QUANTITATIVE SUSTAINABILITY ANALYSIS OF THIRD GENERATION BIOFUELS USING PROCESS DATA FROM MICROALGAE BIOREFINERY

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PhD Thesis Co-Supervision agreement for a Double Degree (DD) PhD between the University of Porto (Doctoral Program in Chemical and Biological Engineering) and the Federal University of Rio de Janeiro (Doctoral Program in Energy and Environmental Planning)

2018

COPPE
Instituto Alberto Luiz Coimbra de Pós-Graduação e Pesquisa de Engenharia
UFRJ

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Planejamento Energético, COPPE, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Planejamento Energético.

Orientadores: Marcos Aurélio Vasconcelos de Freitas
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Rio de Janeiro
Abril de 2018

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TESE SUBMETIDA AO CORPO DOCENTE DO INSTITUTO ALBERTO LUIZ COIMBRA DE PÓS-GRADUAÇÃO E PESQUISA DE ENGENHARIA (COPPE) DA UNIVERSIDADE FEDERAL DO RIO DE JANEIRO COMO PARTE DOS REQUISITOS NECESSÁRIOS PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS EM PLANEJAMENTO ENERGÉTICO.

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RIO DE JANEIRO, RJ – BRASIL
ABRIL DE 2018

Vieira, Monique Branco

Quantitative Sustainability Analysis of Third Generation Biofuels Using Process Data from Microalgae Biorefinery/ Monique Branco Vieira. – Rio de Janeiro: UFRJ/COPPE, 2018.

XX, 124 p.: il.; 29, 7cm.

Orientadores: Marcos Aurélio Vasconcelos de Freitas
Marco Aurélio dos Santos
Nídia de Sá Caetano

Tese (doutorado) – UFRJ / COPPE / Programa de Planejamento Energético, 2018.

Referências Bibliográficas: p. 96 – 117.

1. Biofuels. 2. Microalgae. 3. Biorefinery. 4. Sustainability. 5. High-Value Compounds. I. Freitas, Marcos Aurélio Vasconcelos de *et al.* II. Universidade Federal do Rio de Janeiro, COPPE, Programa de Planejamento Energético. III. Título.

"– Paremos que hay novedad.
¡Mira, mira el Bío-Bío!
– ¡Ah! mama, párate, loca,
para, que nunca lo he visto.
¿Y para dónde es que va?
No para y habla bajito,
y no me asusta como el mar
y tiene nombre bonito.
. . .
– ¿Cómo dices que se llama?
Repite el nombre bonito.
– Bío-Bío, Bío-Bío,
qué dulce que lo llamaron
por quererle nuestros indios.
. . .
– Dime tú que has visto cosas
¿hay otro más grande y lindo?
– No lo hay en tierra chilena,
pero hay unos que no he dicho,
hay más lejos unos lagos
que acompañan sin decirlo
y hacia ellos vamos llegando
y ya pronto llegaremos."

(Gabriela Mistral)

" . . .
Da eternidade,
Sem pressa de chegar ao seu destino.
Ancorado e feliz no cais humano,
É num antecipado desengano
Que ruma em direcção ao cais divino.
. . .
Por isso, é devagar que se aproxima
Da bem-aventurança.
É lentamente que o rabelo avança
Debaixo dos seus pés de marinheiro.
E cada hora a mais que gasta no caminho
É um sorvo a mais de cheiro
A terra e a rosmaninho!"

(Miguel Torga)

Aos meus pais, Renê (in memoriam) e Cléa por serem meus alicerces. Ao Alexandre por ser meu pilar. A vocês dedico esta tese!

Agradecimentos

Foi difícil pensar que esse dia chegaria e que essas folhas poderiam ser preenchidas de gratidão a tantos e tantas que fizeram esse trabalho se tornar possível.

Esta tese nasce de um processo profundo de autoconhecimento, de autodescoberta, de transição de uma fase um tanto turbulenta, que veio a culminar em mares nunca antes navegados. E assim tinha de ser pois "mar calmo não faz bom marinheiro". Literalmente cruzei cordilheiras e atravessei oceanos, aprendi a driblar o mar revolto, a segurar firme no timão, reorientar a proa e foram o mar e a água doce que fizeram esse peixinho aprender a nadar, junto com uns seres muito especiais que encontrei no caminho: as algas!

Esse caminho longo me levou a muitos lugares que nunca poderia pensar, caminhos onde pude resgatar muitas histórias da família "portuguesa com certeza" que foi buscar no Brasil um alento às dificuldades encontradas naqueles tempos, uma história resgatada que não podia e não vai ficar esquecida pelos descendentes que estes ilustres lusitanos produziram. À vocês meus queridos vovôs, eu dedico este caminho inverso, com toda a dedicação, carinho, respeito e admiração com que trataram a nova terra e a nova vida que foram construir no além-mar. A vocês dedico toda a saudade sentida, todas as lembranças deixadas daquele mar tão azul dos Ilhéus Açorianos.

Aos meus alicerces, minha mãe Cléa e o meu pai Renê (*in memoriam*), que me ensinaram através de diversas maneiras que podemos alcançar tudo que quisermos, se assim colocarmos amor e dedicação até nas mais simples das tarefas. Que nossos objetivos se alcançam com muito esforço, nada é fácil, nada será fácil... E assim foi, seu empenho na minha educação e a superação de tantos obstáculos, me impulsionaram a nunca desistir. Agradeço também à minha vovózinha Amélia, ao meu irmão Marcelo, à minha sobrinha Ana Clara e cunhada Cátia, por todo o amor a distância, por toda a preocupação e carinho que me fizeram mais forte. Essa tese eu dedico a vocês!

E ainda foi nestes caminhos longos que encontrei várias pessoas que eu poderia chamar de anjos educadores. Eu não poderia deixar de lembrar do meu amigo e grande chefe por muitos anos, Alfredo Cardoso, que me deu a oportunidade de fazer umas das coisas que eu mais gosto na vida: ensinar! Obrigada por sua amizade de sempre e por vibrar comigo a cada conquista.

Ao meu professor e orientador Marcos Freitas, que me aceitou no IVIG e me deu a oportunidade de começar a trabalhar em um dos lugares mais incríveis que já estive, onde

conheci tanta gente amiga, inteligente, multidisciplinar e as vezes um pouco louquinha, que me fizeram sentir em um nicho tão familiar e acolhedor, que por vezes esquecia que estava no trabalho. Ao meu professor Marcos, eu agradeço imensamente a liberdade que meu deu para voar, ao incentivo de cada dia e a sua forma calma e certeira de resolver os problemas.

Agradeço também ao professor Marcos Santos, que me aceitou como aluna no PPE e apostou que eu poderia chegar até o fim. Agradeço seu apoio com tantas burocracias que se fizeram necessárias e a sua destreza em conseguir resolvê-las tão diplomaticamente.

À minha querida professora Nídia Caetano, a qual me acolheu na Universidade do Porto, aceitando ser minha orientadora e me incentivando a cada novo desafio. Agradeço por acreditar no meu trabalho, sem ao menos me conhecer, por acreditar que poderíamos chegar até aqui hoje. Agradeço pelos seus ensinamentos, pela presteza, disponibilidade em orientar e reorientar os trabalhos em todos os momentos do caminho que estivemos juntas e; claro, pelos cafezinhos sempre fresquinhos do seu gabinete. A senhora é única!

Um agradecimento especial a minha amiga Ágatha, por me apresentar ao IVIG, em um momento em que passava por uma reformulação dos meus desafios e objetivos e, nesse momento, encontrei um time que eu sempre procurei, sem ao menos saber que existia.

À uma amiga muito especial que o PPE e o IVIG me deram, a Mariana Padilha, para mim só Mari, a qual foi e tem sido um grande apoio para tudo, desde as terapias em grupo até ser um dos meus braços que ficou no Brasil, sempre disposta a resolver as burocracias importantes para que eu pudesse defender. E sempre com uma palavra amiga para afagar as aflições. Obrigada por tudo, amiga! Agradeço a todos integrantes do IVIG, em especial ao time dos Estádios: Vania, Neilton, Sylvia, Dutra, Vera, Daniel, Zé e tantos outros que passaram e de alguma forma deixaram, cada a um à sua maneira, uma marquinha na nossa alma.

Agradeço imensamente ao GIBMAR, no Chile. Ao professor Cristian Agurto por ter me aceitado no seu laboratório e ter me dado a oportunidade de realizar este trabalho, oferecendo todo o suporte necessário para que tudo corresse bem. Ao Sergio San Martin, um grande amigo que me ensinou tanto sobre as microalgas, desde fazer cultivos em escala piloto até como retirar silicone das mãos depois da manutenção dos fotobiorreatores. Te agradeço a amizade, companheirismo e paciência durante os 9 meses que passei diariamente aprendendo com você no GIBMAR, ao custo de muito café Pilão, e aos outros 1 ano e meio te incomodando à distancia. Te devo no mínimo uns 2 containers de café Pilão.

Aos demais amigos que fiz no GIBMAR, a Patrícia Honorato, uma grande amiga que ficou para a vida, a qual me ajudou muito no laboratório e também pelas diversões pós-laborais.

Não posso deixar de mencionar, minha amiga Chris Landahur, o Angelo Beratto e o Diego Martinez do GIBMAR. Agradeço por todos os momentos descontraídos que passamos no laboratório, tornando os dias de trabalho muito mais leves e prazerosos. Gracias, chiquillos!

Aos meus amigos de vida, a Babi, minha irmã querida por todo o incentivo de sempre, pelo ombro amigo, pelas longas conversas por vídeo, que me fizeram sentir um pouco mais perto de casa. Às minhas amigas Flávia, Tássia e Ericka, por estarem sempre torcendo por mim, esperando por mim e por sabermos que ficaremos velhinhas juntas. Pelos meus amigos da antiga VM Diretoria: Carol, Fino, Léo e Chico. Meus velhos e eternos amigos, com os quais vivemos muitas aventuras e muitas outras ainda nos esperam na nossa maior idade!

Ao administrativo do PPE, Sandrinha e o Paulo, por sua presteza e agilidade em resolver nossos problemas e por estarem sempre dispostos a ajudar em qualquer circunstância.

À professora Alexandra Pinto, diretora do Programa Doutoral em Engenharia Química e Biológica da FEUP, pela presteza em resolver todas as diligências necessárias para que a defesa pudesse ser realizada em conjunto.

Ao CIETI do ISEP pela cedência do espaço destinado aos trabalhos de gabinete e ao convivio diário que tornaram os dias mais serenos e o trabalho mais leve. Agradeço também a equipe do Laboratório de Tecnologia do ISEP, por sua presteza e disponibilidade em auxiliar nas demandas diárias pelos equipamentos do laboratório.

