 ± 0.42^{b}
C16:0	7.01 ± 1.02^{a}	26.26 ± 2.45^{b}	24.96 ± 1.24^{b}	18.89 ± 2.29^{c}	$20.13\pm1.93^{b,c}$
C18:0	5.1 ± 1.13^{b}	10.8 ± 2.02^{a}	12.85 ± 1.41^{a}	5.96 ± 1.04^{b}	7.31 ± 0.56^{b}
Σ SFA	12.32	41.6	40.1	26.45	28.2
C16:1	0.11 ± 0.02^{b}	1.01 ± 0.24^{a}	$0.53\pm 0.19^{a,b}$	0.35 ± 0.11^{b}	1.14 ± 0.21^{a}
C18:1	77.84 ± 3.42^{c}	$41.08\pm5.45^{a,b}$	$34.9\pm 2.41^{a,b}$	55.88 ± 3.57^{d}	49.63 ± 2.49^{b}
Σ MUFAs	77.95	42.09	35.43	56.23	50.77
C18:2	6.23 ± 1.13^{a}	5.72 ± 1.25^{a}	9.82 ± 1.44^{b}	6.05 ± 1.93^{a}	5.67 ± 1.26^{a}
C18:3	1.98 ± 0.26^{c}	4.24 ± 1.67^{a}	10.53 ± 2.18^{b}	$8.43\pm 0.65^{a,b}$	6.08 ± 0.41^{a}
Σ PUFAs	8.21	9.96	20.35	14.48	11.75

Values are expressed as means ± SD (n=3). SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids. Values followed by different letters on the same line are significantly different ($p < 0.05$).

Comparing the data of this study to the work of Graziani et al., (2013), the amount of α-linolenic acid (C18:3) of the samples growth in SCW is 2-3 times higher, and 5 times greater for SCW at 1.5% in RS. This can be explained by a difference in the culture conditions and growth medium of *G. sulphuraria*. In fact, in the work of Graziani et al. (2013) the microalga was cultivated at 36 °C, while in this test at 27 °C.

Moreover, an interesting result was obtained in terms of polyunsaturated fatty acids (PUFA) concentration, which was higher in the sample SCW 1.5% RS respect to the control (20% vs 8% of the control). The composition and characteristics of lipids and fatty acids of *G. sulphuraria* are regulated by the growth conditions [32]. In our case, the addiction of SCW in the growth media seems to affect the cells metabolism, stimulating the elongation and unsaturation of acyl chains in the algae cells, especially in the sample at 1.5% in RS. However, further studies are required to understand the regulation mechanisms of metabolic flow of fatty acids in *G. sulphuraria*.

Growth optimization of SCW media

The SCW formulation with higher biomass productivity is the one at 2.0% in RS, with 6.05 g L^{-1} of DW. For that reason, the optimization has been performed on this formulated medium.

Central Composite Design (CCD) was used to optimize the utilization of SCW media with the supplementation of organic carbon (glucose) and nitrogen (in forms of ($(NH_4)_2SO_4$). To the best of our knowledge, this is the first study to apply CCD for the biomass optimization of *G. sulphuraria* with a food waste.

The response surface design employed gave 14 combinations of selected nutrients (glucose and ammonium sulfate). In Table 4 is reported the design and the results with the responses. Biomass concentration (DW) was used as response, and was calculated at log phase (12 days). The significance of the model and its second-order equation (2), derived from the multiple regression analysis of the data, was tested by analysis of variance (ANOVA) (Table 5) and p-value lower than 0.05 was considered significant in the analysis. The model fit is also expressed with coefficient of determination (R^2) which was 0.985, indicating that 98.5% of the variability in the Y (response) could be explained by the model. The *p*-value of the model was ($p < 0.005$) which implied that the model was significant, and also the lack of fit is non-significant ($p > 0.05$) proving the validity of the model. The regression equation obtained from the model has been shown in eq. (3)

$$Y= 6.393 + 0.2895\ X1 + 9.269\ X2 - 0.01887\ X1*X1 - 7.073\ X2*X2 + 0.0588\ X1*X2 \quad (3)$$

Where, X1 represent the amount of glucose added and X2 the $(NH_4)_2SO_4$ added (in g L^{-1}) to the formulated SCW media.

Table 4. Growth optimization of SCW media with supplementation of glucose and $(NH_4)_2SO_4$ for *Galdieria sulphuraria* using Central Composite Design (CCD).

Run	Factor Assignment		Biomass (Y)	
	X_1 (Glucose)	X_2 ($(NH_4)_2SO_4$)	Experimental value (g L^{-1})	Predicted value (g L^{-1})
1	0	0	9.92	10.06
2	0	−1	7.45	7.27
3	0	0	10.24	10.06
4	1	0	10.18	10.21
5	−1	0	9.22	8.87
6	0	0	10.01	10.06
7	0	1	10.71	10.42

8	−1	1	9.10	9.28
9	1	−1	7.39	7.21
10	0	0	10.22	10.06
11	1	1	10.65	10.79
12	−1	−1	6.11	6.41
13	0	0	9.69	10.06
14	0	0	10.19	10.06

Coded values; X1 glucose, X2 $(NH_4)_2SO_4$. The three levels (−1, 0 and +1) set for glucose were 0, 5 and 10 g L^{-1} supplemented to the SCW media, while for $(NH_4)_2SO_4$ was 0, 0.4 and 0.8 g L^{-1} respectively.

Based on ANOVA analysis, both the factors showed significant impact on the growth of *G. sulphuraria*. The most significant factor was ammonium sulfate (p=0.005) followed by glucose (p=0.013). In the run n. 12, without the addiction of glucose or nitrogen, the biomass obtained was 6.11 g L^{-1}, while the highest DW value was obtained in run 7 (10.65 g L^{-1}) with a combination of 0.8 g L^{-1} of $(NH_4)_2SO_4$ and 5 g L^{-1} of glucose added. The predicted values are also reported, which are very similar to the experimental values, proving the validity of the model.

The supplementation of inorganic nitrogen source, lead to an increase of biomass yield by 58%. In that way, is possible to obtain a good productivity using SCW as principal nutrient source for *G. sulphuraria* cultivation.

Table 5. Analysis of variance for *G.sulphuraria* biomass optimization using coded values and regression equation.

Source	DF[a]	Adj SS[b]	Adj MS[c]	F-Value	*P*-Value
Model	6	24.2291	4.0382	97.73	0.001
Glucose (X1)	1	2.3188	2.3188	56.12	0.013
$(NH_4)_2SO_4$ (X2)	1	14.6328	14.6328	354.14	0.005
Linear	2	16.9516	8.4758	205.13	0.001
Square	2	5.9442	2.9721	71.93	0.000
X1*X1	1	0.6067	0.6067	14.68	0.006
X2*X2	1	3.4927	3.4927	84.53	0.000
X1*X2	1	0.0552	0.0552	1.34	0.286
Error	7	0.2892	0.0413		
Lack-of-Fit	3	0.0816	0.0272	0.52	0.689
Pure Error	4	0.2077	0.0519		
Total	13	24.5184			

R^2 = 98.51 ([a]DF, degree of freedom; [b]SS, sum of squares; [c]MS, mean squares; F, probability of distribution; *P*, probability).

The biomass obtained in optimized condition is lower than other studies [6,31] where a concentration higher than 12 g L^{-1} was obtained. However different factors should be taken into account, such as the inoculum concentration and the culture conditions (I.e. temperature). Other studies reported a lower productivity than our work when cultivating *G. sulphuraria* on waste material [11]. Moreover, in a recent study where *G. sulphuraria* was grown on fruit-salad wastewater, micronutrients and ammonia where added to promote the complete consumption of the nutrients present in the effluent [10]. This is a similar case as SCW media utilization. An interesting way to exploit the nutrients presents in this type of by-product would be the blend with other food waste (I.e. molasses as carbon source). In that way the supplementation with glucose or ammonium sulphate could be not necessary.

4. Conclusion

SCW can actually be used as alternative and sustainable medium for the cultivation of *Galdieria sulphuraria.* The suitability of this food waste has been tested at different concentrations and compared with SM. When diluted at 2.0% in RS the biomass obtained was higher than the other formulated media. Biochemical composition of biomass reported slightly difference between the algae growth in standard condition respect to the algae growth with SCW media. Fatty acids profile was affected by the new SCW media, obtaining a higher PUFA content respect to the SM. The biomass optimization with SCW media supplemented with glucose and nitrogen led to a good biomass production, proving that this dairy waste can be used as nutrient source for the cultivation of this extremophile red alga. With these results, it is possible to evaluate new economically and environmentally sustainable biotechnological process, using low cost food effluents for the cultivation of *G. sulphuraria*.

References

1. Secchi N, Giunta D, Pretti L, et al. (2012) Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid bacteria. J Ind Microbiol Biotechnol.

2. PANESAR P, KENNEDY J, GANDHI D, et al. (2007) Bioutilisation of whey for lactic acid production. Food Chem 105: 1–14.

3. Sansonetti S, Curcio S, Calabrò V, et al. (2009) Bio-ethanol production by fermentation of ricotta cheese whey as an effective alternative non-vegetable source. Biomass and Bioenergy 33: 1687–1692.

4. Tsolcha ON, Tekerlekopoulou AG, Akratos CS, et al. (2016) Treatment of second cheese whey effluents using a Choricystis -based system with simultaneous lipid production. J Chem Technol Biotechnol 91: 2349–2359.

5. Zimermann JD ar. F, Sydney EB, Cerri ML, et al. (2020) Growth kinetics, phenolic compounds profile and pigments analysis of Galdieria sulphuraria cultivated in whey permeate in shake-flasks and stirred-tank bioreactor. J Water Process Eng 38: 101598.

6. Russo GL, Langellotti AL, Oliviero M, et al. (2021) Sustainable production of food grade omega-3 oil using aquatic protists: Reliability and future horizons. N Biotechnol 62: 32–39.

7. Massa M, Buono S, Langellotti AL, et al. (2019) Biochemical composition and in vitro digestibility of Galdieria sulphuraria grown on spent cherry-brine liquid. N Biotechnol 53: 9–15.

8. Girard J-M, Roy M-L, Hafsa M Ben, et al. (2014) Mixotrophic cultivation of green microalgae Scenedesmus obliquus on cheese whey permeate for biodiesel production. Algal Res 5: 241–248.

9. Abreu AP, Fernandes B, Vicente AA, et al. (2012) Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. Bioresour Technol 118: 61–66.

10. Ende SSW, Noke A (2019) Heterotrophic microalgae production on food waste and by-products. J Appl Phycol 31: 1565–1571.

11. Scherhag P, Ackermann J (2021) Removal of sugars in wastewater from food production through heterotrophic growth of Galdieria sulphuraria. Eng Life Sci 21: 233–241.

12. Pan S, Dixon KL, Nawaz T, et al. (2021) Evaluation of Galdieria sulphuraria for nitrogen removal and biomass production from raw landfill leachate. Algal Res 54: 102183.

13. Moon M, Mishra SK, Kim CW, et al. (2014) Isolation and characterization of thermostable phycocyanin from Galdieria sulphuraria. Korean J Chem Eng.

14. Allen MM (1968) SIMPLE CONDITIONS FOR GROWTH OF UNICELLULAR BLUE-GREEN ALGAE ON PLATES. J Phycol 4: 1–4.

15. Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31: 426–428.

16. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

17. Adams VD (2017) Water and Wastewater Examination Manual.

18. Lie S (1973) THE EBC-NINHYDRIN METHOD FOR DETERMINATION OF FREE ALPHA AMINO NITROGEN. J Inst Brew 79: 37–41.

19. Nazir Y, Shuib S, Kalil MS, et al. (2018) Optimization of Culture Conditions for Enhanced Growth, Lipid and Docosahexaenoic Acid (DHA) Production of Aurantiochytrium SW1 by Response Surface Methodology. Sci Rep 8: 8909.

20. DuBois M, Gilles KA, Hamilton JK, et al. (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem 28: 350–356.

21. Bligh EG, Dyer WJ (1959) A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Can J Biochem Physiol 37: 911–917.

22. AOAC (2005) Official Methods of Analysis of AOAC International.

23. Tsolcha ON, Tekerlekopoulou AG, Akratos CS, et al. (2016) Treatment of second cheese whey effluents using a Choricystis -based system with simultaneous lipid production. J Chem Technol Biotechnol 91: 2349–2359.

24. Oesterhelt C, Schnarrenberger C, Gross W (1999) Characterization of a sugar/polyol uptake system in the red alga Galdieria sulphuraria. Eur J Phycol 34: 271–277.

25. Gross W, Schnarrenberger C (1995) Heterotrophic Growth of Two Strains of the Acido-Thermophilic Red Alga <italic>Galdieria sulphuraria</italic>. Plant Cell Physiol.

26. Rigano C, Fuggi A, Rigano VDM, et al. (1976) Studies on utilization of 2-ketoglutarate, glutamate and other amino acids by the unicellular alga Cyanidium caldarium. Arch Microbiol 107: 133–138.

27. Ho S-H, Li P-J, Liu C-C, et al. (2013) Bioprocess development on microalgae-based CO2 fixation and bioethanol production using Scenedesmus obliquus CNW-N. Bioresour Technol 145: 142–149.

28. Wen Z-Y, Chen F (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnol Adv 21: 273–294.

29. Tischendorf G, Oesterhelt C, Hoffmann S, et al. (2007) Ultrastructure and enzyme complement of proplastids from heterotrophically grown cells of the red alga Galdieria sulphuraria. Eur J Phycol 42: 243–251.

30. Sloth JK, Jensen HC, Pleissner D, et al. (2017) Growth and phycocyanin synthesis in the heterotrophic microalga Galdieria sulphuraria on substrates made of food waste from restaurants and bakeries. Bioresour Technol 238: 296–305.

31. Graziani G, Schiavo S, Nicolai MA, et al. (2013) Microalgae as human food: chemical and nutritional characteristics of the thermo-acidophilic microalga Galdieria sulphuraria. Food Funct 4: 144–152.

32. Wan M, Wang Z, Zhang Z, et al. (2016) A novel paradigm for the high-efficient production of phycocyanin from Galdieria sulphuraria. Bioresour Technol 218: 272–278.

33. Sakurai T, Aoki M, Ju X, et al. (2016) Profiling of lipid and glycogen accumulations under different growth conditions in the sulfothermophilic red alga Galdieria sulphuraria. Bioresour Technol 200: 861–866.

34. Martinez-Garcia M, van der Maarel MJEC (2016) Floridoside production by the red microalga Galdieria sulphuraria under different conditions of growth and osmotic stress. AMB Express 6: 71.

Chapter 4

Production of Omega-3 Oil by *Aurantiochytrium mangrovei* Using Spent Osmotic Solution from Candied Fruit Industry as Sole Organic Carbon Source

This chapter has been published as:

Giovanni L. Russo, Antonio L. Langellotti, Thierry Blasco, Maria Oliviero, Raffaele Sacchi and Paolo Masi . Production of Omega-3 Oil by *Aurantiochytrium mangrovei* Using Spent Osmotic Solution from Candied Fruit Industry as Sole Organic Carbon Source. *Processes* 2021, 9, 1834. https://doi.org/ 10.3390/pr9101834

Abstract

Osmotic dehydration is an important phase in the food industry for the production of dried products, most of all fruits and vegetables. The drying process for the obtainment of candied fruit, lead to a liquid waste called “spent osmotic solution”, characterized with high content of organic compounds, most of all dissolved sugars. The sugar content from this food by-product could be valorised through the growth of biomass with high added value. In this study, the spent osmotic solution from candied fruit industry was used as organic carbon source for the growth and production of docosahexaenoic acid (DHA) by the cultivation of *Aurantiochytrium mangrovei* RCC893. The carbon content of the standard media has been completely replaced by the sugars present in this food by-product. After that, the growth condition of this strain has been optimized through response surface methodologies using a central composite design (CCD), and the optimal combination of spent osmotic solution and nitrogen has been established. Moreover, a scale up trial has been performed using the optimal conditions obtained after CCD to evaluate the scalability of the process.

Keywords: Sustainability, PUFA, food waste, DHA

4.1. Introduction

Omega-3 (ω-3) and omega 6 (ω-6) long chain-polyunsaturated fatty acids (LC-PUFAs) are compounds that has long been studied and discussed. In particular, docosahexaenoic acid (DHA, C22:6n-3) has been widely studied because it is an important fatty acid for human health with a series of benefits. It is the most abundant LC-PUFA in human brain and one of the major components of the central nervous sys-tem, essential for brain growth and development in infants. [1]. For that reason, DHA is used in many adult supplements and infant formulas [2].

Actually, the principal source of DHA is fish oil obtained from fatty fish (i.e. mackerel, salmon and tuna), but it has several disadvantages, most of all the low sustainability for overexploitation of marine biotic resources; contaminations by marine pollutant and characterized by an undesirable fishy smell [3,4]. All these factors have grown the concerns over the long-term sustainability and safety of fish oil increasing the attention on new sources of LC-PUFAs.

Single cell organisms, especially microalgae, received great interests from scientific community due to their capacity to accumulate large amounts of LC-PUFAs in controlled environment.

Thraustochytrids are heterotrophic protists commonly found in marine environments, widely known for their high concentration of ω3 LC-PUFA, most of all DHA [5]. Omega-3 oil obtained from thraustochytrids is a potential alternative to fish oil, because of their high biomass productivity and DHA content which is much higher than the fish source. [6] Among the thraustochytrids, *Aurantiochytrium* (known as *Schizochytrium* until 2007), a protist commonly found in many coastal ecosystems, is one of the highest LC-PUFAs producer. [7] It can produce high amount of lipids (up to 60% of dry weight) and most of that is DHA (up to 55% of total fatty acids) [7,8]. This protist is commercially used in DHA-rich oils and as food ingredient for foods, feeds and nutritional supplement [5] and it is free from common algal toxins (I.e. domoic acid) [9]. Industrial production of DHA by Thraustochytrids requires great amount of glucose and yeast extract (as nitrogen source) that makes the process expensive. In fact, the nutrient source represents a significant portion of the production costs for heterotrophic cultivation [10].

To overcome this issue, many efforts have been carried out to research new sustainable nutrient sources for microalgae cultivation. One of the most promising alternative to standard nutrients, is the utilization of food by-products as medium for the growth of algae biomass [11]. In fact, the use of sugar-rich by-products deriving from agro-food industry can represent a sustainable alternative to reduce omega-3 oil production costs and concurrently valorize food waste.

Spent osmotic solution (SOS) from candied fruit industry is an interesting by-product that could be used for the cultivation of heterotrophic aquatic protists. This waste is generated after osmotic dehydration of fruits (cherry, orange, berries etc.) in order to preserve the aroma, extend shelf-life and to reinforce the sweet taste of fruits [12]. The disposal of this food waste has high economic impact and represents an environmental problem due to high chemical oxygen demand (COD) and low pH. Actually, few attempts have been carried out to recycle or valorize this industrial waste, and the most promising approach is the biotechnological conversion. Aachary and Prapulla, (2009) successfully converted SOS in fructooligosaccharides (FOS) through transfructosylation reaction of fructosyl transferase enzyme produced by *Aspergillus oryzae* MTCC 5154 [12]. However, SOS has never been tested as growth medium for aquatic protist.

Recently, the extremophile red algae *Galdieria sulphuraria*, has been grown using a similar spent brine liquid, resulting in a change of algal biochemical composition and valorizing the food waste [13]. Moreover, *Aurantiochytrium sp*. have been successfully grown on other food by-products thanks to their metabolic feasibility [11,14,15] but the strain RCC893 was never tested on any type of food processing by-product.

Therefore, the purpose of this study is to investigate the potential of spent osmotic solutions from candied fruit industry as carbon source for the cultivation of *Aurantiochytrium mangrovei* RCC893. The growth factors were optimized through response surface methodologies and a scale up trial was also performed. We prove that it is possible to use this food by-product as low-cost carbon source for the production of biomass rich in lipids and DHA.

4.2 Materials and Methods

Organism and cultivation

A. mangrovei (RCC893) was obtained from the Roscoff algae collection (France). A stock culture of an axenic microalga strain was maintained routinely by regular sub-culturing at 2-week intervals on both liquid and agar slants of YEP Medium following the recipe provided by the Roscoff collection. YEP broth was obtained from filtered natural oligotrophic seawater adjusted at pH 6.5. The nitrogen (N) sources were peptone (2 g L^{-1}) and yeast extract (2 g L^{-1}), while the organic carbon source was glucose in a concentration of 20 g L^{-1}. As microelements supplement, 1 mL L^{-1} of metal solution were added to the media, consisting of: $MgSO_4$*7H20 (200 mg L^{-1}), KH_2PO_4 (200 mg L^{-1}), $NaHCO_3$ (100 mg L-1), MnCl2*4H2O (9 mg L^{-1}), Fe3Cl3.6H2O (3 mg L^{-1}), ZnSO4*7H2O (1 mg L^{-1}), CoSO4*5H2O (0.3 mg L^{-1}), and CuSO4*5H2O (0.2 mg L^{-1}) and also 0.1 mg L-1 of thiamine [16]. The protist was cultivated in dark condition at temperature of 24 ± 2 °C. Culture agitation was provided by means of an orbital shaker at 140 rpm.

Spent osmotic solution samples

SOS samples were gently provided by a local factory from Naples, Italy that produces candied fruits. The samples were frozen at -24°C to prevent any kind of fermentation. Prior chemical

analyses, SOS samples were centrifuged at 5,000 g for 15 minutes at 10°C, and then the supernatant was collected and solid fraction discarded.

The chemical composition of SOS was: Dry Weight (DW) 702.11 g Kg^{-1}; Total Ni-trogen (TN) 0.012 g Kg^{-1}; Total sugars 682.23 g Kg^{-1}; Reducing sugars 279.72 g Kg^{-1}; Ash content 0.25 g Kg^{-1}; pH 5-5.3.

Experimental design

The experimental design has been summarized below. It was divided into four parts:

1) Screening test to evaluate the growth of *A. mangrovei* RCC893 using different temperatures and different organic carbon sources in order to define the growth performances and the better operating parameters;

2) Substitution of C source in the standard media using SOS as organic carbon source;

3) Optimization of biomass and DHA production through response surface methodologies (RSM) with different C/N ratio.

4) Scale-up trial in airlift reactor to evaluate the scalability of new SOS medium.

Best growth temperature and organic carbon source

For the determination of the optimal temperature condition, five different temperatures were tested (20, 24, 28, 32 and 36 °C) by means of a shaking thermostatic bath. Three Erlenmeyer flask for each temperature has been prepared with standard medium and a working volume of 100 mL with a rotary speed of 140 rpm was used. Inoculum level of *A. mangrovei* was set at 10% v/v for all the tested temperature.

For the evaluation of organic carbon source, instead, four different type of organic carbon were tested (glucose, fructose, sucrose, and glycerol) by adding 20 g L^{-1} of each to a YEP broth without any other source of organic carbon. Each run was performed in triplicate. For this experiment, a working volume of 120 mL was placed in a 250 mL Erlenmeyer flask and *A. mangrovei* was inoculated into each flask to reach an initial DW of 400 mg L^{-1}. Every 24 h the dry cell weight was evaluated.

Substitution of carbon source with spent osmotic solution

To evaluate the potential of SOS, the sugars present in this food waste was used to replace the glucose in standard media at different percentages of substitution. To achieve that, the carbon content provided by glucose in YEP broth was replaced at 25, 50, 75 and 100% by sugars present in SOS. That means that at 100% substitution, no glucose was added to the media. The differences between the standard media and the one obtained with the food by-product were analysed through ANOVA. The trial was conducted on orbital shaker at 140 RPM at 28 °C in triplicate, and the inoculum level of *A. mangrovei* was set at 10% v/v.

Response surface analysis and formulation of optimized media

Response surface method (RSM) was applied to determine the optimal combination of SOS (g L^{-1}) and yeast extract (nitrogen source) by constructing a three level full factorial central composite design (CCD). The optimization consisted of 14 runs conducted in two blocks with 4 cubic points (or factorial points), 4 axial points (or star points) and 3 center points for each block.

The mathematical relationship of the response (Y) to the significant independent variables X1 and X2 is given by the following quadratic polynomial equation (1):

$$\mathbf{Y} = \boldsymbol{\beta_0} + \sum_{i=1}^{n} \boldsymbol{\beta_i X_i} + \sum_{i=1}^{n} \boldsymbol{\beta_{ii} X_i^2} + \sum_{i=1}^{n} \boldsymbol{\beta_{ij} X_i X_j} \quad (1)$$

Where Y is the predicted response; Xi and Xj are the coded values; β0 the independent coefficient; βi,,j is the linear coefficient associated to each independent factor (Xi,j) and βij and βii are the coefficient for interaction and quadratic effects respectively [9]. The factors selected for this test were yeast extract (YE) and sugars from SOS, both expressed in g L^{-1}. Two responses were taken in exam for this study: biomass productivity (expressed in g/L/day) and DHA productivity (expressed as mg/L/day).

A duplicate for each run were prepared in air-lift bioreactor with a working volume of 300 mL. Culture mixing was provided by means of an air bubbling system equipped with a filter of 0.22 μm to avoid culture contamination.

Finally, the optimized medium was used in a scale up trial with air-lift bioreactor of 5L. The culture oxygenation and mixing was provided through an air bubbling system (flow rate 2.5 L min^{-1}) equipped with a filter of 0.22 μm and the temperature was maintained at 28 ± 1 °C. Growth was carried out in the dark.

Analytical methods

Measurement of dry cell weight and residual nutrients

For all the growth test, every 24 hours aliquots of culture volume were taken and transferred in weighted dry tubes, then centrifuged at 5,000g for 10 min. The supernatants were discarded and the pellet washed with phosphate buffered saline (PBS) and dried overnight in oven at 105°C to obtain the dry cell weight (DCW) [14].

The biomass productivity (g/L/day) was calculated using the following equation:

Productivity = (final DCW content – initial DCW content)/ cultivation time

For the determination of residual sugars, samples were withdrawn from flasks and collected in sterile tubes and filtered prior analysis. The sugar content during cultivation was determined using the Dubois method assay [17].

Fatty acid methyl esters (FAMEs)

Samples were analyzed by GC-MS (Agilent GC7890A-MSD5975C) coupled by an Elementar GC5 combustion oven to an IRMS (Elementar Isoprime 100). In CSIA (Compound Specific Isotope Analysis) mode, samples were prepared according to adapted Bligh and Dyer (1959) and Morrison and Smith (1964) protocols: liquid-liquid lipid extraction from 50 mg samples with a chloroform-methanol-water mixture (2-2-1.8 ratios) with ball mill grinding [18,19]; hydrolysis and fatty acid extraction and transesterification at 100°C during 60 min. with toluene and 7% Boron trifluoride diluted in MeOH (1-1 volume). FAMEs were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., USA). The quantity of DHA was estimated from the peak areas on the chromatogram using nonadecanoic acid (19:0) as an internal standard.

Statistical Analysis

All the analyses were carried out in triplicate, and average values with standard deviation were reported. One-way ANOVA was applied using raw data to test for significant differences among the samples (significance level was always set at $p < 0.05$). The Tukey's test was used as post-hoc analysis, when there were significant differences among the samples. The data were analyzed using IBM© SPSS© Statistics software Ver. 23 (SPSS, Inc., Chicago, IL). The optimization process was evaluated with RSM analysis, performed in 'R' (RStudio with 'R' version 3.0.2, RCore Team, Vienna/Austria).

4.3 Results and discussions

Optimal standard conditions

In figure 1(a) is reported the biomass productivity of *A. mangrovei* RCC893 at different temperatures.

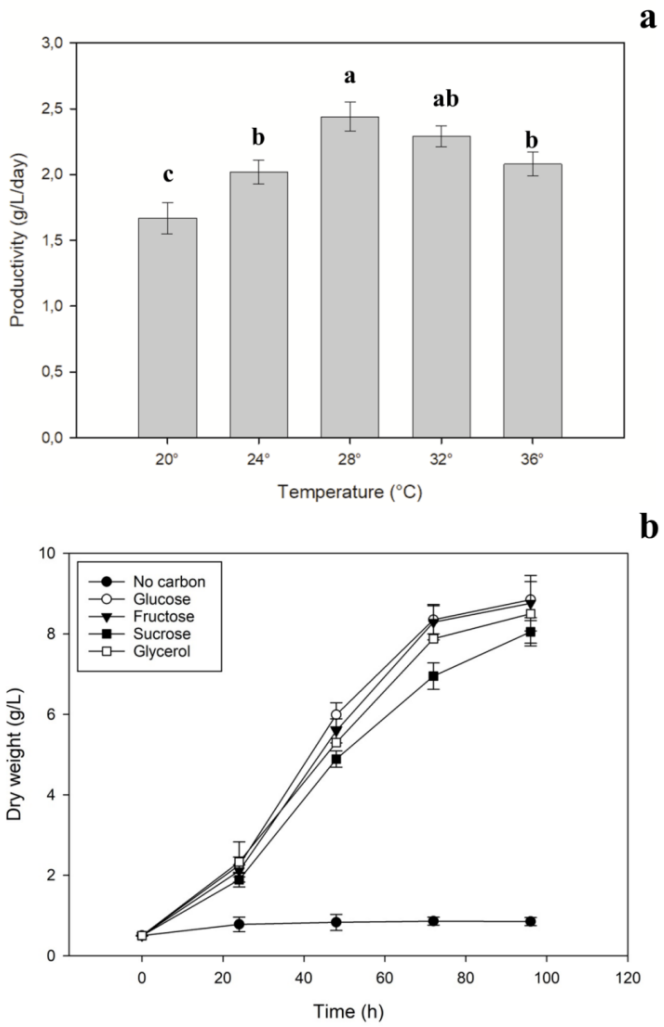

Figure 1. Productivity and growth curves of *A. mangrovei* RCC893 at different growth temperatures (a) and organic carbon source (b) on orbital shaker. Values are reported as mean ± SD. Different letters in the same graph show the statistical difference among the treatments.

At 20 °C the microalga registered the lowest productivity, while the highest biomass production was obtained at 28 °C. Temperatures higher than 32 °C resulted in a lower biomass productivity compared to the one growth at 28 °C. Based on these results, all subsequent tests were conducted at 28 °C.

This result is in line with Nakazawa et al., (2012) that evaluated the growth behavior of *Aurantiochytrium* sp. strain 18W-13a at 10-35 °C and also for Taoka et al., (2009) with strain mh0186 of *A. limacinum* [20,21]. The evaluation of different carbon sources on *A. mangrovei* RCC893 growth is reported in Figure 2(b). No significant differences were observed between glucose, fructose and glycerol which resulted the best carbon sources with the highest final concentrations (between 8.5-8.8 gDW L^{-1}). Media supplemented with sucrose reported a

lower productivity after 72 h of cultivation. However, after 96 h of cultivation, productivity was not statistically different respect to the other sugars. Different works reported a similar screening for different C sources on *Aurantiochytrium* sp. Growth. Yu et al., (2015a), reported a significant growth for *Aurantiochytrium* sp. YLH70 when cultivated with sucrose, but with growth performance lower than glucose and fructose [15]. This result is in line with our study. Mariam et al., (2021) reported glycerol, glucose and fructose as best C source for an indigenous thraustochytrid tested. However, the authors showed that this strain was unable to metabolize sucrose [22]. Moreover, Pahlavanyali et al., (2020) reported that hydrolysis of sucrose in molasses-based medium was necessary in order to improve biomass and DHA production from *Schizochytrium* sp. remarking that sucrose as main organic carbon source could affect negatively the biomass productivity [23].

Utilization of SOS as carbon source

Once the optimal growth condition for *A. mangrovei* RCC893 has been established, the carbon content provided by the glucose (in standard media) has been substituted at four different degrees to evaluate the utilization of SOS as main source of organic carbon respect to the standard conditions with glucose. The results are reported in Figure 2.

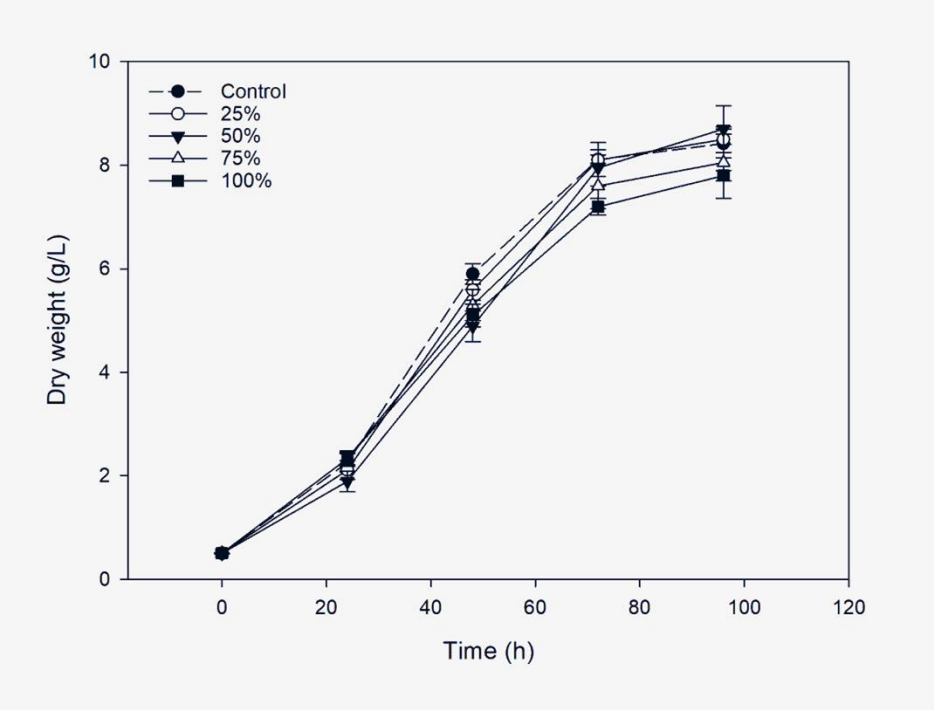

Figure 2. Growth curves of *A. mangrovei* using new media with progressive substitution (25-100%) of standard glucose with sugars from spent osmotic solution. 100% substitution means that the only organic carbon source was sugars from SOS.

In terms of biomass, no significant difference respect to the standard media has been found from 25 to 100% of substitution of glucose in the media with sugars of SOS. That proves the capability of this strain to use the nutrients present in SOS as sole carbon source and without any pre-treatment (i.e. sucrose hydrolysis).

Aurantiochytrium species have been tested several times with alternative cheap substrates for their cultivation [24,25]. In particular, Hong et al., (2011) obtained for *Aurantiochytrium sp*. KRS101 a biomass productivity of 16.7 g L^{-1} day^{-1} in fed-batch mode using sugar cane molasses instead of glucose [24]. Iwasaka et al., (2013) instead, cultivated *Aurantiochytrium* sp. KH105 using waste syrup from fruit industry to obtain DHA and astaxhantin [26]. The authors optimized the waste concentration with CCD to obtain a DW of 8.5 g L^{-1}. Molasses-like substrates resulted very interesting alternative to pure glucose as nutrient source, but inhibitory substances in these by-products must be considered in order to increase the biomass productivity [25]. In our study, SOS do not showed any particular inhibitory effects even at 100% substitution of glucose in standard media.

Response surface results for biomass and DHA productivity

Response surface method was used to optimize the ratio between SOS concentration and yeast extract to assess the best biomass and DHA productivity at different C/N ratio. Experimental design was performed using CCD, fourteen sets of experiments at different concentrations were performed in duplicate to obtain the mean values. In Table 1 the experimental factors, the responses and the predicted values for biomass and DHA productivity are reported.

Table 1. Growth optimization of *A. mangrovei* with spent osmotic solution and yeast extract using Central Composite Design (CCD).

Run	Factor Assignment		Responses		Predicted value	
	X_1 (SOS)	X_2 (YE)	Biomass productivity (g/L/day)	DHA productivity (mg/L/day)	Biomass productivity (g/L/day)	DHA productivity (mg/L/day)
1	0	+1	3.71	337.67	3.59	328.35
2	0	-1	1.99	477.24	2.38	460.59
3	0	0	3.27	402.19	3.52	459.93
4	0	0	3.56	443.12	3.52	459.93
5	+1	0	2.89	394.11	2.73	357.94
6	-1	0	1.77	207.62	1.90	217.70
7	0	0	3.61	436.53	3.52	459.93
8	+1	-1	1.55	389.10	1.48	405.57
9	+1	+1	2.76	166.48	2.91	179.39
10	0	0	3.71	415.48	3.52	459.93
11	-1	-1	1.11	177.88	0.86	171.39
12	0	0	3.39	559.78	3.52	459.93
13	-1	+1	1.82	143.24	1.88	133.09
14	0	0	3.50	463.37	3.52	459.93

Coded values; X1 sugars from SOS (g L^{-1}), X2 yeast extract (g L^{-1}). The three levels (-1, 0 and +1) set for SOS were 14, 35 and 60 g L^{-1}, while for yeast extract was 4, 8 and 12 g L^{-1} respectively.

The experimental results for biomass and DHA productivity were comparable to the predicted values. The significance of the model was tested by ANOVA for biomass (Table 2) and DHA (Table 3) productivity, and p-value lower than 0.05 was considered significant in the analysis.

Table 2. Analysis of variance of central composite design for biomass production using spent osmotic solution.

Source	DF[a]	SS[b]	MS[c]	F-Value	P-Value
Model	6	10.9462	1.82437	23.88	<0.001
Blocks	1	0.0047	0.00475	0.06	0.021
Linear	2	3.4053	1.70266	22.29	0.002
X1 (SOS)	1	1.6275	1.62754	21.31	0.003
X2 (YE)	1	1.7778	1.77778	23.27	0.002
Square	2	7.1934	3.59671	47.08	<0.001
X1*X1	1	4.1712	4.17119	54.60	<0.001

X2*X2	1	0.7717	0.77171	10.10	0.016
2-Way Interaction		0.0419	0.04193	0.55	
X1*X2	1	0.0419	0.04193	0.55	0.483
Error	7	0.5347	0.07639		
Lack-of-Fit	3	0.3699	0.12329	2.99	0.159
Pure Error	4	0.1649	0.04122		
Total	13	11.4810			

[a]DF. degree of freedom; [b]SS. sum of squares; [c]MS. mean squares

Table 3. Analysis of variance for DHA productivity using coded values and regression equation

Source	DF[a]	SS[b]	MS[c]	F-Value	P-Value
Model	6	155540	25923.4	17.50	0.001
Blocks	1	122	122.4	0.08	0.782
Linear	2	34510	17254.9	11.65	0.006
X1 (SOS)	1	30922	30921.7	20.87	0.003
X2 (YE)	1	3588	3588.0	2.42	0.164
Square	2	107076	53538.1	36.13	0.000
X1*X1	1	65612	65612.5	44.28	0.000
X2*X2	1	9320	9320.2	6.29	0.041
2-Way Interaction					
X1*X2	1	3474	3473.6	2.34	0.170
Error	7	10372	1481.7		
Lack-of-Fit	3	1082	360.7	0.16	0.921
Pure Error	4	9290	2322.5		
Total	13	165913			

[a]DF. degree of freedom; [b]SS. sum of squares; [c]MS. mean squares

The model fit for biomass productivity, expressed with coefficient of determination (R^2) was 0.981, indicating that 98.1% of the variability in the Y (response) could be explained by the model. The *p*-value of the model was $p < 0.001$ and the lack of fit was not significant ($p > 0.05$) proving the validity of the model.

The significance of the model for DHA productivity was 0.001, indicating that the model is highly significant. Moreover, the lack of fit was not significant (p=0.921) and the R^2 of the second-order polynomial prediction equation (3) is 0.943 indicating that the DHA variability can be explained by the model for the 94.3% of the total variation.

The experimental results obtained from CCD, were regressed using a quadratic polynomial equation, and the regression equations for biomass (2) and DHA (3) productivity are shown below:

Biomass productivity (g/L/day) = -3.362 + 0.1837 SOS + 0.645 YE - 0.002360 SOS*SOS - 0.0332 YE*YE + 0.00111 SOS*YE (2)

DHA productivity (mg/L/day) = -337 + 28.48 SOS + 64.2 YE - 0.3073 SOS*SOS – 3.65 YE*YE + 0.326 SOS*YE (3)

Based on ANOVA analysis, both the factor (amount of yeast extract and sugars) showed significant impact on biomass productivity of *A. mangrovei*. In figure 3 is reported the three dimensional plot of response surface results, that allows to visualize the interactions between factors on biomass and DHA productivity.

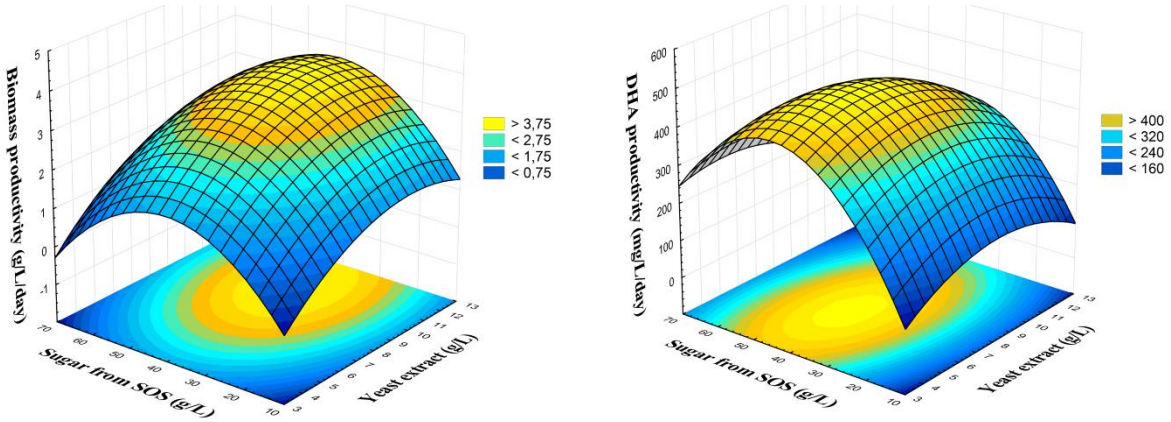

Figure 3. Surface plot for biomass productivity (left) and DHA productivity (right) using central composite design.

As expected, when the factors are at minimum level (14 g L^{-1} of sugar from SOS and 4 g L^{-1} of YE) the biomass productivity registered was minimum.

The stationary point for this model was reached at 41.41 g L^{-1} of sugars from SOS and 10.03 g L^{-1} of yeast extract, which are the best condition to maximize biomass productivity (3.6 g L-1 day^{-1}) for *A. mangrovei* in these conditions. The results showed that exceeding a concentration of 60 g L^{-1} of sugars from SOS lead to an inhibition of the growth (Figure 3). This could be explained by the presence of some inhibitory substance present (or osmotic stress) that affect negatively the biomass growth. This result is in line with those reported by other authors [9,27,28]. In particular Nazir et al., (2018) showed an inhibition of biomass growth and DHA production by *Aurantiochytrium* SW1 with a supplementation of fructose higher than 70 g L^{-1} [9]. Nevertheless, *Aurantiochytrium* BL10 has been reported to grow at concentrations of glucose higher than 120 g L^{-1} [29] and 150 g L^{-1} [30] proving that the capacity of substrate consumption differs among the strains of Thraustochytrids. The optimal concentration of YE instead is in line with other works that reported the best growth performances at 10 g L^{-1} for *A. mangrovei* [30,31].

For DHA productivity, regarding the factors, the sugar content resulted significant (p=0.03) while the YE was not significant (p>0.05), as for the interactions between the two factors. However, from 3D-surface plot (Figure 3) it can be observed that high concentration of YE lead to a lower DHA productivity, while a lower concentration lead to the higher productivity. However, N depletion (reduced YE content), as highly reported in literature, leads to an increase in lipid concentration but gave also high effect in biomass productivity production. For the sugar instead, medium concentrations seem to be optimal for DHA productivity. The DHA productivity obtained in these conditions ranged between 133 and 550 mg L-1 day^{-1}. In our experimental results the best C/N ratio was registered in run 12 (35 g L^{-1} sugar and 8 g L^{-1} YE) with a DHA productivity of 559 mg $L^{-1}day^{-1}$. The model showed that the maximum predicted DHA productivity (490.9 mg L^{-1} day^{-1}) is reached by means of 42.8 g L^{-1} of sugars and 6.05 g L^{-1} of YE. Park et al., (2018) obtained a similar DHA productivity ranging from 0.5 to 0.78 g $L^{-1}day^{-1}$ using orange peel extract as nutrient source. The authors increased the lipid productivity with an addiction of glucose in new formulated media [33]. Liang et al., (2010) also obtained 9.4 g L^{-1} of *Schizochytrium limacinum* using sweet sorghum juice media, with a DHA productivity of 470 mg $L^{-1}day^{-1}$[34].

Finally, combining our results of biomass and DHA optimization using a relative regression equation, the best formulation to increase both lipid and biomass productivity resulted in 38.3

g L^{-1} of sugars and 8.7 g L^{-1} of YE. With this formulation is possible to obtain a predicted biomass productivity of 3.77 g L^{-1} day^{-1} and a DHA productivity of 475 mg L^{-1} day^{-1}.

Scale up trial

In order to assess the applicability and reproducibility of optimized media, an up-scaling experiment was prepared in 5 L airlift bioreactor with 3.5 L of working volume using the SOS nutrient recipe with the highest predicted value in terms of both biomass and DHA productivity.

In figure 4 are reported the growth curves and sugar consumption during cultiva-tion using optimized SOS recipe and a control using glucose at the same organic carbon and YE concentrations.