Ao LEPABE da FEUP, por toda assistência e suporte à pesquisa e ainda ao apoio financeiro. A professora Nídia de Sá Caetano, orientadora dessa tese, é membro integrado do LEPABE – Laboratório de Engenharia de Processos, Ambiente Biotecnologia e Energia, financiado pelo POCI-01-0145-FEDER-006939, Fundo Europeu de Desenvolvimento Regional (FEDER), através do COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) e por fundos nacionais através da Fundação para a Ciência e a Tecnologia.

À Reitoria da Universidade do Porto, por todo o apoio necessário para que o processo de cotutela fosse aprovado com êxito.

À Teresa Mata e ao António Martins, dois outros anjos que apareceram no meu caminho, me ajudaram tanto e me ensinaram bastante sobre escrita de artigos. Obrigada pela ajuda de sempre!

À todo o suporte financeiro, através da concessão de bolsas de estudo de doutoramento e mobilidade, de diferentes agências de fomento: PFRH (Programa de formação de Recursos Humanos) da Petrobrás; Programa EBW Plus da Comissão Européia; Convénio de Desempeño Ciencia, Tecnología e Innovación para la Bioeconomía do governo Chileno.

E por último, mas não menos importante, ao meu grande amor e companheiro de vida: Alexandre Hostand. Agradeço imensamente sua generosidade, seu amor, seu companheirismo de cada dia, sua parceria em todos os momentos e sua forma única de saber lidar com meu peculiar modo de ser e minha nada fácil personalidade. Sem você seguramente eu não conseguiria chegar aqui. Essa conquista certamente também é sua! Te amo!

PETROBRAS
EBW Euro Brazilian Windows
Bioeconomía Ciencia, Tecnología e Innovación
FCT Fundação para a Ciência e a Tecnologia
COMPETE 2020
PORTUGAL 2020
UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional

Resumo da Tese apresentada à COPPE/UFRJ como parte dos requisitos necessários para a obtenção do grau de Doutor em Ciências (D.Sc.)

ANÁLISE QUANTITATIVA DA SUSTENTABILIDADE PARA A TERCEIRA GERAÇÃO DE BIOCOMBUSTÍVEIS UTILIZANDO DADOS DE PROCESSO DE UMA BIORREFINARIA DE MICROALGAS

Monique Branco Vieira

Abril/2018

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Programa: Planejamento Energético

As biorrefinarias de microalgas foram propostas como uma estratégia importante para aumentar a rentabilidade econômica dos bioprodutos, as quais dependem do potencial biotecnológico de cada espécie, associado às condições ambientais e às abordagens tecnológicas. Esse conceito implica na valorização da biomassa residual, convertendo-a em biocombustíveis e/ou compostos de alto valor. Este estudo teve como objetivo analisar a composição bioquímica e a viabilidade técnico-econômica de uma usina de biorrefinagem baseada em *Phaeodactylum tricornutum* cultivada em um fotobiorreator de colunas de bolhas, em escala piloto ao ar livre sob condições naturais no Chile, para produção de biocombustíveis e compostos de alto valor. A biomassa da *P. tricornutum* tem uma composição bioquímica com potencial para uso em uma abordagem integrada de biorrefinagem, principalmente para otimizar a viabilidade ambiental e econômica do processo. Foram propostos e analisados 3 cenários distintos para a produção de biocombustíveis e compostos de alto valor, sendo os mais rentáveis aqueles que consideraram a produção e comercialização de fucoxantina e proteína, devido ao alto valor desses compostos no mercado. A produção de biomassa exclusivamente para a produção de biocombustíveis, mostrou-se não ser viável nas condições consideradas na análise. A hipótese de economia de escala foi um fator crítico para o custo de produção da biomassa e viabilidade dessa abordagem. A análise técnico-econômica é essencial para identificar gargalos econômicos e oportunidades para abordar o portfólio de produtos de microalgas para os diferentes cenários de mercado, a fim de auxiliar os tomadores de decisão e propor melhorias nos processos de cultivo e *downstream*.

Abstract of Thesis presented to COPPE/UFRJ as a partial fulfillment of the requirements for the degree of Doctor of Science (D.Sc.)

QUANTITATIVE SUSTAINABILITY ANALYSIS OF THIRD GENERATION BIOFUELS USING PROCESS DATA FROM MICROALGAE BIOREFINERY

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April/2018

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Microalgae biorefineries have been proposed as an important strategy for enhancing the economic profitability of bioproducts, which depends on the biotechnological potential of each species, associated with environmental conditions and technological approaches. The biorefinery concept applied to microalgae also implies valorization of residual biomass, converting it into biofuels and/or high value compounds. This study aimed to analyze the biochemical composition and the technical and economic feasibility of a biorefinery based on *Phaeodactylum tricornutum* cultivated in an outdoor pilot-scale bubble-columns photobioreactor, under natural conditions in Chile, for production of biofuels and high-value compounds. *P. tricornutum* biomass has a potential biochemical composition for using in an integrated biorefinery approach, mainly in order to optimize the environmental and economic feasibility of the process. Three different scenarios were proposed for the production of biofuels and high-value compounds. The most profitable scenarios were those that considered fucoxanthin and protein production and commercialization, due to the high price of these compounds on the market. The production of biomass as a raw material exclusively targeted for biofuel production showed itself not to be feasible under the conditions considered in the analysis. Furthermore, the assumption of economies of scale was shown to be a critical factor for the biomass price and feasibility of this approach. Technical and economic analysis is essential to identify economic bottlenecks and opportunities for addressing a microalgae product portfolio to the different market scenarios, in order to assist decision-makers and propose improvements in the cultivation and downstream processes.

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Chapter 1

Introduction

1.1 Research Context

The consumption and exploitation of a range of non-renewable resources and use of fossil fuels over many decades as a primary energy source for the world economy have led society to face an increasing environmental problem [1, 2]. Along these lines, the development of new technologies and products should be focused on the efficiency and sustainability of resource use, in order to reduce greenhouse gas emissions, waste generation and depletion of natural resources.

Aiming to reduce carbon dioxide emissions and to replace most of the non-renewable global energy matrix with cleaner energy, several energy alternatives have been employed, such as solar, wind, hydroelectric and biomass. Biomass is defined by the Food and Agriculture Organization of the United Nations (FAO) [3] as mass of organic matter from biological origin, excluding those materials entrenched in geological formations and transformed into fossils. Biomass plays a major role in the climate system due to the capacity of photosynthesis to store CO_2 from the atmosphere in organic material. Biofuel production around the world has increased from 19.651 million tons oil equivalent (toe) in 2005 to 74.847 million toe in 2015 [4]. Biofuels can be derived from several biomass resources, including agricultural crops, and waste from municipal, agricultural and forestry byproducts. Biofuels can also be produced from living microorganisms that generate bio-hydrogen or methane as a result of their metabolism [5, 6].

Currently, biofuels are the main substitute for fossil fuels, of which biodiesel and ethanol are the most produced renewable fuels [7]. Biodiesel is the direct substitute for diesel, and ethanol is the substitute for gasoline. Biofuels can be classified according to their production technology into first, second or third generation biofuels. The main difference between types of biofuel generation is the origin of raw material and variations in the final product process.

In first-generation biofuels, the raw material is mainly derived from food crops.

While the first generation of biofuels can bring some benefits in terms of CO_2 emissions mitigation and energy security, there are serious concerns about land use, loss of biodiversity, competition with the food sector, carbon balances and consumption of water resources [8].

Second-generation biofuels have emerged to address some of the disadvantages of first-generation biofuels, because they use non-food feedstocks, which can be considered as waste, or come from non-arable soils, thus not competing with the food sector. The raw material for these fuels is essentially lignocellulosic biomass [8].

Third-generation biofuels are produced from new sources of biomass, for example modified cultures of microorganisms, using modern genetics and nanotechnology processes. The third-generation biofuels are promising for sustainability, because they do not require agricultural land and potable water resources for growth [9].

In this context, microalgae have been reported as one of the promising alternatives for biofuel production. These microorganisms are eukaryotic or prokaryotic, exhibit high photosynthetic efficiency for biomass production and high growth rates and productivity compared to traditional crops [10]. According to Halim et al. [11], due to the high-lipid content of microalgae, they can be used to produce up to 25 times more oil per hectare than the traditional land crops used for biodiesel production, such as palm oil. Furthermore, microalgae require few nutrients for cultivation, are easily adaptable to different environments, and their cells contain a diverse biochemical composition, comprising carbohydrates, proteins, lipids, fatty acids, and pigments, among others. The biochemical features of microalgae are environmentally modulated and depend on the plasticity and resilience of each species to the culture medium and environmental conditions [12].

A microalgae biorefinery is a holistic approach applied to maximize the whole biomass and its components, such as the biomolecules synthesized by algal cells through its metabolic process. In this concept, the algal biomass can be converted into a variety of added-value ingredients for cosmetics, animal feed, human food, and biofuels, which represent additional benefits of the microalgae carbon sequestration process [13].

The idea of producing biofuels from microalgae is not recent, but huge interest in this issue has grown exponentially in recent decades [13]. In fact, one of the great advantages of using microalgae for this purpose is the remarkable versatility of these microorganisms, which has made this approach more sustainable both from the point of view of economics and of energy, besides the numerous environmental advantages [14–16], which will be discussed throughout the thesis.

The present work intends to demonstrate, under experimental and scenario analysis, the viability and the best technological routes for microalgae-based biofuel development, using a biorefinery approach, in order to support decision and strategic planning for the use of available alternatives in improving profitability and efficiency of resource use.

1.2 Motivation and Thesis Organization

Microalgae-based biofuels and bioproducts have been widely studied around the world. Although microalgae biomass has been considered as a good feedstock alternative for biofuel production, the commercial scale has been shown not to be economically profitable. However, the feasibility of this approach should be considered in a specific scenario, based on real process data for microalgae biomass production and downstream process. However, the evaluation of sustainability and techno-economic analysis of algal biofuels and high-valued compounds is currently performed using theoretical data and process assumptions, or even by laboratory scales, which leads to an unrealistic photosynthetic efficiency when production is extrapolated and scaled-up to the natural environmental conditions of a specific region. Therefore, the lack of information about real data available to perform consistent studies about the feasibility of this approach leads to limitations concerning the reliability of analysis, which affects the costs of the project.

Furthermore, the biochemical composition of microalgae biomass is directly influenced by the physical parameters to which the culture is submitted, such as temperature, solar radiation and pH, among others. Consequently, the natural characteristics of the region where the cultivation system will be carried out must be considered to perform analysis concerning the productivity of the microalga biomass and specific bioproducts produced under those conditions.