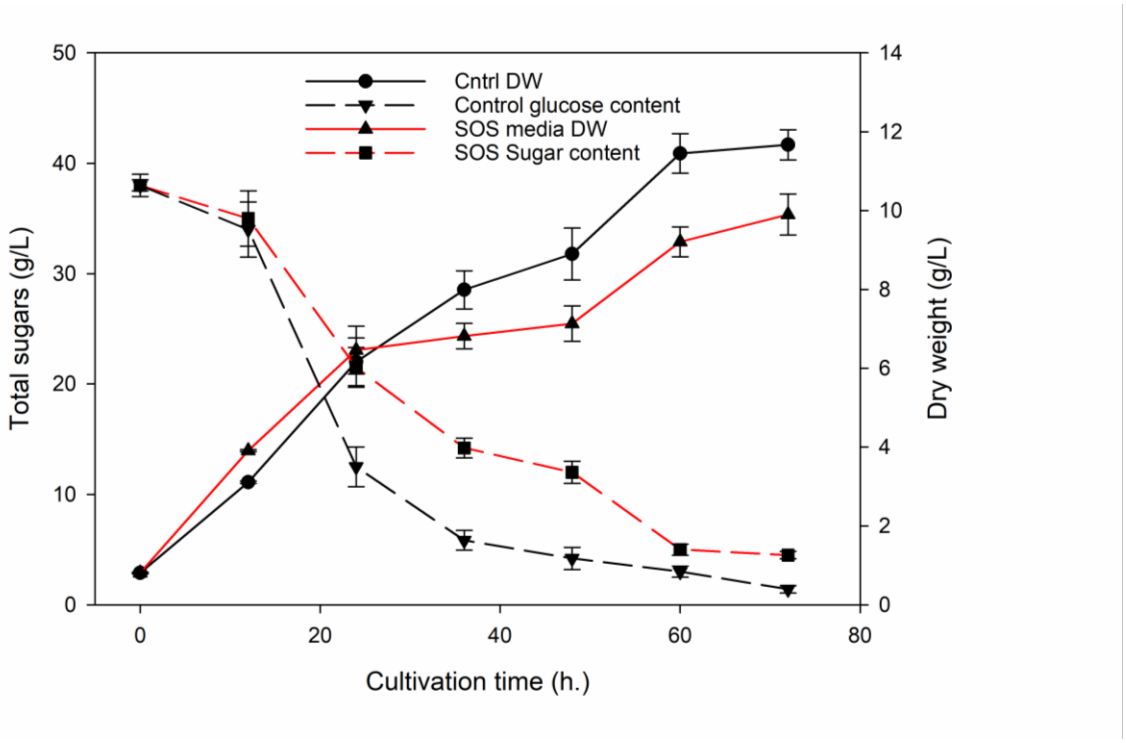

Figure 4. Growth curves and sugar consumption over time for A. *mangrovei* using SOS optimized media compared to their relatives control at the same C/N ratio using glucose. Values are reported as mean ± SD.

The biomass productivity obtained after 72 h was 3.91 ± 0.49 g $L^{-1}day^{-1}$ for the control, while for SOS media recipe 3.30 ± 0.24 g $L^{-1}day^{-1}$. Both the values fit with the predicted growth model developed into the previous experiments.

Sugar consumption followed the biomass increase with values after 72 h of cultivation lower in pure glucose (2.1 g L^{-1}) than in SOS recipe (4.6 g L^{-1}).

To better understand the difference between the standard media and the new formulated media, the fatty acids profile were analyzed and reported in figure 5.

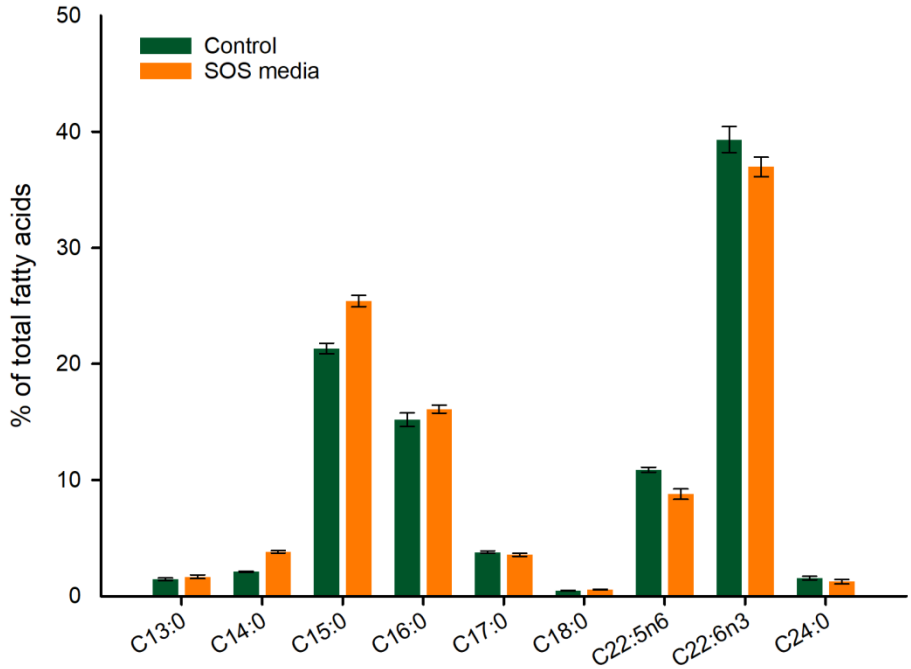

Figure 5. Fatty acid profile (expressed as % of total fatty acids) of new SOS media compared with control using glucose as organic carbon source. Values are expressed as mean (n=3) ± SD.

No relevant difference can be observed in terms of lipid profile between the control and the new media. The only significant difference (p=0.002) can be observed for the C15:0 that resulted in SOS media higher than in the control. Slightly differences in DHA and DPA can be observed in the control respect to the new media resulted not significantly different. This result is in contrast with another study [30] which obtained a biomass lower than 30% when cultivating *Aurantiochytrium* with only sucrose and 50% lower DHA (respect to the control with glucose). However, 36.9% of DHA was obtained in SOS media, proving the possibility to use the food by-product as a cheap organic carbon source for the production of LC-PUFA.

4.4 Conclusion

Spent osmotic solution from candied fruit industry can be employed as efficient organic carbon source for an economical and sustainable DHA production by *A. mangrovei* RCC893. RSM-CCD gave as best predicted nutrient recipe to maximize both biomass and DHA production 41.4 g L^{-1} of sugars from SOS (corresponding to 50 g L^{-1} of SOS) and 8.7 g L^{-1} of YE. The scale up trial using the optimized condition resulted in a biomass productivity of 3.7 g L^{-1} day^{-1} and a DHA productivity of 475 mg $L^{-1}day^{-1}$. This alternative media can reduce the production cost of omega-3 oil from algae, with an additional key advantage of recycling a food industry waste, contributing to a sustainable circular economy development.

References

1. Birch, E.E.; Garfield, S.; Castañeda, Y.; Hughbanks-Wheaton, D.; Uauy, R.; Hoffman, D. Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized trial of long-chain polyunsaturated fatty acid-supplemented infant formula. Early Hum. Dev. 2007, doi:10.1016/j.earlhumdev.2006.11.003.

2. Zeng, Y.; Ji, X.J.; Lian, M.; Ren, L.J.; Jin, L.J.; Ouyang, P.K.; Huang, H. Development of a temperature shift strategy for efficient docosahexaenoic acid production by a marine fungoid protist, Schizochytrium sp. HX-308. Appl. Biochem. Biotechnol. 2011, doi:10.1007/s12010-010-9131-9.

3. Chen, W.; Zhou, P.; Zhu, Y.; Xie, C.; Ma, L.; Wang, X.; Bao, Z.; Yu, L. Improvement in the docosahexaenoic acid production of Schizochytrium sp. S056 by replacement of sea salt. Bioprocess Biosyst. Eng. 2016, doi:10.1007/s00449-015-1517-1.

4. Graham, I.A.; Larson, T.; Napier, J.A. Rational metabolic engineering of transgenic plants for biosynthesis of omega-3 polyunsaturates. Curr. Opin. Biotechnol. 2007.

5. Nakahara, T.; Yokochi, T.; Higashihara, T.; Tanaka, S.; Yaguchi, T.; Honda, D. Production of docosahexaenoic and docosapentaenoic acids by Schizochytrium sp. isolated from yap islands. JAOCS, J. Am. Oil Chem. Soc. 1996, doi:10.1007/BF02523506.

6. Lewis, T.E.; Nichols, P.D.; McMeekin, T.A. The biotechnological potential of thraustochytrids. Mar. Biotechnol. 1999, doi:10.1007/PL00011813.

7. Gao, M.; Song, X.; Feng, Y.; Li, W.; Cui, Q. Isolation and characterization of Aurantiochytrium species: High docosahexaenoic acid (DHA) production by the newly isolated microalga, Aurantiochytrium sp. SD116. J. Oleo Sci. 2013, doi:10.5650/jos.62.143.

8. Barclay, W.; Weaver, C.; Metz, J.; Hansen, J. Development of a Docosahexaenoic Acid Production Technology Using Schizochytrium: Historical Perspective and Update. In Single Cell Oils: Microbial and Algal Oils: Second Edition; 2010 ISBN 9781630670078.

9. Nazir, Y.; Shuib, S.; Kalil, M.S.; Song, Y.; Hamid, A.A. Optimization of Culture Conditions for Enhanced Growth, Lipid and Docosahexaenoic Acid (DHA) Production of Aurantiochytrium SW1 by Response Surface Methodology. Sci. Rep. 2018, 8, 8909, doi:10.1038/s41598-018-27309-0.

10. Humhal, T.; Kastanek, P.; Jezkova, Z.; Cadkova, A.; Kohoutkova, J.; Branyik, T. Use of saline waste water from demineralization of cheese whey for cultivation of Schizochytrium limacinum PA-968 and Japonochytrium marinum AN-4. Bioprocess Biosyst. Eng. 2017, 40, 395–402, doi:10.1007/s00449-016-1707-5.

11. Russo, G.L.; Langellotti, A.L.; Oliviero, M.; Sacchi, R.; Masi, P. Sustainable production of food grade omega-3 oil using aquatic protists: Reliability and future horizons. N. Biotechnol. 2021, 62, 32–39, doi:10.1016/j.nbt.2021.01.006.

12. Aachary, A.A.; Prapulla, S.G. Value addition to spent osmotic sugar solution (SOS) by enzymatic conversion to fructooligosaccharides (FOS), a low calorie prebiotic. Innov. Food Sci. Emerg. Technol. 2009, doi:10.1016/j.ifset.2008.11.013.

13. Massa, M.; Buono, S.; Langellotti, A.L.; Martello, A.; Russo, G.L.; Troise, D.A.; Sacchi, R.; Vitaglione, P.; Fogliano, V. Biochemical composition and in vitro digestibility of Galdieria sulphuraria grown on spent cherry-brine liquid. N. Biotechnol. 2019, 53, 9–15, doi:10.1016/j.nbt.2019.06.003.

14. Ryu, B.-G.; Kim, K.; Kim, J.; Han, J.-I.; Yang, J.-W. Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, Aurantiochytrium sp. KRS101. Bioresour. Technol. 2013, 129, 351–359, doi:10.1016/j.biortech.2012.11.049.

15. Yu, X.-J.; Yu, Z.-Q.; Liu, Y.-L.; Sun, J.; Zheng, J.-Y.; Wang, Z. Utilization of High-Fructose Corn Syrup for Biomass Production Containing High Levels of Docosahexaenoic Acid by a Newly Isolated Aurantiochytrium sp. YLH70. Appl. Biochem. Biotechnol. 2015, 177, 1229–1240, doi:10.1007/s12010-015-1809-6.

16. Lee Chang, K.J.; Dumsday, G.; Nichols, P.D.; Dunstan, G.A.; Blackburn, S.I.; Koutoulis, A. High cell density cultivation of a novel Aurantiochytrium sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils. Appl. Microbiol. Biotechnol. 2013, doi:10.1007/s00253-013-4965-z.

17. DuBois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 1956, 28, 350–356, doi:10.1021/ac60111a017.

18. Bligh, E.G.; Dyer, W.J. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Can. J. Biochem. Physiol. 1959, 37, 911–917, doi:10.1139/o59-099.

19. Morrison, W.R.; Smith, L.M. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. J. Lipid Res. 1964, doi:10.1016/s0022-2275(20)40190-7.

20. Nakazawa, A.; Matsuura, H.; Kose, R.; Kato, S.; Honda, D.; Inouye, I.; Kaya, K.; Watanabe, M.M. Optimization of culture conditions of the thraustochytrid Aurantiochytrium sp. strain 18W-13a for squalene production. Bioresour. Technol. 2012, doi:10.1016/j.biortech.2011.09.127.

21. Taoka, Y.; Nagano, N.; Okita, Y.; Izumida, H.; Sugimoto, S.; Hayashi, M. Influences of culture temperature on the growth, lipid content and fatty acid composition of aurantiochytrium sp. strain mh0186. Mar. Biotechnol. 2009, doi:10.1007/s10126-008-9151-4.

22. Mariam, I.; Kareya, M.S.; Nesamma, A.A.; Jutur, P.P. Delineating metabolomic changes in native isolate Aurantiochytrium for production of docosahexaenoic acid in presence of varying carbon substrates. Algal Res. 2021, doi:10.1016/j.algal.2021.102285.

23. Pahlavanyali, M.; Jalili, H.; Noroozi, M.; Moradi, Y.; Hallajisani, A. The effect of temperature and different carbon and nitrogen sources on the growth and fatty acid profile of a newly isolated microorganism Aurantiochytrium sp . strain SHY. 2020, 19, 3112–3126, doi:10.22092/ijfs.2020.122942.

24. Hong, W.K.; Rairakhwada, D.; Seo, P.S.; Park, S.Y.; Hur, B.K.; Kim, C.H.; Seo, J.W. Production of lipids containing high levels of docosahexaenoic acid by a newly isolated microalga, Aurantiochytrium sp. KRS101. Appl. Biochem. Biotechnol. 2011, doi:10.1007/s12010-011-9227-x.

25. Yin, F.-W.; Zhu, S.-Y.; Guo, D.-S.; Ren, L.-J.; Ji, X.-J.; Huang, H.; Gao, Z. Development of a strategy for the production of docosahexaenoic acid by Schizochytrium sp. from cane molasses and algae-residue. Bioresour. Technol. 2019, 271, 118–124, doi:10.1016/j.biortech.2018.09.114.

26. Iwasaka, H.; Aki, T.; Adachi, H.; Watanabe, K.; Kawamoto, S.; Ono, K. Utilization of waste syrup for production of polyunsaturated fatty acids and xanthophylls by Aurantiochytrium. J. Oleo Sci. 2013, doi:10.5650/jos.62.729.

27. Wong, M.K.M.; Tsui, C.K.M.; Au, D.W.T.; Vrijmoed, L.L.P. Docosahexaenoic acid production and ultrastructure of the thraustochytrid Aurantiochytrium mangrovei MP2 under high glucose concentrations. Mycoscience 2008, doi:10.1007/s10267-008-0415-7.

28. Yokochi, T.; Honda, D.; Higashihara, T.; Nakahara, T. Optimization of docosahexaenoic acid production by Schizochytrium limacinum SR21. Appl. Microbiol. Biotechnol. 1998, doi:10.1007/s002530051139.

29. Chaung, K.C.; Chu, C.Y.; Su, Y.M.; Chen, Y.M. Effect of culture conditions on growth, lipid content, and fatty acid composition of aurantiochytrium mangrovei strain BL10. AMB Express 2012, doi:10.1186/2191-0855-2-42.

30. Yu, X.-J.; Yu, Z.-Q.; Liu, Y.-L.; Sun, J.; Zheng, J.-Y.; Wang, Z. Utilization of High-Fructose Corn Syrup for Biomass Production Containing High Levels of Docosahexaenoic Acid by a Newly Isolated Aurantiochytrium sp. YLH70. Appl. Biochem. Biotechnol. 2015, 177, 1229–1240, doi:10.1007/s12010-015-1809-6.

31. Ju, J.H.; Oh, B.R.; Ryu, S.K.; Heo, S.Y.; Kim, S.Y.; Hong, W.K.; Kim, C.H.; Seo, J.W. Production of Lipid Containing High Levels of Docosahexaenoic Acid by Cultivation of Aurantiochytrium sp. KRS101 Using Jerusalem Artichoke Extract. Biotechnol. Bioprocess Eng. 2018, doi:10.1007/s12257-018-0419-x.

32. Park, W.K.; Moon, M.; Shin, S.E.; Cho, J.M.; Suh, W.I.; Chang, Y.K.; Lee, B. Economical DHA (Docosahexaenoic acid) production from Aurantiochytrium sp. KRS101 using orange peel extract and low cost nitrogen sources. Algal Res. 2018, doi:10.1016/j.algal.2017.11.017.

33. Park, H.; Kwak, M.; Seo, J.; Ju, J.; Heo, S.; Park, S.; Hong, W. Enhanced production of carotenoids using a Thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. Bioprocess Biosyst. Eng. 2018, 41, 1355–1370, doi:10.1007/s00449-018-1963-7.

34. Liang, Y.; Sarkany, N.; Cui, Y.; Yesuf, J.; Trushenski, J.; Blackburn, J.W. Use of sweet sorghum juice for lipid production by Schizochytrium limacinum SR21. Bioresour. Technol. 2010, doi:10.1016/j.biortech.2009.12.087.

Chapter 5

Formulation of New Media From Dairy and Brewery Wastes For a Sustainable Production of DHA-rich oil By *Aurantiochytrium mangrovei*

This chapter has been published as:

Russo, Giovanni L., Antonio L. Langellotti, Vito Verardo, Beatriz Martín-García, Prospero Di Pierro, Angela Sorrentino, Marco Baselice, Maria Oliviero, Raffaele Sacchi, and Paolo Masi. 2022. "Formulation of New Media from Dairy and Brewery Wastes for a Sustainable Production of DHA-Rich Oil by *Aurantiochytrium mangrovei*" *Marine Drugs* 20, no. 1: 39. https://doi.org/10.3390/md20010039

Abstract: Mozzarella stretching water (MSW) is a dairy effluent generated from mozzarella cheese production that does not have a real use and is destined to disposal, causing environmental problems and representing a high disposal cost for dairy producers. Spent brewery yeast (SBY) is another promising food waste produced after brewery manufacturing that could be recycled in new biotechnological processes. *Aurantiochytrium mangrovei* is an aquatic protist known as producer of bioactive lipids such as omega 3 long chain polyunsaturated fatty acids (ω3 LC-PUFA), in particular docosahexaenoic acid (DHA). In this work MSW and SBY have been used to formulate new sustainable growth media for *A. mangrovei* cultivation and production of DHA in an attempt to valorize these effluents. MSW required an enzymatic hydrolysis to enhance the biomass production. The new media obtained from hydrolysed MSW was also optimized using response surface methodologies, obtaining 10.14 g L^{-1} of biomass in optimized medium, with a DHA content of 1.21 g L^{-1}.

5.1 Introduction

Long chain ω-3 polyunsaturated fatty acids (LC-PUFA) have a series of beneficial effects on human health [1]. Among them, docosahexaenoic acid (DHA, C22:6n-3) is an important fatty acid, as it is one of the major components of the central nervous system [2]. Moreover, dietary DHA supplementation has been shown to be important in the prevention of cardiovascular diseases, schizophrenia, and specific types of cancer [3]. Actually, the principal source of DHA is fish oil, but it has several disadvantages, especially the low sustainability, the contamination by marine pollutants, fish allergy issues and an undesirable fishy smell [4,5]. Thraustochytrids, a heterotrophic fungus-like clade of Stramenopiles, represent a potential alternative to fish oil due to their high biomass and DHA productivity, which is much higher than the fish source [6]. Among the thraustochytrids, *Aurantiochytrium* (known as *Schizochytrium* until 2007) is a genus industrially exploited for the production of DHA [7]. *Aurantiochytrium* can produce high amounts of lipids (up to 55% of dry weight) and most of that is DHA (up to 35% of total fatty acids) [7]. For the industrial production of DHA, the price of growth medium represents a significant portion of the production costs, as well as the costs for preparing artificial sea water [8]. For that reason, new biotechnological processes based on the recycling of low cost side-streams from food industries would be an interesting way to produce omega-3 oil with lower production costs. The utilization of aquatic protists for treatment of food waste is gaining attention from the scientific community, thanks to their great metabolic flexibility and bioremediation capacities [9]. *Aurantiochytrium sp*. has been

tested on different types of food waste, showing a high metabolic versatility to utilizing different type of organic and nitrogen sources [10,11].

The dairy industry is one of the main food industries in Italy and Europe, with tons of cheese produced every year. In Italy the main dairy product is the mozzarella cheese, with a production of more than 250,000 tons every year [12]. During the mozzarella manufacturing process, two main side-streams are generated: the cheese whey (CW) and the mozzarella stretching water (MSW). MSW is the effluent generated after the stretching step. It is treated as an effluent by the dairy companies because of its high salinity and high chemical oxygen demand (COD), and therefore it represents a serious environmental issue [13]. Nevertheless, dairy wastewaters are liquids rich in interesting compounds such as lactose (up to 5% *w/v*), proteins (up to 1% *w/v*), and other minor components (mineral salts, lactic acid and vitamins). Dairy by-products could be useful for the formulation of more sustainable microbial media [14], in particular for heterotrophic microorganisms that require high amounts of organic carbon and other nutrients for their growth.

Another promising food waste for biotechnological applications is the spent brewery yeast (SBY). It is an organic waste from brew manufacturing with a high content of proteins, free amino nitrogen (FAN), phosphates and other essential mineral salts [11]. These characteristics make this food waste very promising for microalgal cultivation [15]. In fact, SBY could be used in the formulation of a new medium obtained mainly from food wastes.

To the best of our knowledge, *Aurantiochytrium* cultivation was never tried on dairy wastewater. We found only one paper about testing the saline wastewater from demineralization of CW for the cultivation of *Schizochytrium limacinum* PA-968 [8].

Therefore, the purpose of this study was to investigate the potential of a dairy wastewater (MSW) in combination with SBY as a new sustainable growth medium for *Aurantiochytrium mangrovei* cultivation. The chemical characteristics of MSW have been defined, and the new media optimized through response surface analysis with supplementation of nitrogen from brewery waste. The biochemical composition and lipid content of the obtained biomass have also been determined.

5.2 Results and Discussion

Characterization of MSW

The chemical and physical characterizations of reverse osmosis concentrated MSW (3:1) are reported in Table 1.

Table 1. Chemical and physical characterization of mozzarella stretching water.

Parameters	Value
pH	3.55 ± 0.2
Ash (g L^{-1})	26.2 ± 0.9
Dry weight (g L^{-1})	68.5 ± 1.1
N total (g L^{-1})	0.91 ± 0.09
Protein content (g L^{-1})	3.6 ± 0.2
Lactic acid (g L^{-1})	6.08 ± 0.79
Citric acid (g L^{-1})	1.03 ± 0.19
Free amino nitrogen (mg L^{-1})	0.174 ± 0.03
Reducing sugars (g L^{-1})	23.26 ± 0.4
Lactose (g L^{-1})	22.48 ± 0.7
Total sugars (g L^{-1})	24.12 ± 0.6
COD (mg L^{-1})	33506 ± 21.1
Cl^- (g L^{-1})	14.61 ± 1
Ca^{2+} (g L^{-1})	0.69 ± 0.05
Total P (mg L^{-1})	87.3 ± 1.25
Na^{2+} (g L^{-1})	7.66 ± 0.8
Mg^{2+} (mg L^{-1})	95.76 ± 3.7

Values expressed as mean (n=3) ± SD.

The chemical composition of MSW is not well defined in scientific literature. This wastewater is interesting for the amount of reducing sugars (up to 23 g L^{-1}), and also for a residual content of proteins (3.6 g L^{-1}). The content of free amino nitrogen (FAN) is also relevant (0.174 mg L^{-1}), as it is easily metabolized by aquatic protists, boosting their growth [16].

Moreover, the high amount of ash and chlorides make this waste a candidate for fermentation by marine microorganisms (i.e., *Aurantiochytrium mangrovei*). In fact, a high saline content is a common characteristic for this type of dairy effluent. The phosphorus (P) content reported was 87 mg L^{-1}, which is a good amount for protist cultivation because it is an essential macronutrient for energy transfer and synthesis of phospholipids and nucleic acids [17]. Considering that the P content of standard YEP medium is estimated to be 70–90 mg L^{-1}, the MSW alone can satisfy the P demand of *A. mangrovei*. Magnesium and calcium reported were high if compared to standard medium. However, this amount of Mg^{2+} could positively

affect the cultivation, because magnesium ions act as a cofactor for malic enzyme, which converts malic acid to pyruvate during the transdehydrogenase cycle [18].

MSW showed a low pH value (3.55), probably due to the presence of citric and lactic acid. The latter is produced after natural microbial fermentations of the stretching water by lactic acid bacteria (LAB) or other species of the present microbiota [19]. The presence of organic acids could be interesting for heterotrophic or mixotrophic cultivation of microorganisms. These factors contribute to the high organic load that characterizes the dairy effluents; in fact, the COD found was more than 33 g L^{-1}, which is economically critical for producers that need to dispose of this type of wastewater.

Comparing these results with other dairy wastewater characterizations, we found that MSW has a lower pH value (3.5 vs 4.7–5.1), a higher content of total nitrogen (TN) (900 mg/L vs 140–800 mg/L) and a lower amount of reducing sugars (23.1 g/L vs 30–47 g/L) [20–22].

Nevertheless, the characteristics of MSW showed a promising nutrient composition for fermentations by marine protists. However, the amount of nitrogen and organic carbon are lower with respect to the standard YEP medium used for *A. mangrovei* cultivation.

Screening Tests Results

Evaluation of Organic Carbon Sources

The performance of *A. mangrovei* growth with different organic carbon sources is reported in Figure 1.

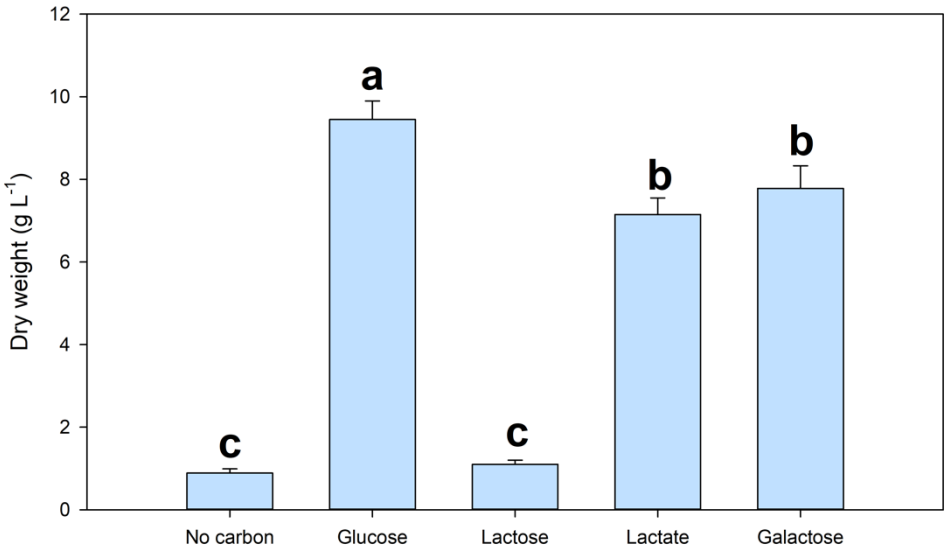

Figure 1. Screening test for different type of organic carbon source for *A. mangrovei* cultivation. The basal medium used for this test is yeast extract-peptone medium (YEP). Different letters mean a significant difference ($p < 0.05$) with $n = 3$.

The protist showed a significant growth when using glucose, lactate and galactose as a source of organic carbon. This result is in line with other studies with the *Aurantiochytrium* species [10,23,24]. However, we found a significant difference ($p < 0.05$) between standard glucose, lactic acid and galactose. For lactose instead, no growth of *A. mangrovei* was observed after 72 h, proving the impossibility to metabolize this disaccharide. In fact, in another work where *Aurantiochytrium sp*. was tested on a media supplemented with demineralized CW, the thraustochytrid showed significant growth only when supplemented with glycerol [8]. Lactate has been tested as it is produced in the dairy wastewater following fermentations by the microbiota. This organic acid can affect positively the fermentation by *A. mangrovei*. A previous study evaluated the growth of *Schizochytrium* sp. using lactic acid instead of glucose [25], and the authors reported that the biomass growth with lactic acid medium was lower than the glucose medium. These findings are in line with our work. For galactose also, our results are in line with another work conducted with *Schizochytrium mangrovei* Sk-02 [24]. The authors reported the capability to metabolize this monosaccharide, but with less efficiency than glucose.

Effect of MSW Hydrolysed and SBY on the Growth of A. mangrovei

Since lactose is not metabolized by *A. mangrovei* (Figure 1) a hydrolysis of MSW was performed in order to increase the bioavailability of the nutrients present. The results of the first screening to evaluate the hydrolysis effect of MSW on *A. mangrovei* growth are reported in Figure 2(A).

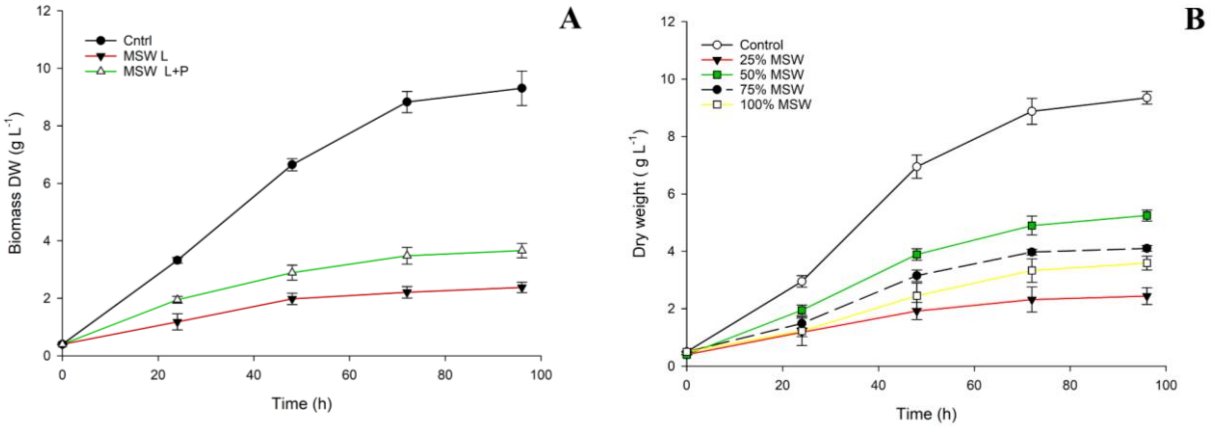

Figure 2. Evaluation of growth performance of *A. mangrovei* with MSW medium with only lactose hydrolysis (MSW L), with sequential lactose-protein hydrolysis (MSW L+P) (**A**) and screening of

MSW hydrolyzed diluted at four different concentrations (25–100% *v/v*) (**B**). All the tests were conducted in triplicate (n = 3). The controls refer to YEP standard medium.

When using a medium in which only lactose was hydrolysed (MSW L), a lower biomass was obtained with respect to the samples' growth with a medium subjected to sequential hydrolysis of proteins and lactose (MSW L+P). In fact, without the complete protein hydrolysis, a lower biomass growth can be observed in terms of dry cell weight (DW) (up to 2.2 g L^{-1}) respect to the 3.63 g L^{-1} obtained on MSW L+P. After the sequential hydrolysis of MSW, the FAN content increased from 0.17 mg L^{-1} to 0.8 mg L^{-1}, and this can explain the growth boost observed.

In fact, small peptides and FAN produced after proteolysis are used more efficiently by aquatic protists, enhancing their growth [9]. Also, the hydrolysis of lactose leads to glucose and galactose formation that can be easily metabolized by *A. mangrovei* (Figure 1a).

In the work of Pleissner et al. [16], a "fungal" hydrolysis with *A. awamori* and *A. oryzae* was used to enhance the nutrient availability of food waste. The authors tested the growth of *A. mangrovei* (called *Schizochytrium mangrovei*), reporting a higher biomass productivity respect to the control, thanks to FAN and glucose released after fungal pre-treatment.

However, the overall growth observed in our screening was very poor with respect to the standard medium. For that reason, we evaluated also the concentration effect of hydrolyzed MSW (lactose and protein hydrolysis) on the biomass production. In Figure 2B are reported the growth curves at different concentrations of MSW.

As we expected, when using this dairy effluent integrally without dilution (at 100% *v/v* of concentration), the growth performance was significantly lower than the control and 50% sample, obtaining only 2.1 g L^{-1} of DW after 96 h of cultivation. At 50% dilution instead, the growth was higher than the 100% and 75% samples, leading to 5.02 g L^{-1} of DW. This could be explained by a saline stress of the salts present in MSW or by other inhibitory substances present [26]. In fact, other authors reported a lower productivity when cultivating thraustochytrids at high salinity content [27,28]. Therefore, all the subsequent cultivation trials have been performed with hydrolyzed MSW diluted at 50% of concentration.

In our work, the ratio of the hydrolysis was the same, but the nutrients present in MSW were not sufficient to obtain a growth similar to the standard media (YEP medium). In fact, the nitrogen content of the standard medium was 0.95 g L^{-1} while the MSW diluted at 50%

contains 0.46 g L^{-1} of N. For that reason, we tested a waste from the brewery industry (SBY) as an alternative nitrogen source that could satisfy the nutritional demand of the microalga. The SBY screening test results are reported in Figure 3**.**

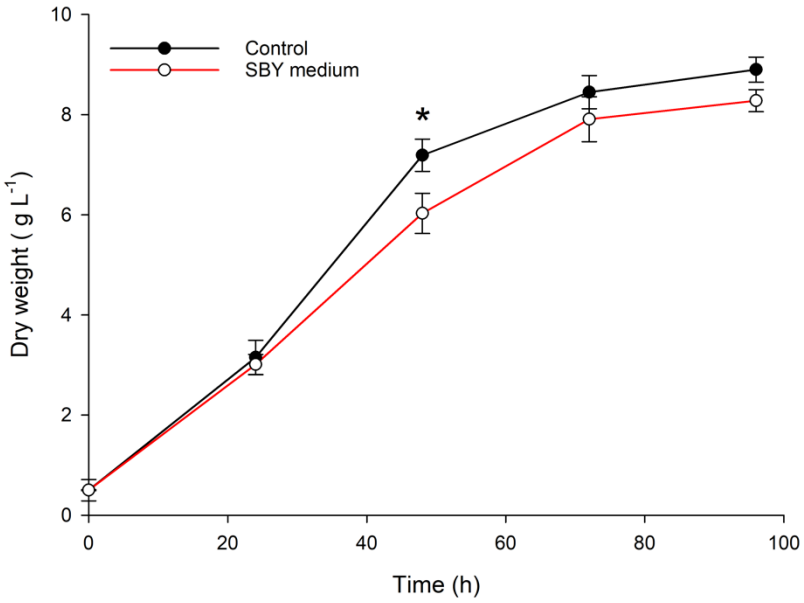

Figure 3. Growth curves of *A. mangrovei* cultivated with spent brewery yeast as the sole nitrogen source compared to standard medium. (*) means a significant difference ($p < 0.05$) with $n = 3$.

Substituting the nitrogen of standard media (from yeast extract and peptone) with nitrogen from SBY, no significant difference after 72 h and 96 h of cultivation was observed. A significant difference ($p = 0.04$) can be observed only at 48 h of cultivation, likely due to a slower uptake of nitrogen from SBY with respect to the standard YE. With this result, SBY could be used to compensate the lack of nitrogen of MSW medium.

This growth performance is in line with a previous work which obtained a biomass productivity (g L^{-1} day^{-1}) of 3.52 ± 1.06 by *Aurantiochytrium* sp. KRS101 when using SBY extracted after autolysis with distilled water as the only nitrogen source [11].

Optimization of New MSW Hydrolysed Media

In order to increase the biomass productivity, MSW medium required a nutrient supplementation. CCD was used to examine the optimal supplementation of organic carbon (glucose) and nitrogen (SBY) in the new MSW media. The response surface design employed gave 13 combinations of selected nutrients (glucose and SBY) with three levels (−1, 0, +1).

Table 2 reports the design and the results with the responses. Biomass concentration (expressed as g L^{-1} of DW) was used as response, and was calculated at log phase (72 h).

Table 2. Experiment design and results of biomass growth optimization with supplementation of glucose and SBY by central composite design.

Run	Factor Assignment		Biomass Dry Weight (Y)	
	X_1 (glucose)	X_2 (SBY)	Experimental Value (g L^{-1})	Predicted Value (g L^{-1})
1	−1	0	7.28	7.75
2	+1	0	10.24	10.06
3	+1	+1	9.79	9.99
4	0	+1	9.87	9.73
5	0	0	9.81	9.77
6	0	0	9.72	9.77
7	+1	−1	7.13	7.09
8	−1	+1	7.85	7.73
9	0	0	9.97	9.77
10	0	−1	6.38	6.78
11	0	0	9.57	9.77
12	0	0	10.14	9.77
13	−1	−1	5.10	4.73

Coded values: X_1 = glucose (g/L); X_2 = SBY (g/L); the three levels (−1, 0 and +1) set for glucose were 0, 15 and 30 g L^{-1}, while for SBY was 0, 2.5 and 5 g L^{-1} respectively.

The significance of the model and its second-order Equation (2), derived from the multiple regression analysis of the data, was tested by analysis of variance (ANOVA) (Table 3) and a *p*-value lower than 0.05 was considered significant in the analysis.

Table 3. Analysis of variance for biomass production using coded values and regression equation.

Source	DF[a]	Adj SS[b]	Adj MS[c]	F-Value	*p*-Value
Model	5	16.45	3.29	23.35	0.003
Glucose (X_1)	1	4.59	4.59	32.60	0.005
SBY (X_2)	1	1.27	1.27	9.07	0.011
Linear	2	5.87	2.93	20.84	0.001
Square	2	10.52	5.26	37.35	0.000
X_1* X_1	1	3.22	3.22	22.91	0.004
X_2*X_2	1	3.28	3.28	23.34	0.002
X_1*X_2	1	0.05	0.05	0.36	0.868
Error	7	0.98	0.14		

Lack of Fit	3	0.87	0.29	9.19	0.129
Pure Error	4	0.11	0.02		
Total	12	17.4376			

R^2 = 97.68 ([a] DF, degree of freedom; [b] SS, sum of squares; [c] MS, mean squares; F, probability of distribution; *p*, probability)

The model fit is also expressed with coefficient of determination (R^2), which was 0.9768, indicating that 97.68% of the variability in the Y (response) could be explained by the model. The *p*-value of the model was ($p < 0.005$), which implied that the model was significant; furthermore, the lack of fit is non-significant ($p > 0.05$), proving the validity of the model. Moreover, the predicted value observed from the model was not significantly different from the experimental value. The regression equation obtained from the model has been shown in Equation (1):

$$Biomass\ dry\ weight\ (gL^{-1}) = 4.736 + 0.194\ Glu + 1.816\ SBY - 0.00384\ Glu * Glu - 0.2433\ SBY * SBY + 0.00067\ Glu * SBY$$

Based on ANOVA analysis, both factors showed significant impact on the growth of *A. mangrovei*. The most significant factor was glucose ($p = 0.003$) followed by SBY ($p = 0.011$).

In the run n. 13, without the addiction of glucose or SBY, the biomass obtained was 5.1 g L^{-1}, while the highest DW value was obtained in run 2 (10.24 g L^{-1}) with a combination of 2.5 g L^{-1} of SBY and 30 g L^{-1} of glucose. With supplementation of SBY and organic carbon, the biomass productivity was doubled. To better understand the RSM results, a 3-D surface plot and a contour plot were elaborated and reported in Figure 4.

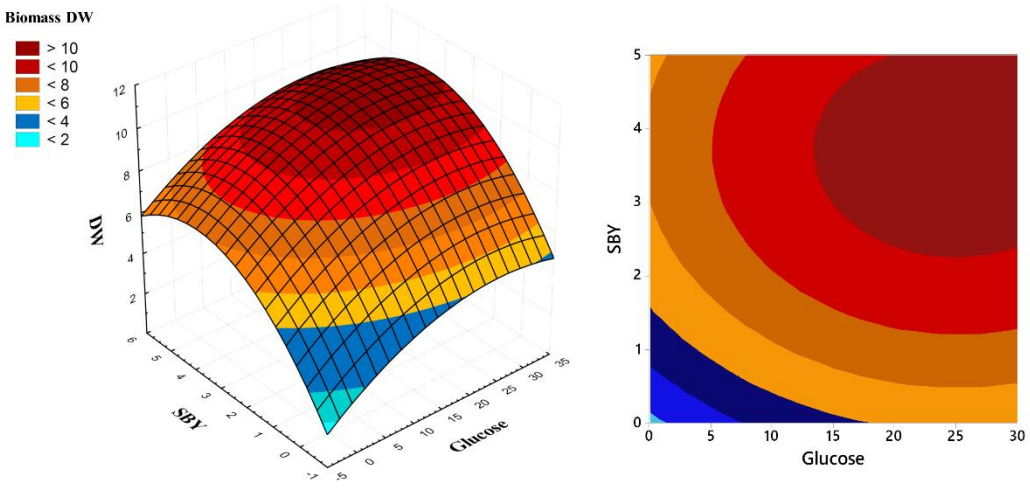

Figure 4. Surface (left) and contour plot (right) of RSM-CCD elaborated for *A. mangrovei* cultivated in MSW medium supplemented with spent brewery yeast and glucose.

From the figure it is possible to observe that a glucose supplementation higher than 15 g L^{-1} and 3.26 g L^{-1} of SBY are useless in terms of biomass productivity. In fact, the biomass DW seems to be stable at 10 g L^{-1} after these values. The optimal concentrations of factors extrapolated from regression equations are: 15.34 g L^{-1} of glucose and 3.22 g L^{-1} of SBY to supplement at MSW medium to obtain a biomass higher than 10 g L^{-1}.

For the optimal concentration of nitrogen, other works reported an optimal concentration of YE at 10–15 g L^{-1} for *Aurantiochytrium mangrovei* [29,30], while for this work it is 3.26 g L^{-1}, suggesting the utilization of hydrolyzed proteins and FAN present in MSW by the microalga as N source.

Model Confirmation and Characterization of Biomass Obtained with New MSW Optimized Medium

Once the optimal formulation of new MSW media was established, we confirmed the predicted biomass of the model cultivating *A. mangrovei* with a supplementation of 15.34 g L^{-1} of glucose and 3.22 g L^{-1} of SBY in MSW media. Moreover, we evaluated the lipid content and the nutrient consumption of the new medium. The results are reported in Table 4.

Table 4. Comparison of biomass dry weight, lipid content, FAN and sugar consumption of *A. mangrovei* between standard media and new MSW medium optimized through CCD.

Parameter	Control	MSW Optimized Media
Biomass DW (g L^{-1})	9.44 ± 0.12	10.07 ± 0.23
Biomass productivity (g L^{-1} day^{-1})	3.14 ± 0.06	3.35 ± 0.08
Total lipids (%DW)	41.1 ± 1.2	38.9 ± 0.88
FAN consumption (%)	80.06	87.24
Sugar consumption (%)	92.61	94.59

All values are expressed an mean (n = 3) ± SD

The biomass obtained was in line with the prediction of the CCD model. No significant differences were observed with the standard media in terms of biomass and lipid productivity. The FAN depletion of new MSW media was higher than the control, while the sugar consumption was very similar. This proves the optimal utilization of nutrients present in MSW media by *A. mangrovei*.

To better understand the differences between the samples, the fatty acids profile was analyzed and reported in Figure 5.

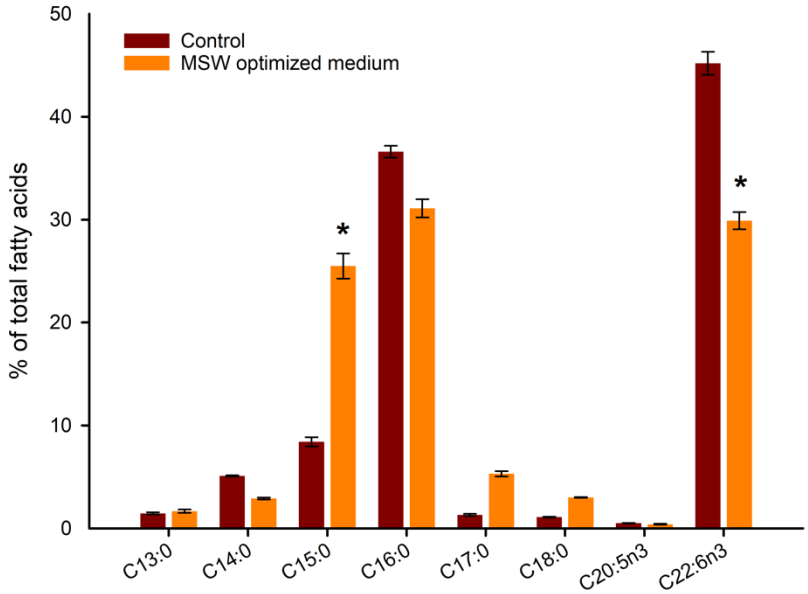

Figure 5. Fatty acids profile of extracted lipid from new MSW optimized media and standard media. (*) means a significant difference ($p < 0.05$) with the control.