Therefore, this study aimed to use upstream equipment on the pilot plant scale, to produce data on yield, energy consumption and processing information for microalgae cultivation. Real data obtained from experimental cases were used to model and perform scaled-up scenarios for microalgae biorefinery components, under natural environmental conditions in Concepción, Chile. These data were used as the foundation to produce process flowsheets that then enabled more realistic scale-up analysis. This study will serve as a benchmark for future upstream and downstream process development.

Chile is highly dependent on external sources for energy production, and it is characterized as having lower energy security. Consequently, the search for natural resources to increase national energy production is an essential task for the establishment of a long-term energy policy. In fact, Chile has a limited potential for producing traditional crops for biofuels, which is explained by its lower area of arable land, which represents only 1.3% of the total national territory [17]. Despite this, Chile has an extensive costal area, corresponding from north to south to 4,270 km, against its 177 km from east to west. Consequently, marine resources play an important role in the Chilean economy and their total potential still remains unclear. Hence, the hypothesis of this thesis is about the evaluation of different scenarios for a microalgae-based biorefinery located in Chile, using real process data obtained from a pilot-plant facility found on site, in order to identify bottlenecks in processes and to achieve improvements in the economic profitability of

this industry.

Furthermore, the main question of this thesis was:

Does the biorefinery approach enhance the techno-economic profitability of microalgae-based biofuels produced under natural Chilean conditions?

The thesis project was developed in three different countries (Brazil, Chile and Portugal), and it was part of a co-supervision agreement for a Double Degree (DD) PhD between the Doctoral Program in Chemical and Biological Engineering at the "Faculdade de Engenharia da Universidade do Porto (FEUP)", Porto, Portugal and the Doctoral Program in Energy and Environmental Planning at the "Programa de Planejamento Energético, Instituto Alberto Luiz Coimbra de Pós-graduação e Pesquisa de Engenharia (COPPE), Universidade Federal do Rio de Janeiro (UFRJ)", Rio de Janeiro, Brazil. The experiments with microalgae biomass production were performed at the "Centro de Biotecnología de la Universidad de Concepción", Concepción, Chile, under collaborative work during the PhD visiting research activities of the student.

This thesis was organized in order to present the results achieved during the project development according to academic articles published, submitted and under submission process. Furthermore, the resulting order of chapter presentation in this thesis introduces the steps of analysis and exploitation of biotechnological potential of the studied microalga under a biorefinery approach, in order to characterize the microalga biomass for the production of biofuel and high-value compounds.

Chapter 1 presents the context of research, motivation and organization of the thesis, the objectives and the limits of the study. This chapter seeks to define the basis on which the thesis will be conducted, as well as the problematic that it intends to solve.

Chapter 2 explores the background and state of the art essential for readers to comprehend the aspects and concepts that will be addressed in the study. However, this chapter will not intend to deepen all the concepts that will be dealt with throughout the chapters; since the presentation of the thesis will be in the form of scientific articles, the specificity of each topic will be addressed at the beginning of each chapter.

Chapter 3 explores the potential of *Phaeodactylum tricornutum* based biodiesel production by analyzing the fatty acids profile of this microalga obtained after cultivating it under natural environmental conditions in Chile. This chapter explores the possibility of using one of the potential products from *P. tricornutum* (biodiesel) and its characterization in order to verify if the lipid profile meets the international standard regulations for biodiesel commercialization. The results of this study have already been published in two indexed peer-reviewed journals:

– BRANCO-VIEIRA, M., SAN MARTIN, S., AGURTO, C., DOS SANTOS, MA., FREITAS, MAV., CAETANO, NS. "Analyzing *Phaeodactylum tricornutum* lipid

profile for biodiesel production, Energy Procedia, v 136, pp 369-373, 2017.

– BRANCO-VIEIRA, M., SAN MARTIN, S., AGURTO, C., DOS SANTOS, MA., FREITAS, MAV., MATA, TM., MARTINS, AA., CAETANO, NS. "Potential of *Phaeodactylum tricornutum* for Biodiesel Production under Natural Conditions in Chile", Energies, v. 11, n. 1, pp. 54, 2017.

Chapter 4 analyses the possibility of using the silica of the diatom cell wall for biomass valorization. This chapter aimed to verify the elemental composition of purified biosilica from residual dried biomass of *P. tricornutum* deriving from a diatom-based biorefinery, in order to explore the opportunity of using the Si content for energy and/or nanoparticle applications, as an alternative for both biorefinery waste valorization and to acquire a high grade and renewable silicon resources. The importance of this chapter is centered on the use of residual microalgae biomass after the exploitation of value-added compounds, which could increase the economic viability of microalgae production and reduce waste generation. The results of this chapter are under submission process to peer-reviewed journals:

– BRANCO-VIEIRA, M., SAN MARTIN, S., AGURTO, C., FREITAS, MAV., MATA, TM., MARTINS, AA., CAETANO, NS. "Diatom-based Biosilica for Energy Applications" (submitted).

Chapter 5 presents the biochemical composition of *P. tricornutum* in order to propose three different biorefinery approaches for biofuels production, associated with high-valued compounds as by-products. After studying the possibility of biodiesel production and the best method for Si content purification from residual biomass, this chapter represents the core of the thesis, due to the biochemical characterization of the microalga strain cultivated under specific conditions found in Concepción (Chile). The knowledge of the biochemical composition of the microalga is an important factor to determine the possible final uses of the biomass. The results of this chapter are under submission process to peer-reviewed journals:

– BRANCO-VIEIRA, M., SAN MARTIN, S., AGURTO, C., FREITAS, MAV., MATA, TM., MARTINS, AA., CAETANO, NS. "Biotechnological Approach for Diatom-based Biorefinery" (submitted).

Chapter 6 analyses the technical and economic feasibility of two proposed biorefineries based on *P. tricornutum* cultivated in an outdoor pilot-scale bubble-columns photobioreactor under natural conditions in Chile, for production of biofuels and high-value compounds, using scale-up scenarios based on real process data. The results of this chapter are under submission process to peer-reviewed journals:

– BRANCO-VIEIRA, M., SAN MARTIN, S., AGURTO, C., FREITAS, MAV., MATA, TM., MARTINS, AA., CAETANO, NS. "Techno-Economic Analysis for *Phaeodactylum tricornutum* based Biorefinery" (submitted).

Finally, **Chapter 7** summarizes the main conclusions of the thesis and explains the prospects for future work.

1.3 Objectives

1.3.1 General Objectives

This thesis aims to analyze the culture conditions and biochemical potential of *P. tricornutum* under natural conditions in Chile for biotechnological application, targeting biofuels and high-value compounds production, using a biorefinery approach. Furthermore, it aims to perform a techno-economic evaluation to verify the feasibility of this concept and identify the bottlenecks in the process.

1.3.2 Specific objectives

The specific objectives of this Thesis are, thus, to:

1. Cultivate *P. tricornutum* in pilot-scale bubble-columns photobioreactors under natural conditions in Chile, and to collect all the related information on this process;

2. Analyse the culture growth and lipids composition of *P. tricornutum* cultivated in a pilot-scale bubble-column photobioreactor, under natural conditions in Chile;

3. Analyse the biochemical composition of *P. tricornutum* in order to address the best alternatives for using the microalgae-bioproducts under a biorefinery approach;

4. Evaluate the technical and economic feasibility of a *P. tricornutum*-based biorefinery plant facility for the production of biofuels and high-value compounds.

Chapter 2

Background

2.1 Microalgae as an Alternative Towards Sustainable Sources

Sustainable practices in manufacturing processes have recently received significant support from the bioeconomy concept, which will henceforth be the driving force for the productive sector to move from a linear economy (i.e. resource extraction and disposal) to a circular economy (maximum efficiency in resource use). Obviously, the challenge should focus on combining economic growth with a rising population, which leads to a steady increase in global energy demands.

World energy consumption has progressively become less significant over recent years in developed countries. The Organisation for Economic Co-operation and Development (OECD) decreased their global primary energy demand from 60% in 1977 to 39% nowadays [18]. However, the challenge from now onwards is focused on developing economies, and these new players have to be considered and integrated as an important part of the global environmental challenges and energy transition. The resolution of these challenges should be focused on the widespread use of renewable resources and the development of new models of energy systems.

Reduction of the ecological footprint and energy transition to renewable systems include a holistic approach that lies in the utilization of a huge diversity of complementary energy sources, such as solar, hydrogen, wind and biofuels. Currently, biodiesel and bioethanol are the only biofuels that are produced and commercialized on an industrial scale. These first generation biofuels are derived from conventional crops, such as soybeans, palm, sugarcane, sorghum, wheat, sugar beet and others that may be preferable [19]. The major concerns about first generation biofuels are their inefficiency and the food competition dilemma. However, second generation biofuels are derived from sources that are not suitable for human consumption, and they can be cultivated in arable and non-arable land, but the implicit point is that they usually require a great quantity of water

or fertilizer to grow, a fact that has led to disappointment in several second generation crops. Finally, the third generation biofuels are derived from highly productive non-edible sources, such as lignocellulosic materials, rice straw and microorganisms like microalgae. Therefore, the third generation biofuels provide a better prospect, due to their non-competition with food, and they contribute to energy security and to mitigating environmental problems.

Microalgae are photosynthetic microrganisms, and their cultivation is at least 10 times more efficient than other biofuel crop production; nor do they need to compete with arable land or freshwater resources [16]. Microalgae are a very diverse group of unicellular, prokaryotic or eukaryotic organisms, present in both aquatic and terrestrial ecosystems [20].

Microalgae are also capable of accumulating large amounts of macromolecules, such as protein, lipids and carbohydrates. Some species show an oil content of 20-50% of their dry weight, while conventional crops, such as soybean and palm, contain less than 5% of oil related to their total biomass [21]. The combination of these characteristics and their high growth rate make microalgae a promising raw material for biofuels and other high-value compounds.

Among microalgae, diatoms are organisms characterized by a cell wall made of amorphous silica (SiO_2) and organic compounds. These organisms are unicellular or eukaryotic, photosynthetic, classified in the class of Bacilariophyceae. This group comprises the most abundant microalgae in the ocean, contributes to about 50% of total phytoplankton primary production [22] and occurs in a range of environments. The organic constituents of their cells make them an important primary food source for higher organisms [23].