Significant differences were observed in the fatty acids profile of MSW optimized media with the relative control in standard conditions. The DHA percentage of MSW medium was

significantly lower than the control (30.6% Vs 45% respectively). This difference could be explained by the nutrient difference between the control and the new medium with different C/N ratios. It has been reported that fatty acids yields decrease when the carbon source is completely depleted, forcing the cells to consume their own reserves of lipids [31]. In fact, in the work of Wang et al. [32], 70 g/L of glucose were added to tofu whey wastewater to provide an extra carbon source in order to enhance lipid accumulation of *Schizochytrium* sp. S31. Supplementation of extra glucose to a food waste medium is also reported in another study with *Aurantiochytrium* sp. KRS101. In that work, the lipids and biomass productivity were enhanced after glucose supplementation [33]. In our case, the focus was not the optimization of DHA yield but the development of a new sustainable medium for *A. mangrovei* cultivation using dairy and brewery waste. Nevertheless, the DHA percentage registered with MSW media was higher than that reported in the literature of *Aurantiochytrium* grown on food waste [32] and in line with another medium based on orange peel extract [33] and on food waste hydrolysate [34]. The content of pentadecanoic acid (C15:0) is also worthy of mention because the percentage observed in MSW medium is significantly higher than the control. In fact, 25.5% of C15 has been observed in the new medium. Odd-carbon fatty acids have been used for anaplerotic therapy for Alzheimer's disease, diabetes, cancer and cardiac disorder [35,36] and represents another high added value molecule. The concentration of C15 found in our study is higher than another work conducted on *Aurantiochytrium* sp. SA-96 [36], where the authors studied the influence of medium components on the production of C15. In fact, the authors found that adding soy milk to the culture medium increased the production of C:15. This could be a similar case as our study.

To further define the high added value compounds obtainable from *A. mangrovei* biomass, we evaluated the carotenoids from the biomass cultivated in standard condition, with MSW optimized medium and with MSW medium at higher luminosity (Table 5).

Table 5. Table of quantification of carotenoid in *A. mangrovei* by HPLC-MS. (Data are given as µg/g DW).

Sample	β-Carotene	Canthaxanthin	Astaxanthin	Violaxanthin
Control	0.34 ± 0.06^{a}	0.62 ± 0.05^{b}	Trace	n.d.
MSW media	2.93 ± 0.05^{b}	0.29 ± 0.04^{a}	Trace	n.d.
MSW media + light	1.85 ± 0.02^{c}	0.27 ± 0.01^{a}	0.28 ± 0.01	3.23 ± 0.08

Values are means ± SD (n=3); n.d.= not detected

The control showed a low amount of carotenoids when compared to the other samples. Thraustochytrids are known to synthesize different carotenoids including β-carotene, astaxanthin, zeaxanthin, cantaxanthin, phoenicoxanthin and echinenone [37]. In our case, the only carotenoids found in *A. mangrovei* cultivated with standard medium are β-carotene and canthaxanthin. For the biomass obtained with MSW media instead, we found a higher content of β-carotene. This difference could be explained by the different composition of the medium, and by the presence of a different saline content that could stress the microorganism, stimulating the pigment production. Moreover, we cultivated *A. mangrovei* in MSW medium with an exposure of high luminosity during cultivation with a light intensity of 200 μmol photons m^{-2} s^{-1} in an attempt to enhance the pigment productivity. Astaxanthin and violaxanthin were detected in that condition, increasing the high added value of the biomass obtained with food waste. In fact, biosynthetic production of carotenoids and pigment from aquatic protists are influenced by several factors, such as light exposition, saline stress and nutrient composition [38]. In scientific literature the carotenoid profile of *Aurantiochytrium mangrovei* is not well described, however the carotenoids found were lower with respect to *Aurantiochytrium sp*. SK4 [39], but in line with *Aurantiochytrium limacinum* ATCC MYA-1381 [40].

Economic Considerations

The utilization of an organic substrate and a nitrogen source are one of the main costs of heterotrophic cultivation. The utilization of pure chemicals such as glucose and yeast extract are not economically feasible for large scale productions of thraustochytrids [41]. Moreover, in order to compete with DHA from fish oil, many companies are focusing on the research of low cost nutrients to use for their fermentation processes. Therefore, the nutrient recovery from food waste and by-product is an important step to achieve. The most expensive carbon source is glucose. In 2010 its cost in the international market reached $500/ton [42], accounting for 23–34% of total production cost for heterotrophic cultivations [33]. However, the main issue for nutrient cost of heterotrophic protists is the nitrogen source, which is more expensive than organic carbon. In fact, the cost of YE in 2010 was assessed to be around 9.2 $/Kg (9,200 $/ton) [43].

A recent study reported that for production of DHA oil from *Schizochytrium sp*., it is possible to reduce the nutrient media cost of >70% using nitrogen from tofu wastewater [32]. In our case, the nitrogen source is completely replaced by the nutrients present in MSW and SBY, with only a small supplementation of glucose. Moreover, the glucose supplementation could be also replaced by other cheap organic carbon sources from food waste, as reported in our previous study [10].

With the process proposed in this study, the nutrient cost could be significantly cut. Also, the artificial seawater of the standard media has been completely replaced by MSW media, which results in a reduction in the cost of artificial seawater and mineral salts. Further studies are required to understand the economic benefit of using these food processing by-products in substitution of the standard medium, most of all a techno-economic assessment of the whole process.

5.3. Materials and Methods

Food Waste Samples and Chemical Characterization

MSW samples were gently provided by a mozzarella cheese factory (*Capurso Azienda Casearia* srl, Gioia del Colle, Bari, Italy) which concentrates the MSW using reverse osmosis in order to reduce the volume to be sent to wastewater treatment plant.

The concentrated MSW samples were taken from the accumulation tanks of the factory, aliquoted and immediately frozen at −20 °C to prevent any fermentation. Prior any analysis, the samples were filtered to remove big solid particulates that could interfere to the biomass growth. Pre-treatment of MSW consisted in a first neutralization from pH 3.5 to 7.0 using NaOH 5 M. After that, the samples were heated to 80 °C for 10 min and then centrifuged at 14,000 g for 7 min to remove the precipitate. [8] The supernatant was collected and sterilized at 121 °C for 15 min.

Spent brewery yeast (SBY) was obtained from an artisanal brewery factory. SBY was aliquoted and frozen at −20 °C. Hydrolysis of SBY was obtained by the standard autolysis method reported by Jacob et al. [44]. Autolysis in the distilled water reported was best in terms of cell growth and economic feasibility. After autolysis, the DW of SBY lysate was 47.5 g L^{-1} and TN was 4.51 g L^{-1}.

Organism and Cultivation

Aurantiochytrium mangrovei (RCC893) was obtained from the Roscoff algae collection (Roscoff, France). A stock culture of an axenic microalga strain was maintained routinely by regular sub-culturing at 2-week intervals on both liquid and agar slants of YEP Medium obtained from half-strength artificial seawater (17.5 g L^{-1} of sea salts) with 30 g L^{-1} of glucose, adjusted at pH 6.5. The nitrogen (N) source was peptone (2 g L^{-1}) and yeast extract (5 g L^{-1}). The algae were cultivated in the presence of light (light intensity, 50 μmol photons m^{-2} s^{-1}) at temperature of 25 ± 2 °C. Culture agitation was provided by means of an orbital shaker at 200 rpm.

Experimental Design

The experimental design consisted of four steps: (1) a screening test to evaluate the behavior of the protist in the presence of various carbon sources and with various enzymatic hydrolysis of MSW; (2) evaluation of different concentrations of MSW as basal medium and screening for SBY as the only nitrogen source; (3) a central composite design (CCD) for the determination of the optimal supplementation of SBY and glucose to MSW medium; and (4) characterization of the biomass obtained in terms of high added value products (lipids and carotenoids).

Screening Tests

For the screening test, three trials were conducted: the first for the evaluation of *A. mangrovei* growth under different types of carbon source; the second to evaluate the growth of *A. mangrovei* in the presence of different concentrations of MSW; the third to evaluate the utilization of SBY as main nitrogen source for *A. mangrovei*.

For the first screening, the carbon sources used were glucose, galactose, lactose and lactic acid, adding the same amount of carbon (in g L^{-1}) of the control with glucose. The second screening was conducted using four concentrations of MSW: 25, 50, 75 and 100% (dilution *v/v*). The dilution was obtained using distilled water without the addition of any other nutrient. The third screening was made using SBY as the only nitrogen source in substitution of standard yeast extract. The salinity of all samples was set at 1.75% (*v/v*) using commercial Aquaforest® sea salts. A working volume of 300 mL was placed in a 500 mL Erlenmeyer

flask for each concentration. *A. mangrovei* was inoculated into each flask to reach an initial concentration of 400 mg L^{-1} of DW. The experiment was carried out at 28 °C and the mixing was provided through an air bubbling system equipped with a filter of 0.22 µm in order to prevent any contamination and to provide oxygenation to the culture.

Hydrolysis of Dairy Wastewater

Enzymatic hydrolyses of MSW were carried out using the method proposed by Bikash et al. [45] with minor modifications. A sequential hydrolysis has been conducted in order to obtain a hydrolysate without lactose and high molecular weight proteins. The protocol used was the following: 300 mL of MSW was heated at 85 °C for 1 h in a water bath in order to stabilize the product. After that, the samples were transferred on an orbital shaker set at 37 °C and a food grade lactase was added to the bottles (186 mg L^{-1}). At the end, the samples were heated at 90 °C for 5 min to inactivate the lactase.

After this first step of hydrolysis, we began the proteolysis phase. The bottles were placed on an orbital shaker at 50 °C, 150 rpm and 12.5 mL of protease from *Aspergillus oryzae* (Merck, Rome, Italy) were added, corresponding to about 16,000 LAPU aminopeptidase units per liter of MSW. Proteolysis was carried out for 3 h. After this period, the enzyme was inactivated at 85 °C for 3 h. The samples obtained were frozen to prevent any fermentation.

Response Surface Analysis and Formulation of Optimized Media

The new media obtained from hydrolysed MSW was optimized using response surface methodology (RSM). RSM is one of the most effective method for the optimization of the fermentation process [46]. This method was applied to formulate the optimal combination of glucose (carbon content) and spent brewery yeast (nitrogen source) to supplement the new hydrolysed MSW medium in order to enhance the biomass production. SBY supplementation was expressed as g L^{-1} of lysate DW.

The RSM has been done by constructing a three level full factorial central composite design (CCD). The optimization consisted of 13 runs conducted in two blocks with 4 cubic points (or factorial points), 4 axial points (or star points) and 3 center points for each block.

The mathematical relationship of the response (Y) to the significant independent variables X_1 and X_2 is given by the following quadratic polynomial equation (2):

$$\mathbf{Y} = \boldsymbol{\beta_0} + \sum_{i=1}^{n} \boldsymbol{\beta_i X_i} + \sum_{i=1}^{n} \boldsymbol{\beta_{ii} X_i^2} + \sum_{i=1}^{n} \boldsymbol{\beta_{ij} X_i X_j} \quad (2)$$

Where Y is the predicted response; X_i and X_j are the coded values; β_0 the independent coefficient; $\beta_{i,j}$ is the linear coefficient associated to each independent factor ($X_{i,j}$) and β_{ij} and β_{ii} are the coefficient for interaction and quadratic effects, respectively [47].

The optimal condition extrapolated from the model was also confirmed by cultivating *A. mangrovei* with the new nutrients parameters, and compared with the prediction of the model.

Analytical Methods

Measurement of Dry Cell Weight

Every 24 h, 10 mL of culture volume were taken and transferred in weighted dry tubes, then centrifuged at 5,000 g for 10 min. The supernatants were discarded, the pellets were washed twice with phosphate buffered saline (PBS) and dried overnight in an oven at 105 °C to obtain the dry cell weight (DW) [11].

Chemical Characterization of Food Waste

For the evaluation of the chemical-physical composition, a different type of analysis has been performed using standard methods to obtain dry weight, ash, salt content, moisture, pH and protein content [17]. The ash content was determined gravimetrically until reaching a constant weight in a muffle furnace at 550 °C. The protein content was evaluated by the Bradford assay [48] using bovine serum albumin (BSA) as the standard (MilliporeSigma, Burlington, MA, USA) and a Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) for the reading of the absorbance.

The determination of reducing sugars was obtained with the dinitrosalicylic assay (DNS) [49], and the lactose has been determined spectrophotometrically following the AOAC method 2006.06 [50]. The pH value was detected using a pH meter (Mettler Toledo, Switzerland). The salinity content was evaluated with a hand refractometer. The Mg^{2+}, Cl^- and Ca^{2+} content were established following ISO standards [51]. FAN content was estimated with the ninhydrin reaction method described by [52].

Lipid Extraction and Fatty Acid Methyl Esters (FAMEs)

The total amount of lipids were extracted according to a method previously established by Cha et al. [53], with minor modifications. 0.1 g of a powdered microalga sample was extracted with 3.33 mL of concentrated HCl (37%). The mixture was shaken using a vortex for 2 min and boiled twice at 100 °C for 20 min to induce cell disruption. The tubes were cooled at room temperature. Lastly, the lipid fraction was extracted three times: once with 4 mL of hexane and twice with 2.5 mL of hexane. The fatty acid methyl esters (FAMEs) were prepared from the total amount of previously obtained lipids by a transmethylation reaction utilizing a methodology previously described with certain modifications [54]. 20 mg of lipid extract was mixed with 50 µL 2N KOH in methanol, 500 µL of n-hexane and 500 µL of methylnonadecanoate (Sigma, St. Louis, MO, USA) as internal standard (1mg/mL). The mixture was vortexed for two min. The upper layer supernatant (FAME extract) was collected and injected into a gas chromatography-mass spectrometer (GC-MS). Microalgal extracts were analyzed according to the method conditions previously described by Conde et al. [55]. The analyses were carried out by using an Agilent 7890A gas chromatograph coupled to a Waters QUATTRO microTM mass spectrometer detector. The separation was achieved on a capillary column DB-5MS (30 m × 0.25 mm; f.t. 0.25 µm) purchased from Agilent Technologies (J&W Scientific, Folsom, CA, USA). The oven temperature was 58 °C for 2 min, 25 °C min^{-1} to 160 °C, 2 °C min^{-1} to 210 °C, 30 °C min^{-1} to 225 °C (held for 20 min). The MS detector operates with an ionization energy of 70 eV and a scanning range of *m/z* 50–550 *m/z*. The conditions were helium as carrier gas at 1.4 mL min^{-1}, the inlet temperature was 220 °C, the detector temperature was 230 °C, 2µL of injection volume (splitless). Data were analyzed using MassLynx version 4.1 (Waters, San Jose, CA, USA).

Determination of Carotenoids in Microalgal Extracts by HPLC/MS Analysis

The extraction was obtained by using an ultrasonic bath (Bandelin, Sonorex, RK52, Berlin, Germany), which operates at a frequency of 35 kHz according to the protocol described previously by Castro-Puyana et al. 2013 [56] with some modifications. Briefly, 10 mg of a sample of microalgal was added to 1.5 mL of ethanol containing 0.1% (*w/v*) of butylated hydroxytoluene. The mixture was centrifuged for 10 min at 10,000 rpm (4 °C). The extracts were collected and filtered through 0.2 µm nylon syringe filters and stored at −18 °C until the analyses.

Microalgal extracts were analyzed by UPLC Acquity coupled XEVO-TQ-S Triple quadrupole mass spectrometry (Waters Corporation, Milford, MA, USA). Carotenoids were separated on an YMC-C30 reversed-phase column (250 × 4.6 mm. 3 µm). The mobile phases consisted of methanol with 5% water and 0.1% formic acid as mobile phase A and methyl tert-butyl ether as mobile phase B. The conditions of the solvent gradient were 60% A to 0% A in 30 min with a flow rate of 1 mL min^{-1}. Analysis parameters were arranged using a positive-ion mode. The parameters of multiple reaction monitoring MRM transitions for all the standards are listed in supplementary material (S1). Additional mass spectrometric parameters were as follows: Source temperature was 150 °C, the desolvation temperature was 500 °C, cone gas flow 150 °C, the source offset was 30 V, the desolvation gas flow was 1000 L/h, the collision gas flow was 0.15 mL min^{-1}, and the collision gas was argon. The data was acquired using MassLynx version 4.1 (Waters, San Jose, CA, USA). Carotenoids were quantified by standards of violaxanthin, astaxanthin, canthaxanthin and β-carotene. The calibration curves were prepared from the limit of quantification (LOQ) to 500–625 mg L^{-1}. All calibration curves revealed a good linearity among different concentrations, and the determination coefficients were higher than 0.9918 in all cases. The method used for analysis showed a limit of detection (LOD) within the range 0.02–2.06 µg L^{-1} and the LOQ was within 0.08–6.85 µg L^{-1} (Supplementary material S2).

Statistical Analysis

All the analyses were carried out in triplicate, and average values with standard deviation were reported. One-way ANOVA was applied using raw data to test for significant differences among the samples (significance level was always set at $p < 0.05$). The Tukey's test was used for post-hoc analysis when there were significant differences among the samples. The data were analyzed using IBM© SPSS© Statistics software Ver. 23 (SPSS, Inc., Chicago, IL, USA). RSM analysis was carried out using Statistica 7.0 package (StatSoft, Tulsa, OK, USA).

5.4. Conclusion

The combination of wastewater from mozzarella manufacturing and brewery waste showed a promising alternative for a more sustainable *Aurantiochytrium mangrovei* cultivation. Pre-treatment of MSW is mandatory to achieve an optimal biomass concentration and lipid

production. Enzymatic hydrolyses achieved good growth performances in terms of biomass produced. However, a supplementation of nitrogen from spent brewery yeast and glucose is required to boost the growth of *A. mangrovei* using MSW.

The optimization with RSM leads to a biomass DW of 10.14 g L^{-1} with 38.9% of lipids and 29.8% of DHA on total FAME. The results are comparable to the relative growth with standard media. These findings suggest that hydrolyzed MSW with SBY can be used in new biotechnological processes in order to reduce nutrient costs for production of biomass that is rich in DHA oil.

References

1. Gharami, K.; Das, M.; Das, S. Essential role of docosahexaenoic acid towards development of a smarter brain. *Neurochem. Int.* **2015**, *89*, 51–62.
2. Birch, E.E.; Garfield, S.; Castañeda, Y.; Hughbanks-Wheaton, D.; Uauy, R.; Hoffman, D. Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized trial of long-chain polyunsaturated fatty acid-supplemented infant formula. *Early Hum. Dev.* **2007**, *83*, 279–284. https://doi.org/10.1016/j.earlhumdev.2006.11.003.
3. Shahidi, F.; Ambigaipalan, P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. *Annu. Rev. Food Sci. Technol.* **2018**, *9*, 345–381. https://doi.org/10.1146/annurev-food-111317.
4. Chen, W.; Zhou, P.; Zhu, Y.; Xie, C.; Ma, L.; Wang, X.; Bao, Z.; Yu, L. Improvement in the docosahexaenoic acid production of *Schizochytrium* sp. S056 by replacement of sea salt. *Bioprocess Biosyst. Eng.* **2016**, *39*, 315–321. https://doi.org/10.1007/s00449-015-1517-1.
5. Graham, I.A.; Larson, T.; Napier, J.A. Rational metabolic engineering of transgenic plants for biosynthesis of omega-3 polyunsaturates. *Curr. Opin. Biotechnol.* **2007**, *18*, 142–147. https://doi.org/10.1016/j.copbio.2007.01.014.
6. Lewis, T.E.; Nichols, P.D.; McMeekin, T.A. The Biotechnological Potential of Thraustochytrids. *Mar. Biotechnol.* **1999**, *1*, 580–587. https://doi.org/10.1007/PL00011813.
7. Gao, M.; Song, X.; Feng, Y.; Li, W.; Cui, Q. Isolation and characterization of *Aurantiochytrium* species: High docosahexaenoic acid (DHA) production by the newly isolated microalga, *Aurantiochytrium* sp. SD116. *J. Oleo Sci.* **2013**, *62*, 143–151. https://doi.org/10.5650/jos.62.143.
8. Humhal, T.; Kastanek, P.; Jezkova, Z.; Cadkova, A.; Kohoutkova, J.; Branyik, T. Use of saline waste water from demineralization of cheese whey for cultivation of *Schizochytrium limacinum* PA-968 and Japonochytrium marinum AN-4. *Bioprocess Biosyst. Eng.* **2017**, *40*, 395–402. https://doi.org/10.1007/s00449-016-1707-5.
9. Ende, S.S.W.; Noke, A. Heterotrophic microalgae production on food waste and by-products. *J. Appl. Phycol.* **2019**, *31*, 1565–1571. https://doi.org/10.1007/s10811-018-1697-6.
10. Russo, G.L.; Langellotti, A.L.; Blasco, T.; Oliviero, M.; Sacchi, R.; Masi, P. Production of Omega-3 Oil by *Aurantiochytrium mangrovei* Using Spent Osmotic Solution from Candied Fruit Industry as Sole Organic Carbon Source. *Processes* **2021**, *9*, 1834. https://doi.org/10.3390/pr9101834.
11. Ryu, B.-G.; Kim, K.; Kim, J.; Han, J.-I.; Yang, J.-W. Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, *Aurantiochytrium* sp. KRS101. *Bioresour. Technol.* **2013**, *129*, 351–359. https://doi.org/10.1016/j.biortech.2012.11.049.
12. Castrica, M.; Ventura, V.; Panseri, S.; Ferrazzi, G.; Tedesco, D.; Balzaretti, C.M. The sustainability of urban food systems: The case of mozzarella production in the city of Milan. *Sustainability* **2020**, *12*, 682. https://doi.org/10.3390/su12020682.
13. Kasmi, M. Biological Processes as Promoting Way for Both Treatment and Valorization of Dairy Industry Effluents. *Waste Biomass Valorization* **2018**, *9*, 195–209. https://doi.org/10.1007/s12649-016-9795-7.
14. Zotta, T.; Solieri, L.; Iacumin, L.; Picozzi, C.; Gullo, M. Valorization of cheese whey using microbial fermentations. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 2749–2764. https://doi.org/10.1007/s00253-020-10408-2.
15. Yuan, X.; Liang, L.; Liu, K.; Xie, L.; Huang, L.; He, W.; Chen, Y.; Xue, T. Spent yeast as an efficient medium supplement for fucoxanthin and eicosapentaenoic acid (EPA)

production by Phaeodactylum tricornutum. *J. Appl. Phycol.* **2020**, *32*, 59–69. https://doi.org/10.1007/s10811-019-01909-3.
16. Pleissner, D.; Lam, W.C.; Sun, Z.; Lin, C.S.K. Food waste as nutrient source in heterotrophic microalgae cultivation. *Bioresour. Technol.* **2013**, *137*, 139–146. https://doi.org/10.1016/j.biortech.2013.03.088.
17. Massa, M.; Buono, S.; Langellotti, A.L.; Martello, A.; Russo, G.L.; Troise, D.A.; Sacchi, R.; Vitaglione, P.; Fogliano, V. Biochemical composition and in vitro digestibility of Galdieria sulphuraria grown on spent cherry-brine liquid. *New Biotechnol.* **2019**, *53*, 9–15. https://doi.org/10.1016/j.nbt.2019.06.003.
18. Ratledge, C. Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* **2004**, *86*, 807–815.
19. Panesar, P.; Kennedy, J.; Gandhi, D.; Bunko, K. Bioutilisation of whey for lactic acid production. *Food Chem.* **2007**, *105*, 1–14. https://doi.org/10.1016/j.foodchem.2007.03.035.
20. Chokshi, K.; Pancha, I.; Ghosh, A.; Mishra, S. Microalgal biomass generation by phycoremediation of dairy industry wastewater: An integrated approach towards sustainable biofuel production. *Bioresour. Technol.* **2016**, *221*, 455–460. https://doi.org/10.1016/j.biortech.2016.09.070.
21. Humhal, T.; Kronusová, O.; Kaštánek, P.; Potočár, T.; Kohoutková, J.; Brányik, T. Influence of nitrogen sources on growth of thraustochytrids in waste water from the demineralization of cheese whey. *Czech J. Food Sci.* **2019**, *37*, 383–390. https://doi.org/10.17221/172/2018-cjfs.
22. Gernigon, G.; Piot, M.; Beaucher, E.; Jeantet, R.; Schuck, P. Physicochemical characterization of Mozzarella cheese wheys and stretchwaters in comparison with several other sweet wheys. *J. Dairy Sci.* **2009**, *92*, 5371–5377. https://doi.org/10.3168/jds.2009-2359.
23. Pahlavanyali, M.; Jalili, H.; Noroozi, M.; Moradi, Y.; Hallajisani, A. The effect of temperature and different carbon and nitrogen sources on the growth and fatty acid profile of a newly isolated microorganism *Aurantiochytrium* sp. strain SHY. *Iran. J. Fish. Sci.* **2020**, *19*, 3112–3126. https://doi.org/10.22092/ijfs.2020.122942.
24. Unagul, P.; Assantachai, C.; Phadungruengluij, S.; Suphantharika, M.; Verduyn, C. Properties of the docosahexaenoic acid-producer *Schizochytrium mangrovei* Sk-02: Effects of glucose, temperature and salinity and their interaction. *Bot. Mar.* **2005**, *48*, 387–394. https://doi.org/10.1515/bot.2005.052.
25. Ju, J.-H.; Ko, D.-J.; Heo, S.-Y.; Lee, J.-J.; Kim, Y.-M.; Lee, B.-S.; Kim, M.-S.; Kim, C.-H.; Seo, J.-W.; Oh, B.-R. Regulation of lipid accumulation using nitrogen for microalgae lipid production in *Schizochytrium* sp. ABC101. *Renew. Energy* **2020**, *153*, 580–587. https://doi.org/10.1016/j.renene.2020.02.047.
26. Song, X.; Ma, Z.; Tan, Y.; Zhang, H.; Cui, Q. Wastewater recycling technology for fermentation in polyunsaturated fatty acid production. *Bioresour. Technol.* **2017**, *235*, 79–86. https://doi.org/10.1016/j.biortech.2017.03.034.
27. Wong, M.K.M.; Tsui, C.K.M.; Au, D.W.T.; Vrijmoed, L.L.P. Docosahexaenoic acid production and ultrastructure of the thraustochytrid *Aurantiochytrium mangrovei* MP2 under high glucose concentrations. *Mycoscience* **2008**, *49*, 266–270. https://doi.org/10.1007/S10267-008-0415-7.
28. Yokochi, T.; Honda, D.; Higashihara, T.; Nakahara, T. Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 72–76. https://doi.org/10.1007/s002530051139.
29. Yu, X.-J.; Yu, Z.-Q.; Liu, Y.-L.; Sun, J.; Zheng, J.-Y.; Wang, Z. Utilization of High-Fructose Corn Syrup for Biomass Production Containing High Levels of

Docosahexaenoic Acid by a Newly Isolated *Aurantiochytrium* sp. YLH70. *Appl. Biochem. Biotechnol.* **2015**, *177*, 1229–1240. https://doi.org/10.1007/s12010-015-1809-6.
30. Ju, J.-H.; Oh, B.-R.; Ryu, S.-K.; Heo, S.-Y.; Kim, S.-Y.; Hong, W.-K.; Kim, C.H.; Seo, J.-W. Production of Lipid Containing High Levels of Docosahexaenoic Acid by Cultivation of *Aurantiochytrium* sp. KRS101 Using Jerusalem Artichoke Extract. *Biotechnol. Bioprocess Eng.* **2018**, *23*, 726–732. https://doi.org/10.1007/s12257-018-0419-x.
31. Wu, S.-T.; Yu, S.-T.; Lin, L.-P. Effect of culture conditions on docosahexaenoic acid production by *Schizochytrium* sp. S31. *Process Biochem.* **2005**, *40*, 3103–3108. https://doi.org/10.1016/j.procbio.2005.03.007.
32. Wang, S.-K.; Wang, X.; Tian, Y.-T.; Cui, Y.-H. Nutrient recovery from tofu whey wastewater for the economical production of docosahexaenoic acid by *Schizochytrium* sp. S31. *Sci. Total Environ.* **2020**, *710*, 136448. https://doi.org/10.1016/j.scitotenv.2019.136448.
33. Park, W.-K.; Moon, M.; Shin, S.-E.; Cho, J.M.; Suh, W.I.; Chang, Y.K.; Lee, B. Economical DHA (Docosahexaenoic acid) production from *Aurantiochytrium* sp. KRS101 using orange peel extract and low cost nitrogen sources. *Algal Res.* **2018**, *29*, 71–79. https://doi.org/10.1016/j.algal.2017.11.017.
34. Patel, A.; Rova, U.; Christakopoulos, P.; Matsakas, L. Mining of squalene as a value-added byproduct from DHA producing marine thraustochytrid cultivated on food waste hydrolysate. *Sci. Total Environ.* **2020**, *736*, 139691. https://doi.org/10.1016/j.scitotenv.2020.139691.
35. Des Rosiers, C.; Labarthe, F.; Lloyd, S.G.; Chatham, J.C. Cardiac anaplerosis in health and disease: Food for thought. *Cardiovasc. Res.* **2011**, *90*, 210–219. https://doi.org/10.1093/cvr/cvr055.
36. Kaya, K.; Kazama, Y.; Abe, T.; Shiraishi, F. Influence of medium components and pH on the production of odd-carbon fatty acids by *Aurantiochytrium* sp. SA-96. *J. Appl. Phycol.* **2020**, *32*, 1597–1606. https://doi.org/10.1007/s10811-020-02111-6.
37. Aasen, I.M.; Ertesvåg, H.; Heggeset, T.M.B.; Liu, B.; Brautaset, T.; Vadstein, O.; Ellingsen, T.E. Thraustochytrids as production organisms for docosahexaenoic acid (DHA), squalene, and carotenoids. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 4309–4321. https://doi.org/10.1007/s00253-016-7498-4.
38. Galasso, C.; Corinaldesi, C.; Sansone, C. Carotenoids from Marine Organisms: Biological Functions and Industrial Applications. *Antioxidants* **2017**, *6*, 96. https://doi.org/10.3390/antiox6040096.
39. Ye, J.; Liu, M.; He, M.; Ye, Y.; Huang, J. Illustrating and Enhancing the Biosynthesis of Astaxanthin and Docosahexaenoic Acid in *Aurantiochytrium* sp. SK4. *Mar. Drugs* **2019**, *17*, 45. https://doi.org/10.3390/md17010045.
40. Kubo, Y.; Shiroi, M.; Higashine, T.; Mori, Y.; Morimoto, D.; Nakagawa, S.; Sawayama, S. Enhanced Production of Astaxanthin without Decrease of DHA Content in *Aurantiochytrium limacinum* by Overexpressing Multifunctional Carotenoid Synthase Gene. *Appl. Biochem. Biotechnol.* **2021**, *193*, 52–64. https://doi.org/10.1007/s12010-020-03403-w.
41. Russo, G.L.; Langellotti, A.L.; Oliviero, M.; Sacchi, R.; Masi, P. Sustainable production of food grade omega-3 oil using aquatic protists: Reliability and future horizons. *New Biotechnol.* **2021**, *62*, 32–39. https://doi.org/10.1016/j.nbt.2021.01.006.
42. Fei, Q.; Chang, H.N.; Shang, L.; Choi, J.; Kim, N.; Kang, J. The effect of volatile fatty acids as a sole carbon source on lipid accumulation by Cryptococcus albidus for biodiesel production. *Bioresour. Technol.* **2011**, *102*, 2695–2701. https://doi.org/10.1016/j.biortech.2010.10.141.

43. Maddipati, P.; Atiyeh, H.K.; Bellmer, D.D.; Huhnke, R.L. Ethanol production from syngas by Clostridium strain P11 using corn steep liquor as a nutrient replacement to yeast extract. *Bioresour. Technol.* **2011**, *102*, 6494–6501. https://doi.org/10.1016/j.biortech.2011.03.047.
44. Jacob, F.F.; Striegel, L.; Rychlik, M.; Hutzler, M.; Methner, F.-J. Spent Yeast from Brewing Processes: A Biodiverse Starting Material for Yeast Extract Production. *Fermentation* **2019**, *5*, 51. https://doi.org/10.3390/fermentation5020051.
45. Ghosh, B.C.; Prasad, L.N.; Saha, N.P. Enzymatic hydrolysis of whey and its analysis. *J. Food Sci. Technol.* **2017**, *54*, 1476–1483. https://doi.org/10.1007/s13197-017-2574-z.
46. Park, H.; Kwak, M.; Seo, J.; Ju, J.; Heo, S.; Park, S.; Hong, W. Enhanced production of carotenoids using a Thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. *Bioprocess Biosyst. Eng.* **2018**, *41*, 1355–1370. https://doi.org/10.1007/s00449-018-1963-7.
47. Nazir, Y.; Shuib, S.; Kalil, M.S.; Song, Y.; Hamid, A.A. Optimization of Culture Conditions for Enhanced Growth, Lipid and Docosahexaenoic Acid (DHA) Production of *Aurantiochytrium* SW1 by Response Surface Methodology. *Sci. Rep.* **2018**, *8*, 8909. https://doi.org/10.1038/s41598-018-27309-0.
48. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
49. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. https://doi.org/10.1021/ac60147a030.
50. Thiex, N. Evaluation of Analytical Methods for the Determination of Moisture, Crude Protein, Crude Fat, and Crude Fiber in Distillers Dried Grains with Solubles. *J. AOAC Int.* **2009**, *92*, 61–73. https://doi.org/10.1093/jaoac/92.1.61.
51. ISO. *NF EN ISO 17294-2 Water Quality—Application of Inductively Coupled Plasma Mass Spectrometry (ICP-MS)—Part 2: Determination of Selected Elements Including Uranium Isotopes*; ISO: Geneva, Switzerland, 2016.
52. Lie, S. The Ebc-Ninhydrin Method for Determination of Free Alpha Amino Nitrogen. *J. Inst. Brew.* **1973**, *79*, 37–41. https://doi.org/10.1002/j.2050-0416.1973.tb03495.x.
53. Cha, T.S.; Chen, J.W.; Goh, E.G.; Aziz, A.; Loh, S.H. Differential regulation of fatty acid biosynthesis in two Chlorella species in response to nitrate treatments and the potential of binary blending microalgae oils for biodiesel application. *Bioresour. Technol.* **2011**, *102*, 10633–10640. https://doi.org/10.1016/j.biortech.2011.09.042.
54. Aued-Pimentel, S.; Lago, J.H.G.; Chaves, M.H.; Kumagai, E.E. Evaluation of a methylation procedure to determine cyclopropenoids fatty acids from Sterculia striata St. Hil. Et Nauds seed oil. *J. Chromatogr. A* **2004**, *1054*, 235–239. https://doi.org/10.1016/j.chroma.2004.07.090.
55. Conde, T.A.; Couto, D.; Melo, T.; Costa, M.; Silva, J.; Domingues, M.R.; Domingues, P. Polar lipidomic profile shows Chlorococcum amblystomatis as a promising source of value-added lipids. *Sci. Rep.* **2021**, *11*, 4355. https://doi.org/10.1038/s41598-021-83455-y.
56. Castro-Puyana, M.; Herrero, M.; Urreta, I.; Mendiola, J.A.; Cifuentes, A.; Ibáñez, E.; Suárez-Alvarez, S. Optimization of clean extraction methods to isolate carotenoids from the microalga Neochloris oleoabundans and subsequent chemical characterization using liquid chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.* **2013**, *405*, 4607–4616. https://doi.org/10.1007/s00216-012-6687-y.

Chapter 6

Techno-economic assessment of DHA-rich *Aurantiochytrium* sp. production using food industry by-products and waste streams as alternative growth media.

This chapter has been published as:

Giovanni L. Russo, Antonio L. Langellotti, Raffaele Sacchi, Paolo Masi. Techno-economic assessment of DHA-rich Aurantiochytrium sp. production using food industry by-products and waste streams as alternative growth media. *Bioresource Technology Reports*, 2022, 100997, ISSN 2589-014X, https://doi.org/10.1016/j.biteb.2022.100997.

.

Abstract

Reducing the production cost of DHA algae oil is a necessary step for obtaining a sustainable omega-3 source able to compete with fish oil. In this study, a techno-economic analysis was carried out for the production of a DHA rich thraustochytrid using food industry by products and waste. Three cultivation scenarios were developed: the first using standard bulk materials as source of nutrients, the second with the utilization of brewery by-product and spent osmotic solutions; the third scenario using a mix of dairy wastewater, brewery by-product and spent osmotic solution. The operating costs were reduced by an average of 35% with food wastes scenarios, increasing up to 8% the return on investment respect to the standard cultivation method. Depending from plant dimension, sensitivity analysis showed a decrease in unit production cost up to 38% when using food industry side-streams.

6.1. Introduction

Long chain polyunsaturated fatty acids (LC- PUFA) are bioactive compounds with a series of health benefits by human consumption. In the recent years there was a rising interest for health improvements via supplementation of omega-3 oil. For that reason, a boost of product demand is expected in the next 10 years [1]. The prevalent LC-PUFA are the eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) that are essential fatty acids because human body cannot synthesize them on its own. The main producers of these fatty acids are the marine organisms, accounting for the 82.8% of the market share for omega-3 oil [1]. In fact, a traditional source of LC-PUFA is represented by cold-water fish. However, the large-scale production of fish oil is no longer sustainable for the marine environment [2]. Moreover, the fish oil is affected from ocean pollution, with bio-toxin hazard risks [3], food allergies and other issues relatives to the stability of the oil [4]. For all these reasons, the research of alternative source of LC-PUFA is mandatory to satisfy the rising demand of omega-3 oil which could be sustainable and economically viable for producers.

Aquatic protists are well known to be the most productive culture for omega-3 oil production, in particular heterotrophic marine protists [5]. The attention of scientific community on this topic developed in the last years a specific “Omega-3 biotechnology” trend from aquatic protist [5–7]. The most used protist for industrial production of DHA-rich oil is the Thraustochytrid *Aurantiochytrium sp*. (formerly known as *Schyzochitrium*) [5]. These aquatic

protists are efficient producer of oil, accounting for 35-50% of lipid (on dry weight basis) of which 30-50% is DHA [5]. As heterotrophic protists, thraustochytrids are cultivated in biofermenters with glucose or sugar molasses as carbon source, coupled with yeast extract or ammonium sulphate as nitrogen source. However the costs regarding large scale production, including capital and energy costs for cultivation, are still the main challenge [8]. In fact, despite the high lipid and biomass productivity of these protists, the high requirement of organic carbon and nitrogen add relevant feedstock costs to the whole process.

For that reason, many studies have been conducted using alternative low cost organic and nitrogen sources. One of the most promising alternative low cost medium is represented by food by-products and wastes (FBWs) [5,9,10]. These wastes are well segregated streams rich in nutrients that could be exploited by thraustochytrids for the production of omega-3 oil with a reduced production cost. Many studies focused on the utilization of typical food waste such as restaurant, canteen and bakery wastes for cultivation of microalgae [9,11]. However, in our opinion, a better option would be the exploitation of food industry side-streams that are easily to use in biotechnological terms. In fact, for food waste in "sensu stricto" many pre-treatments are required prior their utilization as culture medium, and that would increase the investment costs [9,12]. Moreover, previous studies demonstrated that side-streams such as dairy wastewater, sugar molasses and brewery wastes have been successfully used for the cultivation of *Aurantiochytrium sp*. [13–16] without particular pre-treatments. However, these studies are limited to a lab scale, without considering an industrial scale up, and in particular, without considering the economic impact of using FBWs as a source of nutrients instead of standard bulk materials.

The sustainability of cultivation for biomass rich in lipids can be measured from an economic standpoint through techno-economic analysis (TEA) [17]. TEA studies are important in minimizing economic risks at early stages of technology development. In literature there are few works that have performed a TEA for the production of aquatic protists rich in PUFA [18,19]. Most of the other TEA found are based on the obtainment of biodiesel [17,19–21] and other valuable compounds from aquatic protists [22].

To the best of our knowledge, no papers have ever been published concerning the TEA of thraustochytrids cultivation, especially considering a scenario with food waste-based media.

For that reason, in order to evaluate the reduction of cultivation costs of heterotrophic protists rich in omega-3, in this study we present for the first time a TEA study for *Aurantiochytrium*

sp. industrial cultivation using FBWs as alternative media in comparison with standard cultivation medium. The cost-efficiency of FBWs as raw material for DHA-rich biomass cultivation has been established through software simulation.

6.2. Materials and methods

Simulation description and assumptions

In this study SuperPro Designer® 12.0 was used for the development of process design and simulations of a biotechnological process for the obtainment of biomass powder rich in DHA. Three different cultivation models were developed for *Aurantiochytrium sp*.: the first scenario (model A) is the "standard" cultivation process using classic bulk materials for heterotrophic cultivation (i.e. glucose and yeast extract); the second scenario (model B) is represented by a mix of spent osmotic solutions (SOS) from candied fruit industry and spent brewery yeast (SBY); the third scenario (model C) is a combination of dairy wastewater, SOS and SBY.

The basic assumptions for this simulation are reported in Table 1.

Table 1. Basic assumption of the simulation proposed.

Parameter	**Value**	Reference
Plant dimension	150 m^3	This study
SOS sugar content	70% of DW	[13]
SBY nitrogen content	10% of DW	[14,16]
Biomass/carbon yield	28%	[13]
Maximum specific growth rate (μ max)	0.04h^{-1}	[13,14]
Biomass DHA	18% of DW	[16]
Medium C/N ratio	20 (w/w)	[14]
Aurantiochytrium powder selling value	US$ 20/Kg	Alibaba.com
Glucose cost	0.55 $/Kg	[37]

The plant is located in Italy with a 15-years lifetime, including 20 months of construction and 4 months of start-up period. The operation mode was set to be in batch with a processing capacity of 150 MT per batch of cultivation medium.

The cultivated protist selected is *Aurantiochytrium sp*. with an initial concentration of 0.8 g L-1 of dry weight (DW) and a lipid content of 40% of DW and 18% of DHA.

For the standard cultivation, the growth medium was composed of 40 g L-1 of glucose, 15 g L-1 of artificial sea salts and 10 g L-1 of yeast extract (YE). This medium composition was established by literature data and our previous experiments [13,14], taking in consideration the best carbon yield conversion. *Aurantiochytrium* is grown in biofermenters equipped with an impeller and a bubbling system. It should be noted that the selected approach is not claimed to be the best or most optimized process, but is one of the more likely options to be feasible on a large scale using methodology currently used in industrial bioprocessing.

The fermentation parameters used for this simulation are the following: temperature = 28°C; power consumption 0.5 kW/m3; reaction time = 48 h. The cultivation time of thraustochytrids range from 48 to 120 h, however from our experience and from literature data, it can be seen that generally the highest biomass productivity occurs during the first 48 hours of cultivation [13,23]. For that reason, in order to increase the batch numbers during the year and maximize the profits, we set a batch cycle of 48 hours in the simulation.

Process description

The elaboration of the three models was performed after our previous studies and experience. In Figure 1 is reported the standard cultivation process of *Aurantiochytrium sp*. using SuperPro Designer.

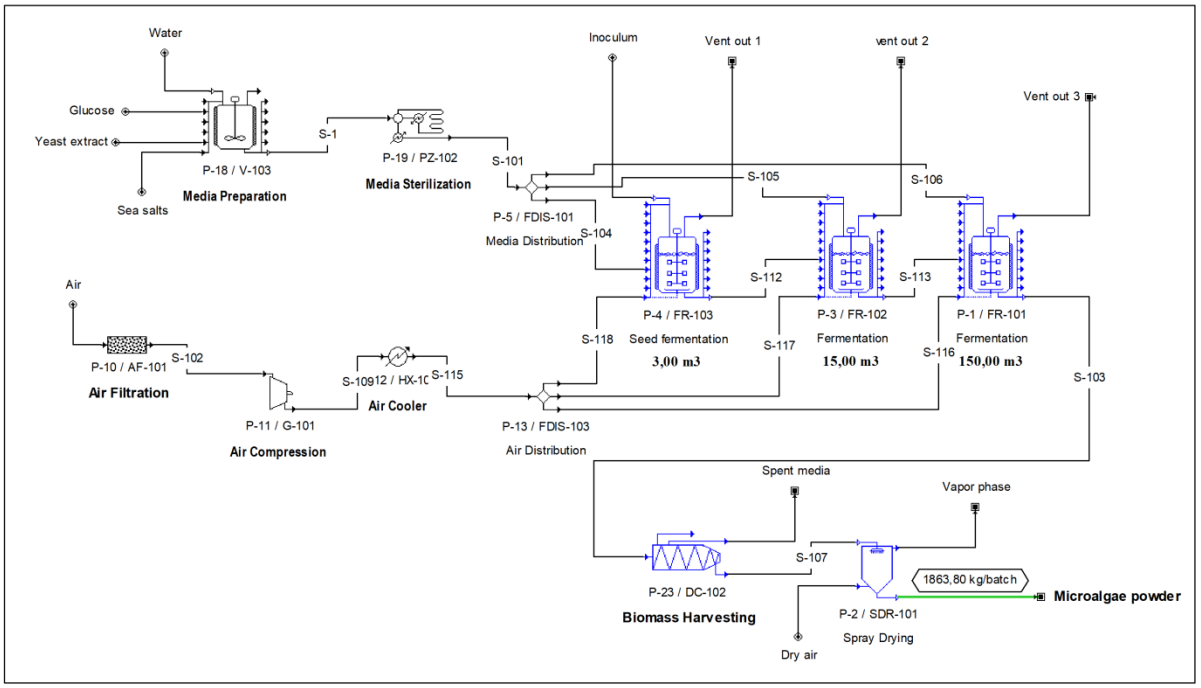

Figure 1. Process flow diagram for standard cultivation of *A. mangrovei* (model A)

In the standard model, the nutrients used are bulk materials: glucose as main organic carbon source and yeast extract as nitrogen and mineral salts source. The process first involves the mixing of all nutrients in a blending tank with artificial seawater (obtained with water and commercial sea salts), after which the medium obtained is sterilized and separated into three bio-fermenters. The first (the seed reactor) is sized to be 3 m^3 in volume using an inoculum from previous fermentations; the second reactor is 15 m^3 and the third is 150 m^3. The biofermenters are set to be used up to 80% of their volume. After cultivation period, the biomass is collected by means of a decanter centrifuge and pulverized by means of a spray dryer. The spent media in this model is destined for disposal, but could be recycled in other fermentation processes.

In Figure 2 and 3 are reported the alternative cultivation models using FBWs medium.