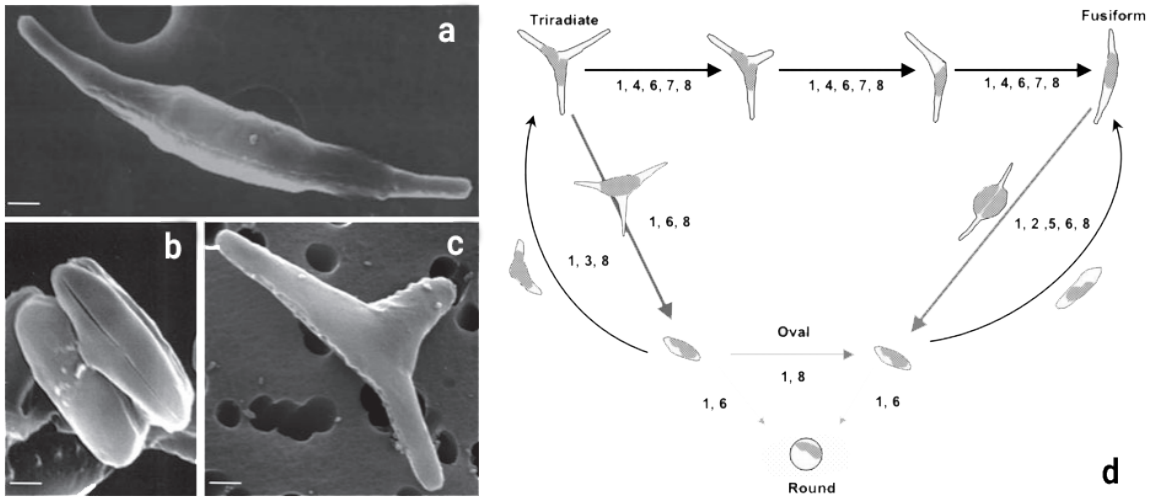


Figure 1: *P. tricornutum* morphotypes: (a) fusiform cell; (b) two oval cells, on valve top view; (c) triradiate cell; (d) representation of transitional forms between morphotypes.

Source: De Martino et al. [24]; Bartual et al. [25]

Described for the first time by Bohlin [26], the diatom *P. tricornutum* can be found in freshwater and marine environments, and it exists in three different morphological forms: fusiform, triradiate and oval (Figure 1). This microalga shows higher growth rates and yield, and as they are easy to cultivate they have been exploited in biotechnology fields as an alternative for producing diverse raw materials for many applications, such as animal feed, biopharmaceuticals [27] [28], delivery system for recombinant protein expression [27] and biofuel production [29].

The *P. tricornutum* is highly dependent on the light source [30], but it has the capacity to respond to environmental variations and to deal with light stresses due to its sophisticated mechanisms, particularly a highly efficient photoprotective one [31]. Similar to other diatoms, *P. tricornutum* has a highly silicified cell wall, and an external layer of diatoms called the frustule. Therefore, these organisms can survive without making silicified hard and porous cell walls and are capable of growing without silicon in the culture medium [24]. *P. tricornutum* can be genetically transformed and constitutes an attractive model to study because of its small genome [32, 33] which has already been fully sequenced. These microalgae are important components of the marine ecosystem, playing a significant role, mainly for global carbon fixation and biogeochemical cycling of minerals.

2.2 Carbon Capture and Fixation

Microalgae are photosynthetic organisms with the capacity to convert solar radiation into chemical energy, according to general Equation 1:

$$6CO_2 + 6H_2O \xrightarrow[Sunlight]{} C_6H_{12}O_6 + 6O_2 \tag{1}$$

The photosynthetic process is divided into two distinct phases: light dependent reactions or light reactions which occur only in the presence of light intensities; and carbon fixation reactions or dark reactions, occurring both in the presence and absence of light [34].

The photosynthetic organisms absorb the electromagnetic radiation in a wavelength varying between 400 and 720 nm by photosynthetic pigments, classified in groups (for microalgae), as chlorophylls, carotenoids and phycobilins, which are different due to their chemical composition and light absorption capacity [35, 36].

The photosynthesis process takes place on the cytoplasmic organelle, namely the chloroplast, which is found only in superior plants and photosynthetic organisms (including microalgae and cyanobacteria). In chloroplasts, chlorophylls and other pigments are inserted into the photoactive complexes, in pairs, called photosystem I (PSI) and photosystem II (PSII), which use light energy to excite the electrons in a pair

of chlorophylls modified at the center of the photosystem. These excited electrons are raised to a higher energy level and transferred into an electron transport chain (Figure 2) [37, 38]. The chlorophylls present in reaction center of PSI and PSII are called P680 and P700, respectively, due to the wavelength they are able to absorb. The electron flow is initiated in the PSII complex, through the excitation of electrons present in the P680 chlorophyll dimer. The replacement of electrons on these modified chlorophylls comes from the oxidation of the water molecule, through a process that is not yet elucidated, releasing one molecule of oxygen for every two molecules of oxidized water [34]. PSII contains different co-factors for electron transfer to the chlorophyll dimer P700 of the PSI complex. Once in the PSI complex, the electrons accepted by the P700 chlorophyll dimer are oxidized by the chlorophylls of the antenna complex to reduce a molecule of NADP+ to NADPH [39], NADPH [39], which will be used in the Calvin cycle, also known as dark reactions of photosynthesis [37, 40].

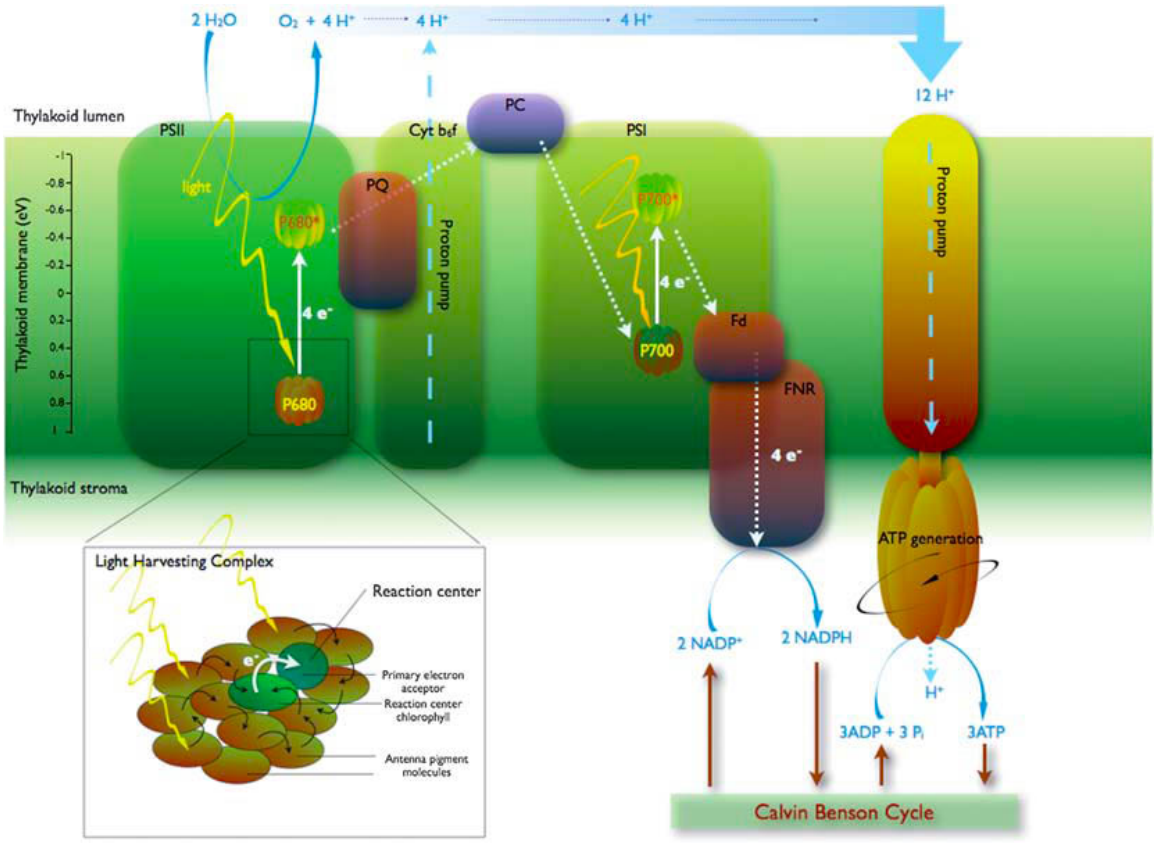


Figure 2: Representation of the light reactions of photosynthesis. The functional units are represented as oval shapes; photosystem II (PSII), plastoquinone (PQ), plastocyanin (PC), cytochrome b6f complex (Cyt b6f), photosystem I (PSI), ferredoxin (Fd), ferredoxin-NADP reductase (FNR) and ATP synthase. P680 and P700, refer to the reaction centres of photosystem II (PSII) and I (PSI) respectively, the asterisk (*) indicates the excited state. The inset shows the light harvesting complex (LHC).

Source: Williams & Laurens [41].

The independent light and dark reactions of photosynthesis lead to the fixation of carbon itself, incorporating it into organic molecules. The main step of the dark reactions is catalyzed by the RuBisCO enzyme (Ribulose-1,5-bisphosphate carboxylase/oxygenase), which is responsible for the carboxylation of ribulose sugar 1,5 bisphosphate and its subsequent reduction in other organic molecules necessary for the metabolism of the microalgae [41].

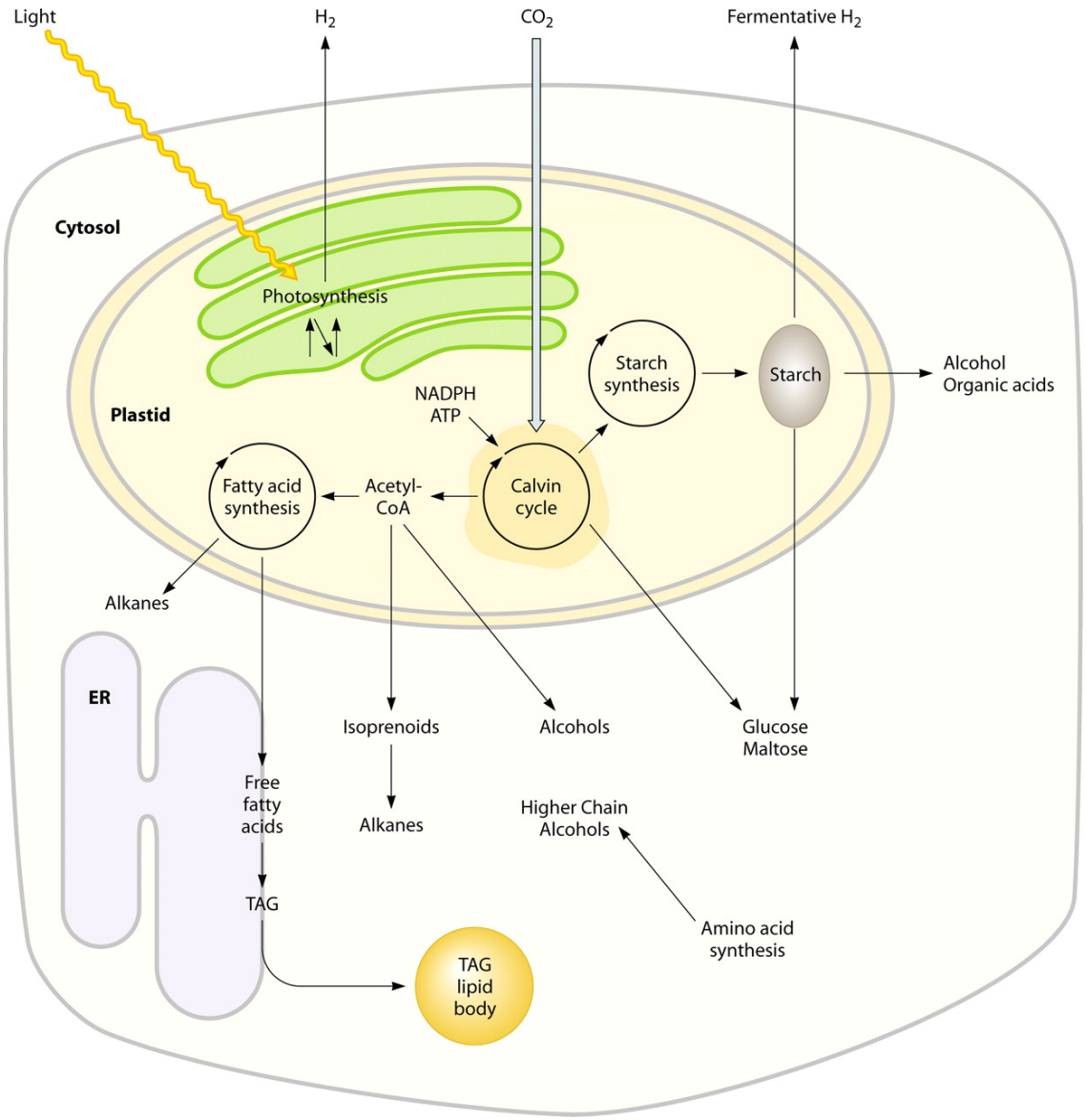


Figure 3: Main metabolic pathways of microalgae for production of different biofuel feedstocks.

Source: Radakovits et. al [42].

Considering the above explanation, microalgae are able to produce different types of organic molecules that can be transformed into biofuels. The main sources of microalgae energy reserves are in the form of lipids and carbohydrates, which can be converted into

biodiesel and bioethanol, respectively.

The main types of lipids present in microalgal cells can be classified into neutral lipids (triglycerides, cholesterol) and polar lipids (phospholipids). The main routes of lipid synthesis in plant cells are: (a) the formation of Acetyl-CoA in the cytoplasm, (b) elongation and desaturation of carbonic chain in the formation of fatty acids and (c) the biosynthesis of triglycerides [43].

Carbohydrates are one of the most important sources of energy for cells. Algae have relatively high photoconversion efficiency, and are able to store a large amount of carbohydrates (potentially more than 50% of their dry weight). In general, algal carbohydrates are composed of starch, glucose, cellulose/hemicellulose and various other types of polysaccharides. Conventionally, starch and cellulose are the polysaccharides used for biofuel production, especially bioethanol [14].

The conversion of acetyl-CoA and CO_2 into malonyl-CoA, considered the first phase in the biosynthesis of fatty acids, occurs in the chloroplast. The reaction has two steps and is catalyzed by an enzymatic complex. The elongation of fatty acids occurs through the condensation of malonyl-CoA and Acetyl-CoA molecules; after repeated reactions, fatty acids with 16 or 18 carbons are formed in their chain. The first step in the formation of triglycerides is the condensation of glyceraldehyde-3-phosphate with Acyl-CoA and the formation of lysophosphatidic acid. This reaction is catalyzed by the enzyme glycerol-3-phosphate acyltransferase, which a low specific activity in the pathway of the synthesis of triglycerides, and is suggested as the limiting point of it. After that, phosphatidic acid, diacylglycerol and triglycerides are synthesized by a series of catalytic reactions [14] (Figure 3).

2.3 Cultivation Systems

Microalgal biomass production can be performed in the laboratory or on an industrial scale, using closed (photobioreactors) or open systems (open ponds). The opens systems are usually 10 to 50 cm in depth in order to allow the natural light penetration and atmosphere gas diffusion to the culture medium [11, 44] (Figure 4), while photobioreactors (PBRs) have been developed under a wide range of configurations, designed to obtain higher biomass productivities, such as: flat plates, horizontal tubular, concentric type air-lift, helical tubular, flat plate air-lift, bissolar, semi-spherical [45–49].

PBRs have some advantages over open systems. Despite its higher production cost, a PBR normally presents higher photosynthetic efficiency. In addition, PBRs present better relation area/surface than open ponds, which leads to higher biomass productivities. Another concern is about the possibility of controlling culture parameters and contaminations at PBRs, while in open ponds it is more difficult and the culture is usually performed under natural conditions [43].

Figure 4: Different types of microalgae culture system. The image shows the open culture systems in upper quadrants, while in lower quadrants the closed culture systems or photobioreactors appear.

Source: Spirulina Source [50]

Tubular PBR have been widely used for microalgae cultivation, both on pilot or industrial scale. These systems consist of a set of transparent tubes, generally composed of some type of plastic or glass. The tubes' diameter is quite variable, ranging between 2 and 10 cm, and the three-dimensional arrangement adopted can be helical, inclined, vertical, horizontal or bubble columns [43].

Microalgae cultivation using PBR allows the use of natural, artificial and/or residual CO_2 to feed the culture medium, so it is an alternative process for mitigating industrial carbon emissions coupled with biomass production [51]. Some studies have estimated that one kilogram of dry algae biomass produced consumes about 1.83 kg of CO_2, which corresponds to 54.9-67.7 tons annually of CO_2 absorbed by 30 to 37 tons of microalgae biomass per hectare [52].

After biomass production, the constituent elements of microalgae can be extracted and used as raw materials for the production of various products. For the production of biofuels, in general, Figure 5 represents the life cycle steps, starting with microalgae species selection for culturing, followed by biomass harvesting, extraction of the target components and finally biofuel production.

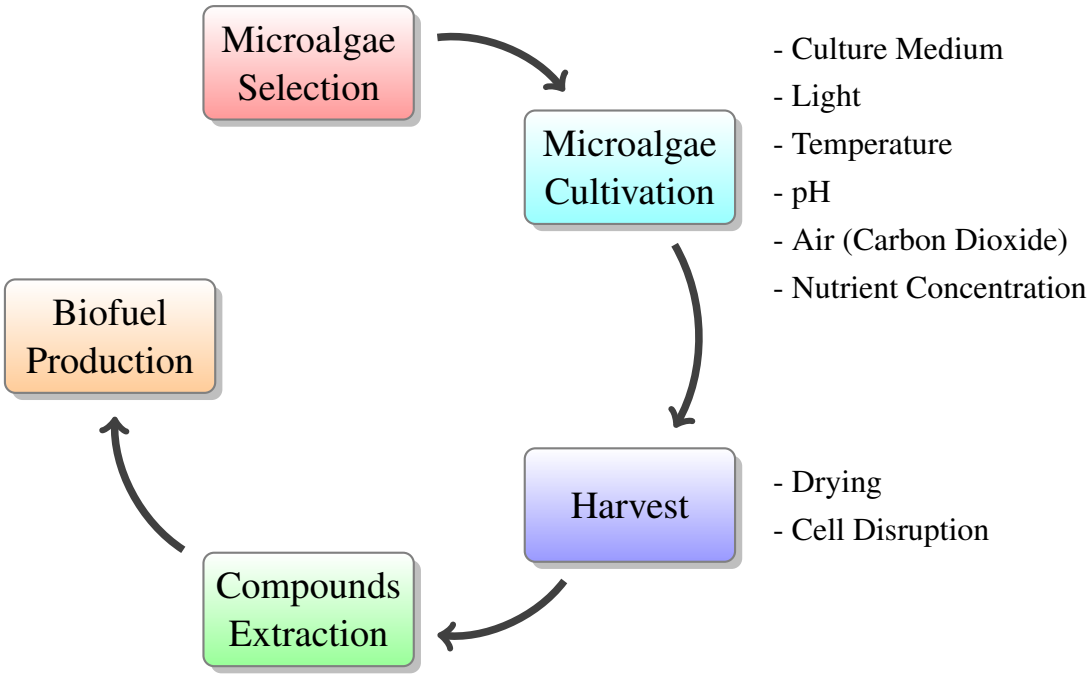


Figure 5: Steps for production of biofuels from microalgae.

2.4 Microalgae-based High-value Compounds and Biofuels

Although microalga-based biofuel production has been shown to be quite promising, the costs related to the process are still not competitive. One of the suggested proposals to reduce these costs is by increasing the biomass price through valorization of by-products of the process, using a biorefinery concept. This approach contributes to reducing the environmental impacts and to increasing the profitability of the value chain. The use of microalgae for biofuel production generates a significant amount of residual biomass, which can be used in integrated biorefineries for the extraction of added-value products and other biofuels.

Basically, there are several definitions for the concept of biorefinery. The International Energy Agency (IEA) defines it as the sustainable processing of biomass with a spectrum of added-value products and energy [53]. Broadening this concept, Zhu [13] defines biorefinery as an industrial process in which biomass is converted into different biochemical compounds, materials and energy, based on an oil refinery where multiple fuels and products originate from a primary resource.

Despite all the different concepts applied to biorefineries, one issue appears to be intrinsic in all, which is the fact that the production chain involved in this concept is used in a way that maximizes the resource use, valorizing waste, reducing losses and environmental impacts. In a microalgae biorefinery, many bio-products can be extracted

and produced, depending on the considered microalga, their biochemical composition and the culture conditions. Microalgae are lipid-rich species, carbohydrates, proteins and other valuable compounds. In this context, several high-value products can be extracted and produced from microalgae for a range of industries. For biofuel production, the versatility demonstrated by these organisms is quite large. Lipids extracted from microalgae cells can be converted mainly into biodiesel; while carbohydrates, including starch and cellulose, can be transformed into bioethanol through the fermentation process and residual biomass into biomethane by anaerobic digestion [15]. In addition, the chemical, biochemical and thermochemical conversion processes can be used for the production of biobutanol, bio-oil, syngas and biokerosene [16] (Figure 6).