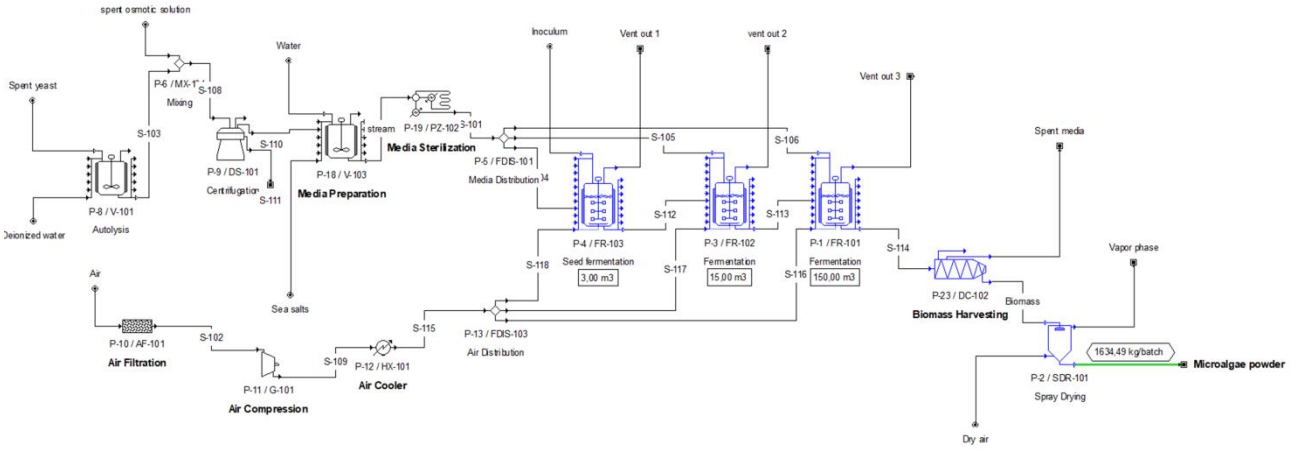

Figure 2. Process flow diagram for alternative cultivation medium of *A. mangrovei* (model B).

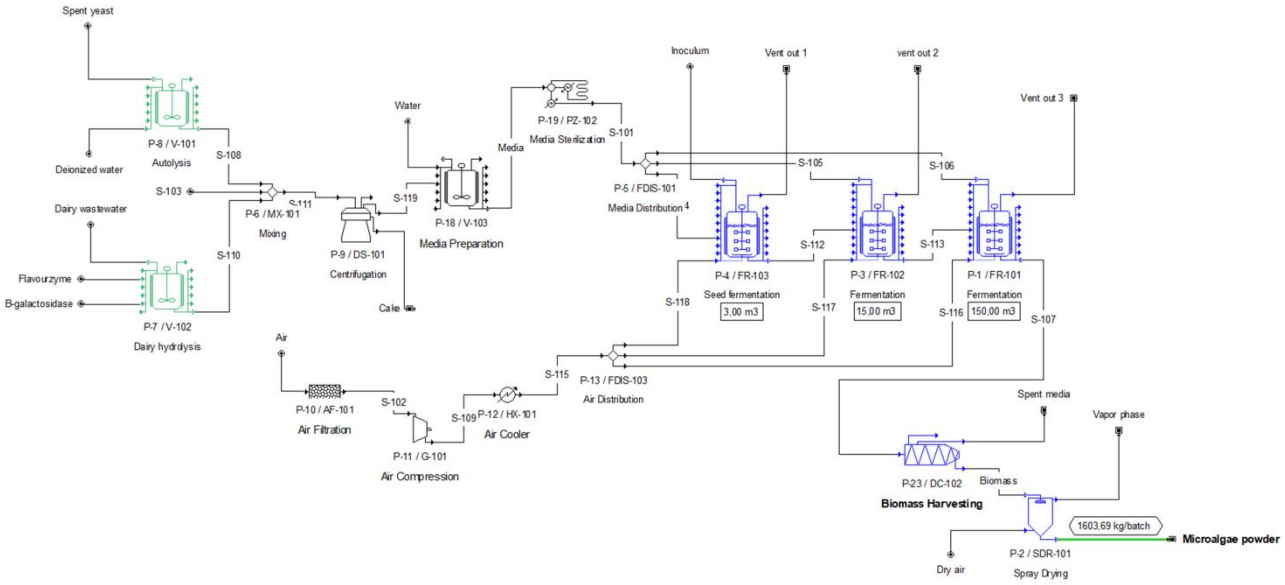

Figure 3. Process flow diagram for alternative cultivation medium of *A. mangrovei* (model C).

For the model B, the nutrients were derived from a combination of FBWs: SOS as main organic carbon source and SBY as source of nitrogen and mineral salts. This process was elaborated after our previous study [13]. The SBY was treated with an autolysis reaction using deionized water as reported by [14], conducted in a blending tank with a capacity of 3 m^3 with a process duration of 24 h at 50°C. The wastes are then centrifuged in a disk-stack centrifuge in order to separate the cake from the liquid. The obtained liquid is then mixed in a blending

tank with water and sea salts. The rest of the process follows the same procedures as the standard model (model A).

In the model C instead (figure 3), a combination of dairy wastewater, SOS and SBY has been evaluated. Also for this process, the elaboration was made after our previous study [16].

In this case the dairy wastewater was subjected prior to enzymatic hydrolysis. Specifically, the lactose and proteins present have been hydrolyzed to make these nutrients more bioavailable for *Aurantiochytrium sp*. cultivation. The hydrolysis process follows the method used in our previous study, where the hydrolyzed dairy wastewater gave good growth performance for *A. mangrovei* [16]. After that, the dairy wastewater is mixed with the SOS and SBY and the stream is centrifuged using a disk stack centrifuge in order to obtain the liquid without solid residue. The obtained stream was then diluted with water in a tank as the dairy wastewater gives better performance when diluted to 50% [16]. The next part of the process follows that of Model A.

Economic analysis

The economic parameters set in the simulation software are reported in Table 2.

Table 2. Economic evaluation parameters for the entire simulation.

Parameter	**Value**
Year construction starts	2022
Construction period (months)	20
Start-up period (months)	4
Project lifetime (years)	15
Inflation rate (%)	4
NPV interest (%)	6
Depreciation period (years)	10
Loan interest (%)	12
Income taxes (%)	20

The economic performance of the three models was measured by calculating the fixed capital investment (Table 2) and total capital cost (TCC). TCC was calculated on the basis of the

equipment purchase cost obtained from the simulation software including piping, instrumentation and other indirect costs items (e.g., construction and engineering). The equipment size and costs (Table 3) were estimated using SuperPro Designer built-in cost model and Power Law model. The cost rule used was the following (equation 1):

$$Cost = C_0 * \left(\frac{Q}{Q_0}\right)^a$$

Where C0 is the investment cost for a piece of equipment with Q0 process capacity; "a" value is a scaling factor (constant number) describing the economic and financial impact of changing the size of biofermenters.

The operating cost includes the raw material cost, labor and utility cost. The raw materials costs were estimated from various sources present in literature data. The labor and utility cost instead, were estimated from SuperPro Designer.

Profitability and sensibility analysis

For the profitability analysis the payback time, gross margin, net present value (NPV), internal rate of return (IRR) and return on investment (ROI) were estimated using SuperPro Designer.

The NPV was calculated using the following equation (2):

$$NPV\ (USD) = \sum_{t-1}^{T} \frac{Ct}{(1+d)^t} - C0$$

Where C0 is the initial investment, t is the lifetime in years; Ct is the net cash flow during period t and d is the discount rate. The IRR is a value that indicates the efficiency or yields of an investment, and represents the discount rate at which the NPV value is zero.

The NPV and IRR are essential values to assess the profitability of an investment or a project [24].

The ROI instead describes the rate of the cash return and is calculated using the following equation (3):

$$ROI\ (\%) = \frac{Annual\ net\ profit}{Capital\ cost} * 100$$

A sensibility analysis was also carried out using SuperPro Designer and Microsoft Excel 2016 to evaluate the economic behavior of the new processes scenarios in face of some uncertainties [25]. The uncertainty considered was biomass powder processing flow rate, with a range from 1040 Kg/batch to 3080 kg/batch.

Statistical analysis

The data were analyzed using SPSS software, version 23 (IBM Corp., Armonk, NY, USA) and SuperPro Designer version 12.0 for the simulation process. One-way analysis of variance (ANOVA) was applied using raw data to test for significant differences among the models. $p < 0.05$ was considered statistically significant. Tukey's test was used for post-hoc analysis when there were significant differences among the samples.

6.3. RESULTS AND DISCUSSION

Capital cost

In Table 3 the equipment costs of the three cultivation models are reported.

Table 3. Equipment costs and specifications

Code	Equipment	Cost (US$)		
		Model A	**Model B**	**Model C**
FR-101, FR-102, FR-103	Fermenters	810,000	810,000	812,000
DC-102	Decanter centrifuge	200,000	200,000	200,000
SDR-101	Spray dryer	150,000	150,00	150,000
PZ-102	Pasteurizer	90,000	90,000	90,000
V-101	Autolysis tank	-	30,000	20,000
V-102	Dairy reaction tank	-	-	50,000
V-103	Blending tank	21,000	21,000	21,000
DS-101	Disk-stack centrifuge	-	40,000	40,000
HX-101	Heat exchanger	8,000	8,000	8,000
AF-101	Air filter	8,000	8,000	8,000
G-101	Centrifugal compressor	2,000	2,000	2,000
Unlisted equipment		323,000	340,000	350,000
Total		1,613,000	1,700,000	1,750,000

Model A is the scenario with the lowest equipment cost (1,613,000 USD), while Model C is the one with the highest cost (1,750,000 USD). In fact, model C requires two food waste hydrolysis treatments, which add equipment that are not necessary for model A, thus increasing the total equipment cost for this alternative cultivation model. However this cost difference is not high, as the fermenters (required in all models) represent the largest cost portion (50-55% of the total equipment costs). This is in agreement with other papers which state that biofermenters represent the highest cost portion for this type of fermentation processes [5,9,26].

However, the difference in capital costs between the various models is more marked if other parameters are also taken in consideration. For this, the direct fixed capital cost (DFCC) was also evaluated and reported in Table 4.

Table 4. Total capital investment of the proposed plants

Item	Cost (US$)		
	Model A	**Model B**	**Model C**
Equipment purchase cost	1,613,000	1,700,000	1,750,000
Installation	559,000	590,000	599,000
Process piping	484,000	510,000	525,000
Insulation	45,000	51,000	53,000
Electrical	161,000	170,000	175,000
Buildings	726,000	765,000	788,000
Yard improvement	161,000	170,000	175,000
Auxiliary facilities	645,000	680,000	700,000
Engineering	976,000	1,029,000	1,058,000
Construction	1,708,000	1,801,000	1,851,000
Total plant cost	7,564,000	7,977,000	8,198,000
Contractor's fee	378,000	399,000	410,000
Contingency	756,000	798,000	820,000
Direct fixed capital cost	8,699,000	9,173,000	9,428,000

The DFCC takes in consideration also other parameters related to capital costs such as electrical, building and engineering costs. The DFCC of model A was 8,699,000 USD while for model C was 9,428,000 USD, denoting a significant increase in capital costs for models that use food waste as a source of alternative nutrients.

Comparing the capital costs with a model that provides the reuse of food waste for the heterotrophic cultivation of *Chlorella pyrenoidosa*, our costs are significantly lower [12]. In fact in the work of Pleissner et al., despite a cultivation volume (100 m^3) lower than that of our study (150 m^3), the equipment costs reported were more than 3 million USD. This is mainly due to the various pre-treatments (grinding, separation etc.) that food wastes must undergo before their utilization as a nutrient source. Our study, on the other hand, demonstrates the greater economic feasibility in the utilization of FBWs streams compared to traditional food waste for the cultivation of biomass.

Operating cost results

As expected, the initial investment costs for a process that involves the use of FBWs are higher than a standard process that uses bulk material. However, the material costs of the proposed models have been elaborated and reported in Table 5.

Table 5. Raw material cost of the three cultivation models

Item	Cost (US$)		
	Model A	**Model B**	**Model C**
Glucose	556,920	0	0
Sea salts	82,620	82,620	0
Water	55,538	56,089	28,044
Yeast extract	1,296,675	0	0
Spent osmotic solution	0	39,780	25,245
Spent brewery yeast	0	13,005	10,710
Enzymes	0	0	315,945
Total	1,991,753	191,494	351,900

Without the use of bulk nutrients, raw material costs are drastically reduced in the biomass cultivation with FBWs. In fact, in model B, the costs of materials are reduced by about 10 times respect to the standard model A (US$ 191,494 vs US$ 1,991,753 respectively). In model B the nutrients are mainly represented by the SOS and SBY, which fully replace the glucose and yeast extract of model A. The costs of the SOS concern only those of transport to the plant, while those of the SBY are 0.068 US$/kg according to [14].

In model C, on the other hand, the total costs are US$ 351900, which is almost double that of model B. This is due to the enzymes used in the hydrolysis process of dairy wastewater that

represents the 89% of raw material costs. This is in accord with [12] which stated that the enzymes to treat food wastes are the largest portion of material costs. Nevertheless, the raw material costs of model B if compared to model A are still drastically lower. These data denote that by replacing bulk nutrients with FBWs there is a consequent economic advantage in terms of growth medium costs for *Aurantiochytrium sp*. cultivation.

To better understand all the costs related to the processes, the annual costs of the three plants have been elaborated and reported in Figure 4.

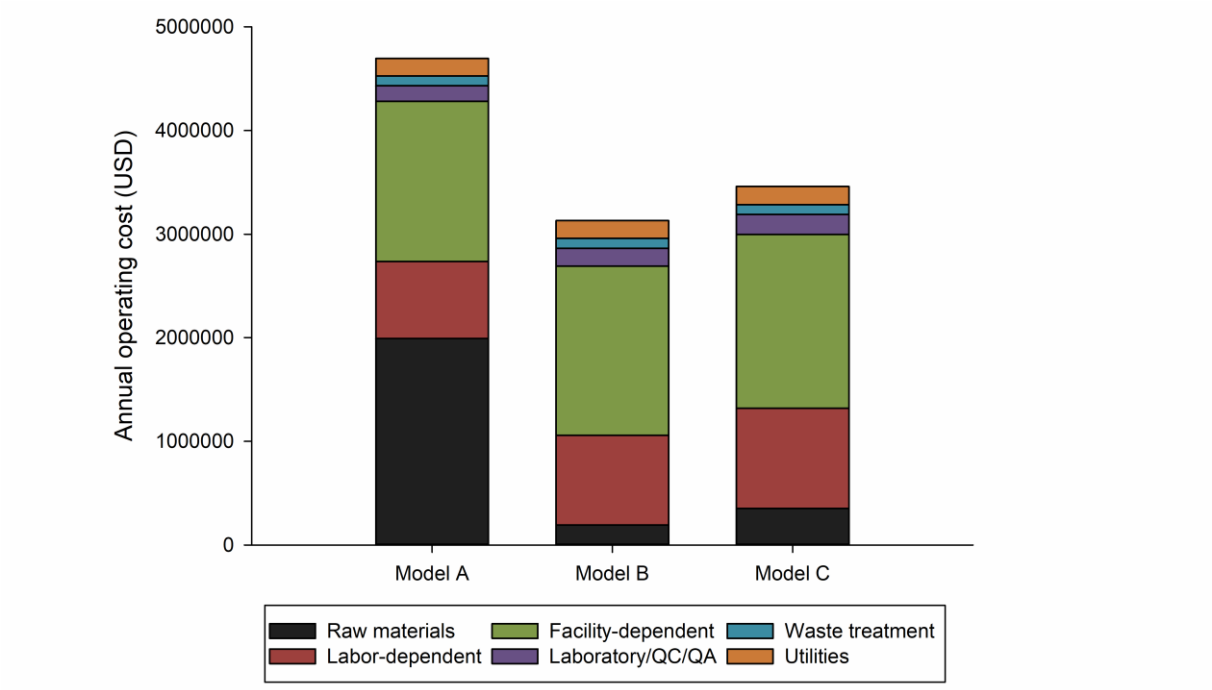

Figure 4. Annual cost breakdown of the three models proposed.

When FBWs are used as alternative nutrient sources, the annual costs are significantly lower than the model with bulk materials. This is mainly due to the decrease in raw material costs for the model B and C. This demonstrates the real convenience of using these FBWs as low cost nutrient sources for the cultivation of biomass with high added value.

In the work of Pleissner et al., [12], the annual operating cost for the cultivation of *C. pyrenoidosa* was almost 10 million €, which is about double the cost of model A, and three times the cost of FBW-based models B and C. The authors reported that two-third of the operational cost is associated to the demand of amylase and protease used to hydrolyze food waste. In our case instead, the high percentage of operating cost are the facility-dependent, which take into account the costs of depreciation and maintenance. This is in agreement with

other studies that confirm the facility-dependent costs as the major portion of total operating costs [27].

Acien et al., (2012) reported that the raw material costs for autotrophic microalgae represent only 2.7% of the total production costs [28], which is definitely far from the reported values in this study. In fact, the recycle of FBWs seems to be much more advantageous for heterotrophic cultures rather than for autotrophic ones. In the work of Kwan et al., (2015), the raw materials for the cultivation of *C. pyrenoidosa* accounts for the 46% of the total operating costs [9], which is in line with our study.

Economic assessment summary and profitability analysis

The economic summary of the three models is reported in Table 6.

Table 6. Economic assessment summary

	Model A	**Model B**	**Model C**
Total capital investment (US$)	9,407,000	9,753,000	10,043,000
Operating cost (US$)	4,694,000	3,132,000	3,441,000
Revenue (US$)	5,703,000	5,002,000	4,907,000
Batch size (Kg MP)	1,863.8	1,634.49	1,603.69
Cost basis annual rate (Kg MP/yr)	285,161	250,077	245,365
Unit production cost (US$/Kg MP)	16.46	12.52	14.02
Unit production revenue (US$/Kg MP)	20	20	20
Gross margin (%)	17.7	37.39	29.88
Return on investment (%)	17.65	24.56	20.88
Payback time (years)	5.67	4.07	4.79
Internal rate of return (%)	15.81	23.93	19.65
Net present value (US$)	5,557,000	11,316,000	8,579,000

MP= main product, referring to biomass.

As already mentioned above, the highest capital investment is that of model C. The revenues of model A resulted US$ 5,703,000 while for model B and C were US$ 5,002,000 and US$ 4,907,000 respectively. This difference in revenues detected between the three models is due to a different growth performance by biomass between standard bulk materials and alternative FBWs media. In fact, as reported in previous studies, a lower productivity of thraustochytrids is generally observed when a food waste-based medium is used instead of standard cultivation medium [13,16]. This difference has been reported in order to evaluate a scenario that is as realistic as possible. Nevertheless, the lower production costs are enough to compensate this

difference with model A. Indeed, the difference in operating costs also translates into a higher profit margin, which is 17.7% for standard cultivation model vs the 37.39% of the model B and 29.88% of model C. The revenues are made by assuming that the biomass is sold for $ 20 per kg. Setting a price for this type of product is difficult as it depends on the quality of the biomass. In our case the price was established after research on market price of Alibaba website in order to obtain a realistic selling price.

In scientific literature, the prices of powdered biomass are not well defined and proven. For heterotrophic *C. pyrenoidosa* a selling price of 36 €/kg (circa 40 US$/kg) is stated. In the PUFACHAIN project report, a selling price for PUFA-rich biomass was estimated at US$ 17 to US$ 36 per kg of dried biomass, depending on production capacity. The authors stated this potential cost price for algae biomass in a market scenario for Southern Europe, which is comparable to our model located in Italy [29]. Thus, our assumption of 20 US$/kg of biomass could be considered a reasonable price.

The results of profitability analysis were also resumed in Table 6. The model B resulted the scenario with the major economic feasibility, with a payback time of 4.07 years and a NPV of 11.31 million US$. The standard cultivation scenario reported a NPV of US$ 5,557,000 with a payback time of 5.67 years. NPV is a vital parameter to understand if a project is either worth investing or not. In our case, the scenarios with FBWs resulted the most lucrative and strong processes to invest in respect to the standard cultivation method. In particular, model B reported the highest NPV value, which suggests that this scenario would be the most promising process. However, the model C has the potential to use a dairy effluent during the process as source of nutrient. The use of an effluent can represent a potential source of revenue for this type of plant, as the treatment of this type of wastewater represents a high cost for the dairy producers, which could pay a waste treatment fee [9]. This additional source of income could make the process even more cost-effective.

Sensitivity analysis

In Figure 5 are reported the results of the sensitivity analysis conducted with SuperPro Designer with a batch throughput ranging from 1000 to 3000 Kg/batch of biomass powder.

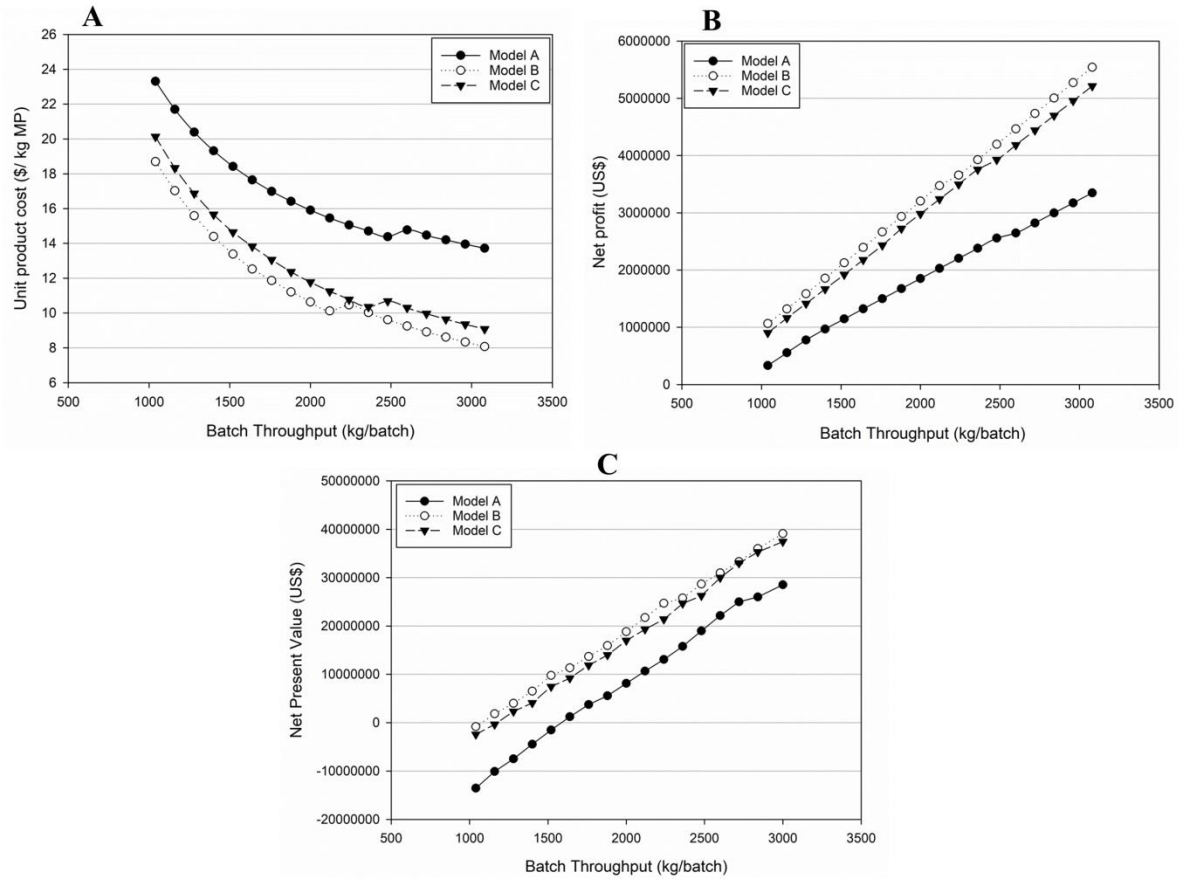

Figure 5. Sensitivity analysis based on unit product cost (A), net profit (B) and net present value (C) as function of batch throughput.