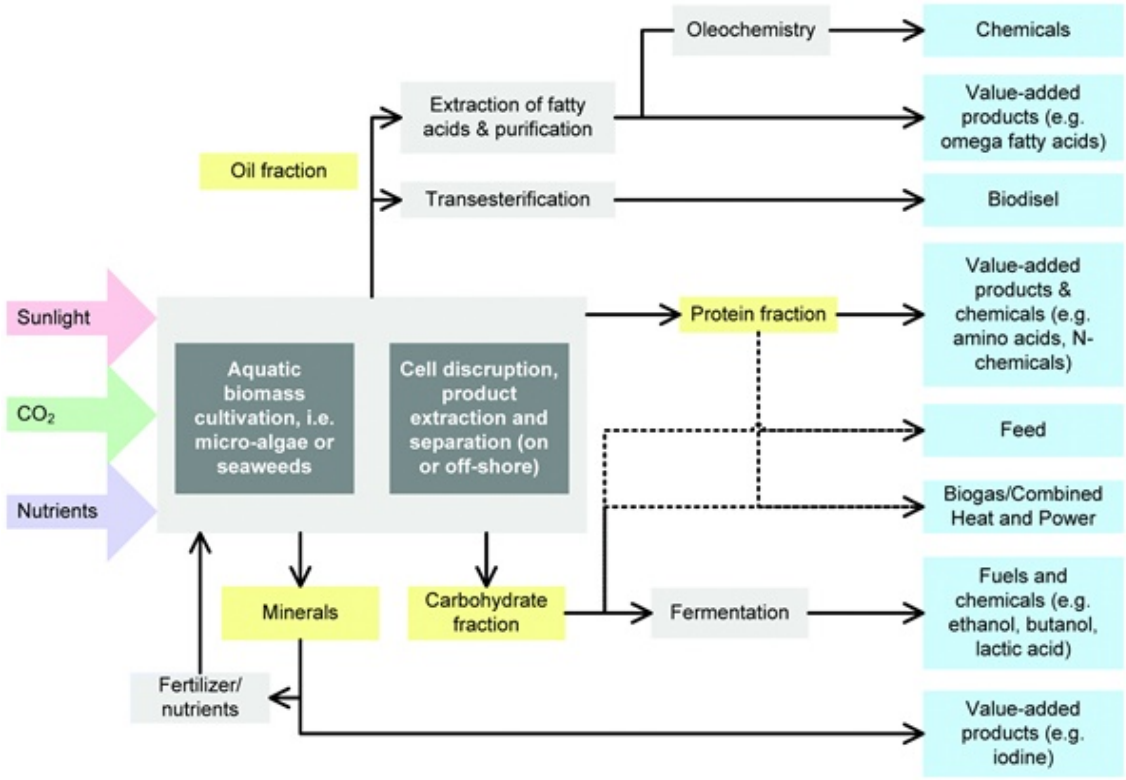


Figure 6: Sustainable analysis for cultivation of microalgae under a biorefinery concept.

Source: Koutinas et al. [54]

Some microalgae products could be metabolically induced by changes in the culture conditions, such as environmental conditions of the cultivation region (solar radiation, wind, evaporation and temperature, among others) or also could be strategically manipulated, such as nutrient availability, salinity and CO_2 concentration, which may vary according to the desired bioproduct and the tolerance and resilience of the microalgae species [43].

As an example of this process, microalgae cultivated under limited availability of nitrogen usually accumulate a higher lipid content, while those cultivated under sulfur restrictions can produce hydrogen gas [55]. Therefore, for applying this method, it is necessary to know the metabolism of the microalga in order to define which product will be targeted, since the biomass production is directly related to the initial parameters and conditions of cultivation systems.

Biodiesel can be characterized as a mixture of alkyl esters of fatty acids obtained by a transesterification reaction in which the triglycerides react with methanol or ethanol. The process for producing biodiesel from microalgae and traditional crops is quite similar. For the production of biodiesel, two steps are necessary: extraction of the oil and the transesterification process. For the extraction of lipids and fatty acids from microalgae, organic solvents are used, such as hexane, ethanol (96%) or a mix of hexane-ethanol (96%) [56].

The transesterification reaction is used to convert the microalgae oil to esters. The transesterification reaction takes place under multiple steps, including reversible hydrolysis, in which triglycerides are converted to diglycerides, then diglycerides are converted to monoglycerides and, finally, monoglycerides are converted to fatty acids and glycerol (as by-product). This is followed by re-esterification with alcohol (e.g. methanol), with a stoichiometric molar ratio of 3:1 (alcohol: fatty acid).

Furthermore, bioethanol is produced through carbohydrate fermentation, mainly starch hydrolysis. Microalgae are organisms that have a stock of carbohydrates, which can be hydrolyzed into simple sugars and used for bioethanol production through yeast fermentation. The final product, ethanol, can be obtained from the distillation process.

Compared with plant biomass, microalgae have special properties for the production of bioethanol: cell walls are formed by a large amount of polysaccharides with low concentrations of lignin and hemicelluloses, a fact that favors the hydrolysis of the cell wall in glucose [57] without the need for enzymatic pretreatment. Microalgae generally contain large amounts of carbohydrates in their constitution, and the fact that they do not have organs (such as roots, leaves and fruits) facilitates the process of hydrolysis of complex carbohydrates into simple ones.

Additionally, studies have shown that ethanol production increased by 60% after lipid extraction from microalgae biomass, when compared to carbohydrate extraction and ethanol production with intact cells of the *Chlorococum sp* microalgae [57]. This fact can be explained due to the cell wall breaking process of during lipid extraction, providing hydrolyzed carbohydrates for fermentation and bioethanol production.

Finally, the technology for biogas production from anaerobic digestion of residual biomass has already reached a mature and technically feasible stage. However, there are several factors that affect the success of digestion, such as: temperature, humidity, nutrient content and pH. In addition, the efficiency of the process will depend on the microalga

strain, biomass pre-treatment and digester technology.

A study conducted with microalgae has shown that the production of methane after the extraction of lipids, carbohydrates and proteins from microalgae is quite feasible [15]. Chisti [7] demonstrated that residual biomass provided about 9,360 MJ of energy per metric ton after removing 30% of the oleic content of microalgae, which corresponds to a considerable amount of energy and can contribute to sustainable production of algae biodiesel.

Despite of this, the production of biogas from microalgae biomass is still on a pre-commercial scale since some drawbacks exist. Firstly, the amount of energy demanded by the digesters and the occupied surface to generate 1MJ of methane is higher than for the generation of 1MJ of microalgae biodiesel [58]. Another concern about microalgae is that they have high protein content, which results in low C:N ratios, which can affect the digestion process. In order to increase methane production, co-digestion has been used, in which products with high C:N ratio are added to the algal biomass [59].

Therefore, the other chapters of this thesis will discuss the biochemical potential of *P. tricornutum* for production of biofuels associated with the commercialization of co-products, in order to verify the viability of this approach.

Chapter 3

Potential of *Phaeodactylum tricornutum* for Biodiesel Production under Natural Conditions in Chile

Diatoms are very diverse and highly productive organisms, found in a wide variety of environments. This study aims to analyze the growth and lipid composition of *Phaeodactylum tricornutum*, cultured in an outdoor pilot-scale bubble column photobioreactor under natural conditions in Chile for biodiesel production. Results showed that *P. tricornutum* cultures reached their highest biomass concentration (0.96 ± 0.04 kg m^{-3}) after 14 days of culturing, at the stationary phase, with a volumetric productivity of 0.13 kg m^{-3} d^{-1}. Biomass samples showed a total lipid content of 9.08 ± 0.38 wt%. The fatty acid methyl ester analysis revealed a composition of 24.39% C16-C18 fatty acids, 42.34% saturated fatty acids, 21.91% monounsaturated fatty acids and 31.41% polyunsaturated fatty acids. These findings suggest that *P. tricornutum* oil can be used as an alternative raw material for the production of biodiesel capable of meeting international quality standards.

3.1 Introduction

The production of biofuels offers new opportunities to mitigate climate change and promote energy security. Furthermore, the complementation of fossil fuels, used for many decades as a primary energy source, leads to a circular economy approach by closing the carbon cycle [60]. In this context, microalgae have attracted significant interest as feedstock for different types of renewable fuels such as biodiesel, methane, hydrogen and ethanol, among others [61].

Microalgae photosynthetic metabolism produces valuable compounds using solar energy, water, carbon dioxide and other available compounds. Through this metabolic process, they are capable of generating raw materials to use in biofuels, food and feed production [20]. Their high areal productivity and lipid content offer several advantages over traditional crops. Also, the possibility of using non-arable land and non-potable

water (such as sea or wastewater), reduces the competition with food crops and secures sustainability [62]. Moreover, the production of microalgae biodiesel can be associated with other co-products, increasing their economic feasibility and sustainability [63]. Also, it is possible to effectively combine microalgae cultivation with wastewater treatment, reducing operating costs and simultaneously allowing potential waste valorization [64].

Among eukaryotic phytoplankton, diatoms (Bacillariophyceae) represent the largest population of microalgae in the ocean [65]. They are responsible for nearly 40% of marine primary productivity [66] and represent the richest group of algae species, with approximately 100,000 known species in both marine and freshwater environments. However, only 12,000 of them have been described so far [67]. Despite their abundance, diversity and simplicity to cultivate, only a few of them have been cultured for biotechnology or production purposes [68].

The marine diatom *Phaeodactylum tricornutum* is an ecologically significant, polymorphological and unicellular microalga. It is well-known and modelled for studying the physiology, biochemistry and genomics of diatoms, whose complete genome information is already available [69]. This species can accumulate diverse metabolites such as carotenoids, proteins, carbohydrates, silica and polyunsaturated fatty acids (PUFA), some of them largely used for aquaculture, animal feed and human consumption [20, 61, 68]. In particular, it is a potential source of eicosapentaenoic acid, 20:5n3 (EPA), an essential PUFA, and fucoxanthin, a major pigment in diatoms and one of the most abundant carotenoids in nature, especially in marine environments, which are known to be essential nutrients for animals [70].

Recently, *P. tricornutum* has been considered as a potential candidate for biodiesel production due to its high growth rate under optimum conditions and its lipid content, ranging from 20 to 30 dry wt% under photoautotrophic conditions [65]. Moreover, this marine diatom grows in saline water and does not compete with freshwater sources, which makes it more commercially attractive. Its components, fucoxanthin and PUFA, are regarded as economically valuable co-products that can be extracted to effectively offset the costs of its cultivation for biodiesel production [70].

The success of mass culture of *Phaeodactylum* to obtain highly valuable products, such as PUFA and lipids for biodiesel production requires the optimization of growth conditions, particularly temperature, light and nitrogen deficiency [65]. These factors are strongly influenced by the type of reactor used for this process. Nowadays, large-scale production of microalgae can be done both in open ponds and photobioreactors (PBR), being the production costs considerably lower in the first.

Concerning the culture of some *Phaeodactylum* species, it is mandatory the use of closed PBR, as they require strict control of temperature in the range of 20–25 °C, which is difficult to maintain in open ponds [71]. PBRs for microalgae cultivation have a wide range of configurations, designed to obtain high biomass volume per unit, according to

each culture purpose. Photobioreactors are usually structured in flat plates, alpha-helix, horizontal tubular, concentric air-lift, helical tubular, air lift flat plates, semispherical and bubble column designs [72, 73].

Bubble columns are used as an alternative to conventional PBR designs due to their low-cost production and operational simplicity. Furthermore, this type of PBR is compact and effective for producing large quantities of biomass and scaling up lab and pilot-scale culture into higher volumes [74]. However, there is scarce information regarding the characterization of microalgae grown in outdoor pilot-scale bubble columns, operated under natural solar irradiance and temperature, capable of increasing the efficiency and sustainability of the process [15, 74–76]. Studies about this issue are needed for establishing the foundations of more efficient large-scale microalgae cultivation using this system [77].

P. tricronutum has been cultured for its long-chain PUFA. In general, nutrient limitation, in particular nitrogen, is the most effective method for improving microalgal lipid content, although the response is species-specific [78]. Several studies of the influence of culture conditions on lipid production have been performed, both for laboratory [70, 71] and outdoor conditions [66, 79]. The general conclusion arising from these studies is that the biochemical composition of microalgae and their growth rate are determined by environmental factors, such as temperature, light availability, nutrients and salinity. In addition to environmental factors, the growth phase or culture time also have significant effect on the biochemical composition of microalgal cells [70]. However, no definitive conclusions on the most adequate conditions to obtain higher quantities of certain compounds can be obtained from the literature.

In this study the culture growth and lipid composition of *P. tricornutum* cultivated in outdoor pilot-scale bubble columns PBR is analyzed. The experiments were performed in Concepción, Chile, which enjoys a temperate Mediterranean, bordering on Oceanic climate.

3.2 Materials and Methods

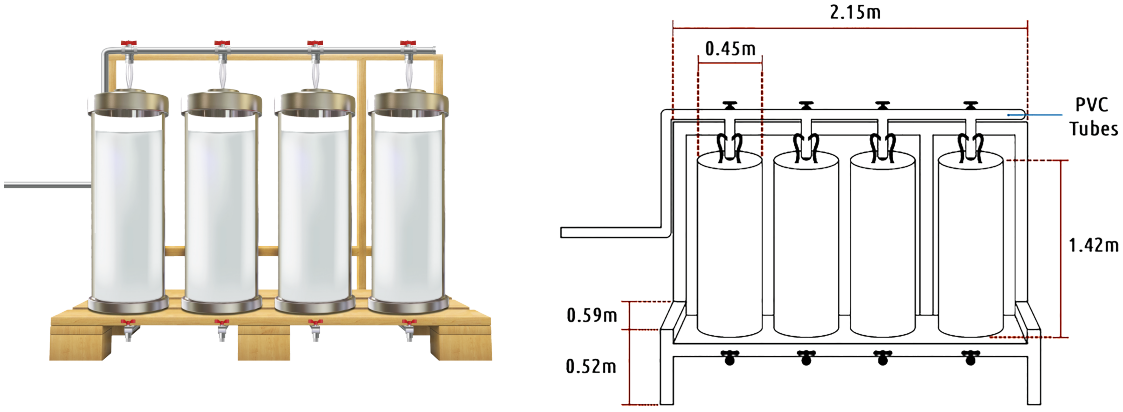
3.2.1 Culture Conditions

The cultures of the diatom *P. tricornutum* Bohlin (originating from Cañar Blanco, La Serena, IV Region, Chile), initially stored at the laboratory's culture collection, were maintained by sub-cultivation in 250 mL Erlenmeyer flasks with 100 mL culture medium, aerated with atmospheric air supplied by an air blower with a flow rate of 120 L/min, 0.18 bar (LA-120A, Nitto Kohki Co. Ltd., Tokyo, Japan) at 23 ± 1 °C under artificial light with 200 μmol photons m^{-2} s^{-1} in 16:8 (light:dark) cycles. The Walne culture medium [80], supplemented with 0.017 g L^{-1} of silicate, was prepared with natural seawater, filtered

and autoclaved for 45 min at 20 psi and 121 °C. After 7 days of sub-cultivation the cultures were transferred into 1 L Erlenmeyer flasks under the same conditions, until the exponential growth phase was observed. The cultures were scaled-up into 20 L plastic carboys containing sterile Walne medium and silicate, under the same conditions of sub-cultures for approximately 14 days.

3.2.2 Outdoor PBR Culture Management

Phaeodactylum tricornutum samples were batch cultured in 800 L bubble column PBR, in outdoor conditions in Concepción, Chile, from January to March 2016. The PBR used for outdoor *P. tricornutum* cultivation consisted of four parallel acrylic tubes, each one with a length of 1.40 m and 0.45 m wide, capable of containing a 200 L volume, placed vertically on a woody base. The base of the tubes was conical and composed of PVC tubes that allowed samples to be harvested at the end of the cultivation period. A plastic cover closed the upper end of the tubes. The area occupied by the PBR was 1.27 m^2 (Figure 7).



(a) (b)

Figure 7: Bubble column PBR used for outdoor *P. tricornutum* cultivation. (a) Schematic illustration of the PBR; (b) Sketch map of the PBR front view containing all sizes.

The air bubbling within the culture was generated by an air blower with a flow rate of 200 L min^{-1}, at 0.2 bar relative pressure (LAM-200, Nitto Kohki Co. Ltd., Tokyo, Japan) connected to the tubes by PVC tubes coupled to the cultures by rubber hose/glass capillary system. The PBR was oriented in an East/West direction; the latitude and longitude of the site were 36°50'02.1" S and 73°01'49.3" W, respectively.

Initially, the tubes were filled-up with 170 L of natural seawater. Sodium hypochlorite (NaClO) 0.03 g L^{-1} was added to natural seawater as a disinfectant agent, maintaining air bubbling overnight. After 24 h of treatment, the NaClO was neutralized with 100 mL of $Na_2S_2O_3$ 50 g L^{-1}. The natural seawater was enriched with a commercially modified Guillard's f/2 formulation [81] with silicate (Pentair, Aquatic Eco-Systems, Minneapolis,

MN, USA). The 20 L plastic carboys containing *P. tricornutum* culture were utilized as inoculum for each 200 L column. The cultures were monitored every day until the stationary growth phase was reached using an YSI 556 Multi-Probe System (YSI Inc., Yellow Springs, OH, USA) that measures the salinity, dissolved oxygen, temperature, and pH. The natural illumination was measured every minute by a photosynthetically active daily-averaged irradiance (PAR) sensor (QSPL-2100 Quantum Scalar Laboratory sensors, Biospherical Instruments Inc., San Diego, CA, USA).

Aliquots of 50 mL were sampled regularly from each culture and measured at 750 nm (OD_{750}), 680 nm (OD_{680}) and 550 nm (OD_{550}) in a HALO DB-20 UV/VIS double beam spectrophotometer (Dynamica Scientific Ltd, Newport Pagnell, UK). Optical density at OD_{750} served as proxies of dry biomass, calculated with previously determined equations $M = 0.89 \times OD_{750} + 0.38$ ($R^2 = 0.97$, M = biomass (g L^{-1})).

The photoinhibition was calculated by the relative measurement of the quantity of chlorophyll per mass of biomass through the ratio of OD_{680}/OD_{550}, for monitoring the possible culture contamination by bacteria and other organisms. This measurement can also be used as an indication of cell bleaching due to photoinhibition [82]. Cellular light scattering is usually determined for measuring culture absorption at 550 nm because algae can hardly absorb light at such point, whereas at 680 nm, the optical density corresponds to both scattering and light absorption of chlorophyll-a. For healthy cells, this ratio should be above 1.0 [83].

Growth dynamics of the microalgae was quantified by the growth rate and the progressive sum of daily growth rate. Growth rate was determined using Equation 2,

$$\mu = \frac{\left[\ln\left(\frac{X_2}{X_1}\right)/\ln 2\right]}{\Delta t} \tag{2}$$

where μ is the growth rate (day^{-1}), X is microalgae biomass concentration at different time points (kg m^{-3}), and Δt is the difference between two time points (day).

The progressive sum of daily growth rate ($\sum\mu$) is determined using Equation 3,

$$\sum \mu = \mu_{(n-1)} + \mu_{(n+1)} \tag{3}$$

where $\sum\mu$ is the accumulated growth rate (day^{-1}), $\mu_{(n-1)}$ is the growth rate at time 1, and $\mu_{(n+1)}$ is the growth rate at time 2.

After 14 days of cultivation, the microalgae were harvested by overnight flocculation, using 0.5 M NaOH at pH 10.40, followed by biomass centrifugation at 4,000 rpm for 5 min (Rotofix 32A, Hettich Zentrifugen, Tuttlingen, Germany). Then, they were stored at -20 °C until lyophilized at -70 ± 2 °C for about 72 h (FDU-7008, Operon, Yangchon-eup, South Korea). The total dry biomass was measured using an analytical balance (ABJ

220-4M, Kern, Balingen, Germany) and stored at -20 °C until the remaining biochemical analysis was done.

3.2.3 Microalgae Lipid Analysis

For lipid extraction and transesterification, the following methods were performed at room temperature and pressure: (1) Soxhlet extraction using hexane as solvent; (2) Bligh and Dyer [84] method as modified by Mata et al. [85]; (3) direct Bligh and Dyer extraction and transesterification of lipids.

Lipid Extraction with Hexane

Ten grams of dry algae were placed in a cellulose thimble (25 mm I.D., 28 mm O.D., 100 mm length) inside a Soxhlet extractor, using hexane as solvent, without any pre-treatment. The extraction was done at 60 °C for 24 h using 250 mL of hexane. After the solvent was removed, the extracted lipid was gravimetrically quantified using an analytical balance (Kern ABJ 220-4M).

Lipid Extraction with Methanol/Chloroform

Lipids of *P. tricornutum* were quantified after extraction using the Bligh and Dyer [84] method as modified by Mata et al. [85]. Chloroform, methanol and distilled water were added in ratios of 1:2:0.8 (v/v), respectively, into 300 mg of dried biomass. The resulting mixture was sonicated for 30 min (Ultrasons 6L, Selecta, Barcelona, Spain). A second extraction step was then performed by adding the co-solvents at ratios of 2:2:1.8 (v/v) of chloroform, methanol and distilled water respectively. The samples were sonicated for another 30 min, and then centrifuged at 3,000 rpm for 15 min (4000R Benchtop Refrigerated Laboratory Centrifuge, Centurion Scientific Ltd, West Sussex, UK). After centrifugation and resting, the lower layer was carefully recovered and transferred into another pre-weighted glass tube using a syringe. After the chloroform evaporated at room temperature, the extracted lipids were gravimetrically weighted (Kern ALJ 220-4) in order to estimate the total lipid content.