This analysis was carried out to evaluate the effect of potential future growth improvements on the biomass productivity. As shown in the figure, by increasing the biomass productivity per single batch, there is a significant decrease in production costs. It is also possible to observe the difference in production costs between the standard model (Figure 5A) and those involving the use of FBWs.

The difference in net profit with increasing biomass produced was also assessed (Figure 5B). In this case too, with an increase to 3000 Kg/batch of powder biomass, it is possible to maximize profits and make these alternative models more economically feasible. Sensitivity analysis was then also performed for NPV (figure 5C). Model A reported a negative NPV value up to 1583 Kg/batch, after which it increases with a positive trend. For model B and C instead, the reported NPV values are positive even at low batch throughput values, denoting an increased profit perspectives than the standard cultivation model.

It has been reported that the production yield, together with factors related to the bioprocessing system, represent the greatest factor of uncertainty in the development of new

in emerging technology systems [30]. In fact, improved biomass productivity increases the economic feasibility of the process considerably, reducing production costs by half in the new FBWs medium scenarios. For this reason, it is also important to optimize biomass productivity in the new growth conditions.

Potential revenues and economic aspects of obtained biomass

Powdered *Aurantiochytrium* biomass is an important source of high-value bioproducts. First of all, DHA is the component of greatest commercial interest obtainable from this biomass. *Aurantiochytrium* is universally recognized as an alternative source of DHA respect to fish oil and terrestrial plants [5,31]. The PUFA market is currently dominated by fish oil, but algae oils are gaining greater market share every year, aided also by an increased consumer awareness about sustainable bio-based products [31]. In this work we considered a biomass at 40% in lipids, and 18% in DHA. This means that for every Kg of *Aurantiochytrium* produced, it is possible to extract 400 g of oil with 35-40% DHA. In terms of production costs, to obtain 1 liter of *Aurantiochytrium* oil produced under our conditions, the costs would range from US$ 30 for Model B to US$ 41 for Model A. However, the costs of lipids extractions were not considered in this evaluation, which in any case are not too high. In fact, the costs of extraction with supercritical CO2 are around 2.3 €/kg, which has a relatively low impact on production costs [32]. In any case, the production costs to obtain DHA oil with alternative FBWs scenarios would be reduced of 27%. Furthermore, the defatted biomass represent an element with economic potential as it could be used for biofuel production [33]; animal feeding supplements [34]; biogas generation via anaerobic digestion [35] or used as source of antioxidants [36]. This defatted cake could be sold at a price of 66 US$ dry tonne−1 which is the estimated price for animal feeding meal [33]. These factors altogether can contribute to further reducing the costs related to the production of biomass and DHA and make the oil competitive with the fish oil.

6.4. Conclusion

Costs related to feedstock and raw materials have a great impact on the overall economics of *Aurantiochytrium* sp. production. However, raw material costs can be reduced by up to 10 times using FBWs. The combination of SBY and SOS as nutrient sources resulted the most economic feasible process.

The results of this study may serve to lay the foundations for future developments in the field of omega-3 biotechnology for thraustochytrids. Moreover, further optimizations on downstream processing are needed to lower the production costs and open future perspectives for implementing a biorefinery production model able to increase sustainability of the whole process.

References

1. Grandviewresearch Omega-3 market size & share Available online: https://www.grandviewresearch.com/industry-analysis/omega-3-market (accessed on Dec 4, 2021).

2. Olsen, R.E.; Waagbø, R.; Melle, W.; Ringø, E.; Lall, S.P. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds; Turchini, G.M., Ng, W.-K., Tocher, D.R., Eds.; CRC Press, 2010; ISBN 9780429151033.

3. Tan, K.; Ma, H.; Li, S.; Zheng, H. Bivalves as future source of sustainable natural omega-3 polyunsaturated fatty acids. Food Chem. 2020, 311, 125907, doi:10.1016/j.foodchem.2019.125907.

4. Lenihan-Geels, G.; Bishop, K.S.; Ferguson, L.R. Alternative sources of omega-3 fats: Can we find a sustainable substitute for fish? Nutrients 2013.

5. Russo, G.L.; Langellotti, A.L.; Oliviero, M.; Sacchi, R.; Masi, P. Sustainable production of food grade omega-3 oil using aquatic protists: Reliability and future horizons. N. Biotechnol. 2021, 62, 32–39, doi:10.1016/j.nbt.2021.01.006.

6. Gupta, A.; Barrow, C.J.; Puri, M. Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. Biotechnol. Adv. 2012.

7. Ji, X.J.; Ren, L.J.; Huang, H. Omega-3 biotechnology: A green and sustainable process for omega-3 fatty acids production. Front. Bioeng. Biotechnol. 2015.

8. Hall, C.A.S.; Benemann, J.R. Oil from algae? Bioscience 2011.

9. Kwan, T.H.; Pleissner, D.; Lau, K.Y.; Venus, J.; Pommeret, A.; Lin, C.S.K. Techno-economic analysis of a food waste valorization process via microalgae cultivation and co-production of plasticizer, lactic acid and animal feed from algal biomass and food waste. Bioresour. Technol. 2015, doi:10.1016/j.biortech.2015.09.003.

10. Pleissner, D.; Lam, W.C.; Sun, Z.; Lin, C.S.K. Food waste as nutrient source in heterotrophic microalgae cultivation. Bioresour. Technol. 2013, 137, 139–146, doi:10.1016/j.biortech.2013.03.088.

11. Sloth, J.K.; Jensen, H.C.; Pleissner, D.; Eriksen, N.T. Growth and phycocyanin synthesis in the heterotrophic microalga Galdieria sulphuraria on substrates made of food waste from restaurants and bakeries. Bioresour. Technol. 2017, 238, 296–305, doi:10.1016/j.biortech.2017.04.043.

12. Pleissner, D.; Smetana, S. Estimation of the economy of heterotrophic microalgae- and insect-based food waste utilization processes. Waste Manag. 2020, doi:10.1016/j.wasman.2019.10.031.

13. Russo, G.L.; Langellotti, A.L.; Blasco, T.; Oliviero, M.; Sacchi, R.; Masi, P. Production of Omega-3 Oil by Aurantiochytrium mangrovei Using Spent Osmotic Solution from Candied Fruit Industry as Sole Organic Carbon Source. Processes 2021, 9, 1834, doi:10.3390/pr9101834.

14. Ryu, B.-G.; Kim, K.; Kim, J.; Han, J.-I.; Yang, J.-W. Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, Aurantiochytrium sp. KRS101. Bioresour. Technol. 2013, 129, 351–359, doi:10.1016/j.biortech.2012.11.049.

15. Humhal, T.; Kastanek, P.; Jezkova, Z.; Cadkova, A.; Kohoutkova, J.; Branyik, T. Use of saline waste water from demineralization of cheese whey for cultivation of Schizochytrium limacinum PA-968 and Japonochytrium marinum AN-4. Bioprocess Biosyst. Eng. 2017, 40, 395–402, doi:10.1007/s00449-016-1707-5.

16. Russo, G.L.; Langellotti, A.L.; Verardo, V.; Martín-García, B.; Pierro, P. Di; Sorrentino, A.; Baselice, M.; Oliviero, M.; Sacchi, R.; Masi, P. Formulation of New Media from Dairy and Brewery Wastes for a Sustainable Production of DHA-Rich Formulation of New Media from Dairy and Brewery Wastes for a Sustainable Production of DHA-Rich Oil by Aurantiochytrium mangrovei. 2021, doi:10.3390/md20010039.

17. Somers, M.D.; Chen, P.; Clippinger, J.; Cruce, J.R.; Davis, R.; Lammers, P.J.; Quinn, J.C. Techno-economic and life-cycle assessment of fuel production from mixotrophic Galdieria sulphuraria microalgae on hydrolysate. Algal Res. 2021, doi:10.1016/j.algal.2021.102419.

18. Chauton, M.S.; Reitan, K.I.; Norsker, N.H.; Tveterås, R.; Kleivdal, H.T. A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: Research challenges and possibilities. Aquaculture 2015, 436, doi:10.1016/j.aquaculture.2014.10.038.

19. Sano Coelho, R.; Cuellar, M.C.; Franco, T.T.; van der Wielen, L.A.M. Techno-economic assessment of heterotrophic microalgae biodiesel production integrated with a sugarcane bio-refinery. Biofuels, Bioprod. Biorefining 2021, 15, doi:10.1002/bbb.2174.

20. Morowvat, M.H.; Ghasemi, Y. Maximizing Biomass and Lipid Production in Heterotrophic Culture of Chlorella vulgaris: Techno-Economic Assessment. Recent Pat. Food. Nutr. Agric. 2018, doi:10.2174/2212798410666180911100034.

21. Sari, Y.W.; Kartikasari, K.; Widyarani; Setyaningsih, I.; Lestari, D. Techno-economic assessment of microalgae for biofuel, chemical, and bioplastic. In Microalgae; 2021.

22. Ferreira da Silva, A.; Brazinha, C.; Costa, L.; Caetano, N.S. Techno-economic assessment of a Synechocystis based biorefinery through process optimization. In Proceedings of the Energy Reports; 2020.

23. Hong, W.-K.; Yu, A.; Oh, B.-R.; Park, J.M.; Kim, C.H.; Sohn, J.-H.; Kondo, A.; Seo, J.-W. Large-Scale Production of Microalgal Lipids Containing High Levels of Docosahexaenoic Acid upon Fermentation of <i>Aurantiochytrium</i> sp. KRS101. Food Nutr. Sci. 2013, doi:10.4236/fns.2013.49a1001.

24. Farid, M.A.A.; Roslan, A.M.; Hassan, M.A.; Hasan, M.Y.; Othman, M.R.; Shirai, Y. Net energy and techno-economic assessment of biodiesel production from waste cooking oil using a semi-industrial plant: A Malaysia perspective. Sustain. Energy Technol. Assessments 2020, doi:10.1016/j.seta.2020.100700.

25. Woinaroschy, A.S.T. Simulation and optimization of citric acid production with SuperPro designer using a client-server interface. REV. CHIM 2009, 9, 979–983.

26. Mupondwa, E.; Li, X.; Boyetchko, S.; Hynes, R.; Geissler, J. Technoeconomic analysis of large scale production of pre-emergent Pseudomonas fluorescens microbial bioherbicide in Canada. Bioresour. Technol. 2015, 175, 517–528, doi:10.1016/J.BIORTECH.2014.10.130.

27. Petrides, D.; Carmichael, D.; Siletti, C.; Koulouris, A. Biopharmaceutical process optimization with simulation and scheduling tools. Bioengineering 2014, doi:10.3390/bioengineering1040154.

28. Acién, F.G.; Fernández, J.M.; Magán, J.J.; Molina, E. Production cost of a real microalgae production plant and strategies to reduce it. Biotechnol. Adv. 2012.

29. Van der Voort, M.P.J.; Spruijt, J.; Potters, J.; de Wolf, P.L.; Elissen, H.J.H. Socio-economic assessment of Algae-based PUFA production. Public Output Rep. PUFAChain Proj. 2017.

30. Parsons, S.; Abeln, F.; McManus, M.C.; Chuck, C.J. Techno-economic analysis (TEA) of microbial oil production from waste resources as part of a biorefinery concept: assessment at multiple scales under uncertainty. J. Chem. Technol. Biotechnol. 2019, doi:10.1002/jctb.5811.

31. Cvejic, J.H.; Langellotti, A.L.; Bonnefond, H.; Verardo, V.; Bernard, O. Microalgae as a source of edible oils. In Lipids and Edible Oils; 2020.

32. Fiori, L.; Manfrini, M.; Castello, D. Supercritical CO2 fractionation of omega-3 lipids from fish by-products: Plant and process design, modeling, economic feasibility. Food Bioprod. Process. 2014, doi:10.1016/j.fbp.2014.01.001.

33. Ou, L.; Thilakaratne, R.; Brown, R.C.; Wright, M.M. Techno-economic analysis of transportation fuels from defatted microalgae via hydrothermal liquefaction and hydroprocessing. Biomass and Bioenergy 2015, doi:10.1016/j.biombioe.2014.11.018.

34. Xiao, F.; Xing, J.; Li, H.; Xu, X.; Hu, Z.; Ji, H. Effects of the defatted Schizochytrium sp. on growth performance, fatty acid composition, histomorphology and antioxidant status of juvenile mirror carp (Cyprinus carpio var. specularis). Aquac. Res. 2021, doi:10.1111/are.15150.

35. Yang, Z.; Guo, R.; Xu, X.; Fan, X.; Luo, S. Fermentative hydrogen production from lipid-extracted microalgal biomass residues. Appl. Energy 2011, doi:10.1016/j.apenergy.2010.09.009.

36. Cai, X.; Yan, A.; Fu, N.; Wang, S. In vitro antioxidant activities of enzymatic hydrolysate from schizochytrium sp. and its hepatoprotective effects on acute alcohol-induced liver injury in vivo. Mar. Drugs 2017, doi:10.3390/md15040115.

37. Globenewswire Available online: https://www.globenewswire.com/news-release/2019/12/11/1959460/0/en/Glucose-Glucose-Syrup-Market-in-the-EU-2007-2025-Analysis-and-Outlook-Germany-to-Remain-the-Largest-Market-Despite-Recession-Fears.html (accessed on Dec 5, 2021).

38. Industryarc Available online: https://www.industryarc.com/Report/18940/yeast-extracts-market (accessed on Dec 5, 2021).

Chapter 7

Conclusion and future perspectives.

Aquatic protists are considered a promising sustainable source of long chain polyunsaturated fatty acids (LC-PUFA) and other commodities. In particular, heterotrophic protists have shown high PUFA productivity compared to autotrophic organisms. However, the costs related to feedstocks and raw materials showed a great impact on the overall economics of heterotrophic biomass production. For this reason, the search for alternative sources of nutrients for fermentation processes is necessary to develop new sustainable processes. For this reason, the reuse of food by-products and waste (FBWs) for a sustainable cultivation of aquatic protists has been evaluated in this thesis.

In **Chapter 3**, the use of second cheese whey (SCW) has been evaluated as the main source of nutrients for the growth of the extremophilic microalga *Galdieria sulphuraria*. SCW proved to be an ideal growth medium for this microalga, but it required a dilution to 60% and nitrogen supplementation to boost the biomass growth. The nutrient composition has been also optimized using response surface methodologies (RSM) in order to establish the optimal nitrogen supplementation. Fatty acids profile was affected positively by the new SCW media, obtaining a higher PUFA content respect to the standard cultivation medium.

Another FBWs tested was the spent candied osmotic solution (SOS) coming from the processing of candied fruit. In **Chapter 4**, SOS was used as the main source of organic carbon for the growth of *Aurantiochytrium mangrovei*. This thraustochytrid has been reported to be an excellent producer of DHA, with a certain metabolic versatility. In fact, the SOS was used in this study as sole organic carbon source for the cultivation of *A. mangrovei*, without significant differences with the standard cultivation medium. In that case also, the optimal nutrient composition has been determined with RSM. With the optimized condition, the biomass productivity registered was 3.7 g L^{-1} day^{-1} and DHA productivity 475 mg $L^{-1}day^{-1}$.

Given the high efficiency of this protist in using FBWs to produce DHA, it was also used in a subsequent study. In **Chapter 5**, the use of mozzarella stretching water (MSW) as a growth medium was evaluated for the cultivation of *A. mangrovei*. The protist was found to be unable to metabolize the lactose of which MSW is rich, and for this reason the medium was subjected to enzymatic hydrolysis. Since the MSW is not able to supply alone the nitrogen quota necessary for the growth of the protist, a supplementation of spent brewery yeast (SBY) was

carried out. SBY is another FBWs that could be valorized after biomass cultivation. In fact it is a waste rich in nitrogen and mineral salts. A blend of the two FBWs was carried out and optimized via central composite design. The optimization leads to a biomass DW of 10.14 g L^{-1} with 38.9% of lipids and 29.8% of DHA on total fatty acids, proving the suitability of this new culture media made from FBWs.

In **Chapter 6**, the insights of this thesis were combined in a techno-economic model. Projections were made on biomass production costs for a hypothetical facility located in Italy with a production volume of 150 m^3 for each batch. In this study we compared the standard cultivation process using bulk materials with an alternative process using only FBWs as growth medium. Our estimations indicated that using FBWs as only nutrient sources for cultivation of Thraustochytrids rich in DHA, decreases the production costs from 16.46 US$/Kg (from standard cultivation) to 12.52 US$/Kg using a medium made from SOS and SBY. Moreover, the net present value (NPV) and return on investment (ROI) were increased from US$ 5,557,000 and 17.65% of the standard cultivation to US$ 11,316,000 and 24.56% with the proposed model using only FBWs. Altogether, this thesis successfully designed and applied FBWs valorization for the cultivation of biomass rich in high added value compounds. In particular the reuse of FBWs has been designed for an industrially relevant aquatic protist strain (*A. mangrovei*) proving its effectiveness in reducing production costs. The results of this thesis may serve to lay the foundations for future developments in the field of omega-3 biotechnology from aquatic protists using FBWs. Further studies should be made to evaluate in a biorefinery model all the possible utilizations of co-products coming from the biomass fractionation in order to increase both sustainability and economic profitability of the process. All these efforts will be able to shift this product from the high value market of food supplements to the market of bulk commodities, competing efficiently with the fish oil, to date the main source of LC-PUFA for both human and animal consumption.

List of publications:

Scientific Journals

- Massa, M., Buono, S., Langellotti, A. L., Martello, A., Russo, G. L., Troise, D. A., Fogliano, V. (2019). **Biochemical composition and in vitro digestibility of Galdieria sulphuraria grown on spent cherry-brine liquid**. *New biotechnology*, *53*, 9-15.
- Giovanni L. Russo, Langellotti, A. L., Genovese, A., Martello, A., & Sacchi, R. (2020). **Volatile compounds, physicochemical and sensory characteristics of Colatura di Alici, a traditional Italian fish sauce**. *Journal of the Science of Food and Agriculture*, *100*(9), 3755-3764.
- Giovanni L. Russo, Antonio L. Langellotti, Maria Oliviero, Raffaele Sacchi and Paolo Masi. **Sustainable production of food grade omega-3 oil using aquatic protists: reliability and future horizons**. *New Biotechnology*, 2020, 62, 32–39
- Giovanni L. Russo, Antonio L. Langellotti, Maria Oliviero, Raffaele Sacchi and Paolo Masi. (2021). **Valorization of second cheese whey through cultivation of extremophile microalga *Galdieria sulphuraria*.** *AIMS environmental science*, 2021. 8(5):435-448
- Giovanni L. Russo, Antonio L. Langellotti, Thierry Blasco, Maria Oliviero, Raffaele Sacchi and Paolo Masi. (2021). **Production of omega-3 oil by *Aurantiochytrium mangrovei* using spent osmotic solution from candied fruit industry as sole organic carbon source**. *Processes*, 9(10):1834
- Giovanni L. Russo, Langellotti, A. L., Verardo, V., Martín-García, B., Di Pierro, P., Sorrentino, A., Oliviero M., Sacchi R., Masi, P. (2022). **Formulation of New Media from Dairy and Brewery Wastes for a Sustainable Production of DHA-Rich Oil by *Aurantiochytrium mangrovei***. *Marine Drugs*, *20*(1), 39.
- Giovanni L. Russo, Antonio L. Langellotti, Raffaele Sacchi, Paolo Masi. (2022). **Techno-economic assessment of DHA-rich *Aurantiochytrium* sp. production using food industry by-products and waste streams as alternative growth media.** *Bioresource Technology Reports*, 100997 (https://doi.org/10.1016/j.biteb.2022.100997)

BOOK CHAPTERS:

- B. García, M. Razola-Díaz, G. L. Russo, E. Giambanelli, A. L. Langellotti, A. Gómez-Caravaca, V. Verardo. **Chlorella As Valuable Ingredient For Functional Foods**. Book Title: Super and Nutraceutical Foods: Composition and Technology. NovaPublisher, March 2021. ISBN: 978-1-53619-082-3

Poster & oral presentations

- Cedric Dufloer, Antonio L. Langellotti, Giovanni L. Russo**,** Jelena Cvejic, Thierry Blasco. **Compound specific stable isotope analysis of n-3 PUFA from different microalgae products**. Algaeurope 2019, Paris.
- Giovanni L. Russo, Vito Verardo, Cédric Dufloer, Thierry Blasco, Paolo Masi, Raffaele Sacchi, Jelena Cvejic, Antonio L. Langellotti. **From food by-products to n-3 LCPUFA through microalgae: the SUSPUFA project.** Algaeurope 2019, Paris.
- Giovanni L. Russo. Poster presentation with title: **Sustainable production of health-promoting n-3 LC-PUFA using agro food industry by-products**

through microalgae. European Research Night – Meet me tonight, September 2019. Portici.

- Giovanni L. Russo., Crussy P., Oliviero M., Baselice M., Masi P., Blasco T., Sacchi R. **Cultivation of *Aurantiochytrium mangrovei* using spent osmotic solution as carbon source.** Poster presentation, Algaeurope 2020, Online event.
- Russo, G. L., Langellotti, A. L., Verardo, V., Martín-García, B., Di Pierro, P., Sorrentino, A., Oliviero M., Sacchi R., Masi, P. **Mozzarella By-Product As New Culture Medium For The Pufa Producer Aurantiochytrium Mangrovei**. Algaeurope 2021.
- Giovanni L. Russo**. Bioconversion of agri-food waste in high added value molecules through microalgae cultivation.** Ecomondo conference 2021.

Ringraziamenti.

Per questo lungo viaggio ringrazio innanzitutto i miei tutor Prof. Raffaele Sacchi, il Dr Luca Langellotti ed il gruppo di lavoro alla sezione acquacoltura del CAISIAL (Marcone, Mietta e gli altri colleghi che mi hanno affiancato in questi anni) che hanno permesso la realizzazione di questo percorso formativo;

Ringrazio la mia famiglia per il sostegno fisico e morale che mi forniscono ogni giorno.

Sono cresciuto, sono caduto, non mi sono ancora rialzato, ma con la conclusione di questo percorso adesso posso raccontare a me stesso quello che sono in grado di realizzare.

Per questo il ringraziamento più grande va a quella parte di me che nonostante tutte le delusioni, la solitudine, i soprusi con cui bisogna convivere nella società, riesce ancora a dare un colpo di reni per spingermi ad andare avanti, inesorabilmente.