Direct Bligh and Dyer extraction and Transesterification of Lipids

Samples were tested by the Bligh and Dyer [84] method after placing 100 mg of lyophilized biomass in a glass vial with 3 mL of chloroform–methanol 2:1 (v/v), 1 mg mL^{-1} Tricosanoic acid (C23:0) as lipid standard, and 0.5 mg mL^{-1} butylated hydroxytoluene (BHT). The mixture was then shaken overnight at room temperature. After extraction, the mixture was centrifuged at 5,000 rpm (Hettich Zentrifugen Rotofix 32A, Hettich Instruments, Massachusetts, USA) for 5 min at room temperature. The

mixture was transferred to another funnel to allow the separation of organic and aqueous layers. The bottom layer was then collected, and the solvent was evaporated using N_2. After the solvent evaporated, 1.5 mL of NaOH was poured into the flask, and the extracted lipids were heated to 100 °C for 5 min.

The transesterification process was carried out using 2 mL of BF3 in methanol (2 wt %) at 100 °C for 30 min in order to determine fatty acid methyl ester (FAME) content. Then, 1 mL of isooctane was added after the mixture cooled, followed by shaking for 30 s. After, five milliliters of saturated NaCl was added before centrifugation. The upper phase was carefully transferred to 2 mL amber vials and stored at -20 °C.

GC Analysis

Transesterified products were analyzed by a gas chromatograph–mass spectrometer (GC 6000 Vega Series 2, model 6300-03b, Carlo Erba Instruments Ltd, Wigan, UK), equipped with a HP-FFAP cross-linked FFAP (25 m length, 0.32 mm diameter, 0.52 μm film thickness) column. A solution of 1 mg mL^{-1} of FAME mix (Food Industry FAME Mix, Restek, Bellefonte, PA, USA) was used as the internal standard for FAME analysis. Samples (1 μL) were injected at an initial oven temperature of 100 °C. After injection, the oven was heated at 100 °C/5 min to 240 °C at 4 °C/min and held at final temperature for 20 min. The carrier gas was nitrogen (N_2) at 100 kPa. The injector temperature was 225 °C, and FID detector temperature was set at 250 °C. The acquisition data were performed with the Autochro Data Module (Younglin Instrument, Anyang, South Korea), and the Autochro-3000 Software (Younglin Instrument, Anyang, South Korea). FAME in samples were identified by comparing the retention times (RT) and area (mVs) of FAME peaks with those of internal standards.

The percentage of each FAME present on the dry sample was calculated using Equation 4,

$$\text{FAME yield (wt\%)} = \frac{\text{Crude lipid yield (wt\%) x FAME content (\%)}}{100} \quad (4)$$

3.2.4 Statistical Analysis

Statistical analyses between samples and replicas were determined using mean and standard deviation, using Excel and STATISTICA® 7.0 software (StatSoft, Inc., Palo Alto, CA, USA, 2004).

3.3 Results and Discussion

3.3.1 Biomass Production

The objective of the culture system was to decrease costs and human intervention during the experimental period. Hence, the microalgae culture was performed with complete nutritional medium from the starting point, without further addition of nutrients.

The diatom *P. tricornutum* was grown in the commercial culture medium f/2. The culture was monitored daily by measuring biomass concentration, temperature, pH, salinity and dissolved oxygen concentration. The highest biomass concentration obtained in the early stationary phase was 0.96 ± 0.04 kg m^{-3} at day 14 of cultivation (Figure 8).

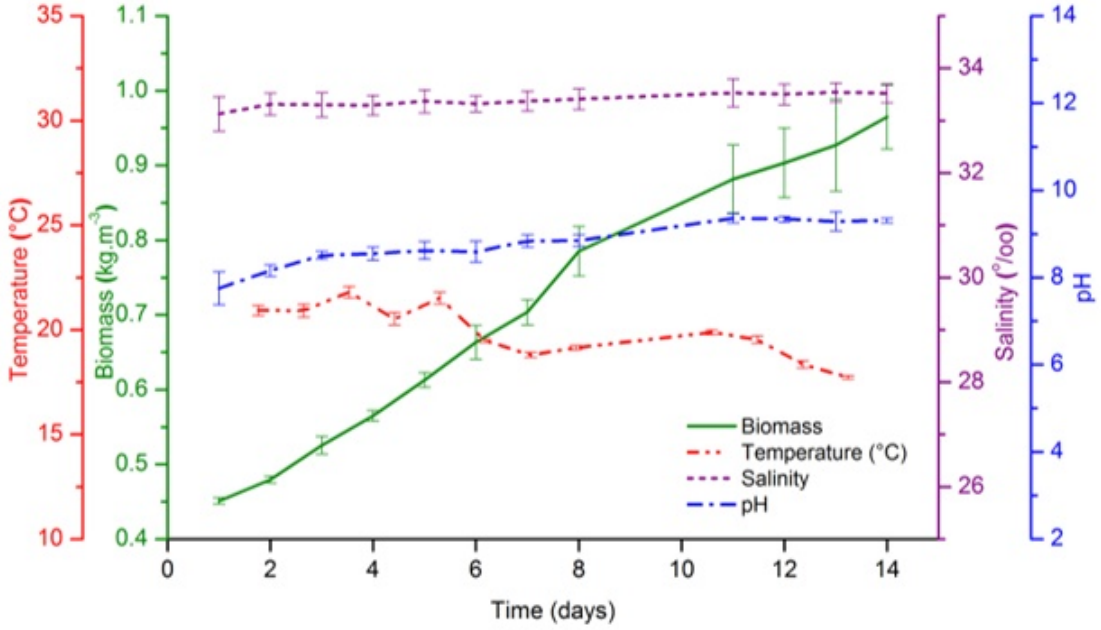


Figure 8: Variation of parameters for *P. tricornutum* batch culture during 14 days. Green line is the biomass concentration; red line is the culture temperature variation; purple line is the salinity of the culture and blue line is the culture's pH variation. Error bars are standard deviation.

The temperature varied from 17 to 21 °C, pH ranged from 7 to 9 and salinity remained almost constant during the entire cultivation period. On day 11, when temperature decreased, it was not possible to observe a significant variation in biomass concentration. At this point the culture reached the stationary phase and was harvested at day 14 (Figure 2).

The maximum volumetric productivity was 0.13 kg m^{-3} d^{-1} and the maximum areal productivity was 0.08 kg m^{-2} d^{-1} observed at day 8 of cultivation (Figure 9). Cumulated specific growth is shown in Figure 9, where it is possible to observe the variations that occurred in the microalgae growth. The stationary phase began after 8 days of cultivation, when the end of cellular divisions was observed and cumulated growth rate of about 0.97

day^{-1} (Figure 9). Furthermore, the maximum growth rate of 0.17 day^{-1} was also measured at day 8 of cultivation (Figure 10b). The range of maximum production and growth rates were observed at days 6 and 8 of cultivation. At this point, temperature started to decrease (see Figure 8) along with culture production which seemed to be strongly influenced by temperature variability (Figure 9). In fact, *P. tricornutum* showed substantial variability in biomass production during the year, showing higher productions in summer than in winter [86].

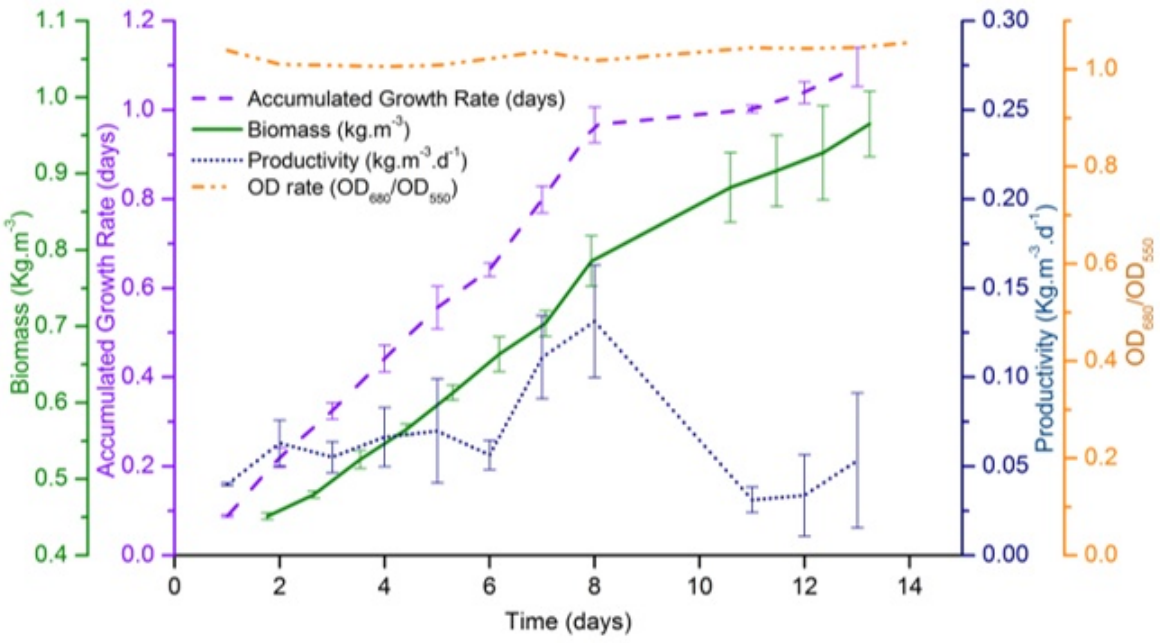


Figure 9: *P. tricornutum* biomass growth analysis during a batch culture regime. Green line is the biomass concentration; violet line is the cumulated growth rate; dark blue line is the culture productivity and orange line is the OD_{680}/OD_{550} rate. Error bars are standard deviation.

Benavides et al. [66] have compared the biomass productivity of *P. tricornutum* grown outdoors at different biomass concentrations in open ponds and closed PBR. They have concluded that in general the productivity is higher in PBR because light is more efficiently used in this type of system. These authors have obtained optimal biomass concentrations of 0.6 kg m^{-3} and 1.0 kg m^{-3} in open ponds and PBR respectively. These concentrations are similar to the optimal biomass concentrations observed in this work.

Usually, the biomass concentration and productivity of microalgae cultures are dependent on culture and climate conditions. In outdoor cultures, the PBR's location determines the values regarding temperature and solar irradiance, while dissolved oxygen and pH are dependent on PBR's design and operating conditions [86–88]. Indoor cultures, with smaller volumes and controlled parameters, have shown better performance, with higher production in comparison to the outdoor cultures with no controlled parameters [76, 77]. However, this study showed higher average values of biomass productivity and

concentration than some indoor experiments. For example, a study conducted by Song et al. [61] have achieved its highest biomass concentration (0.5 kg m^{-3}), growth rate (0.5 day^{-1}) and biomass productivity (0.23 kg m^{-3} d^{-1}) for *P. tricornutum* in an indoor 3 L bubble column PBR, under controlled light irradiance and temperature, batch regime and 8 days of culture.

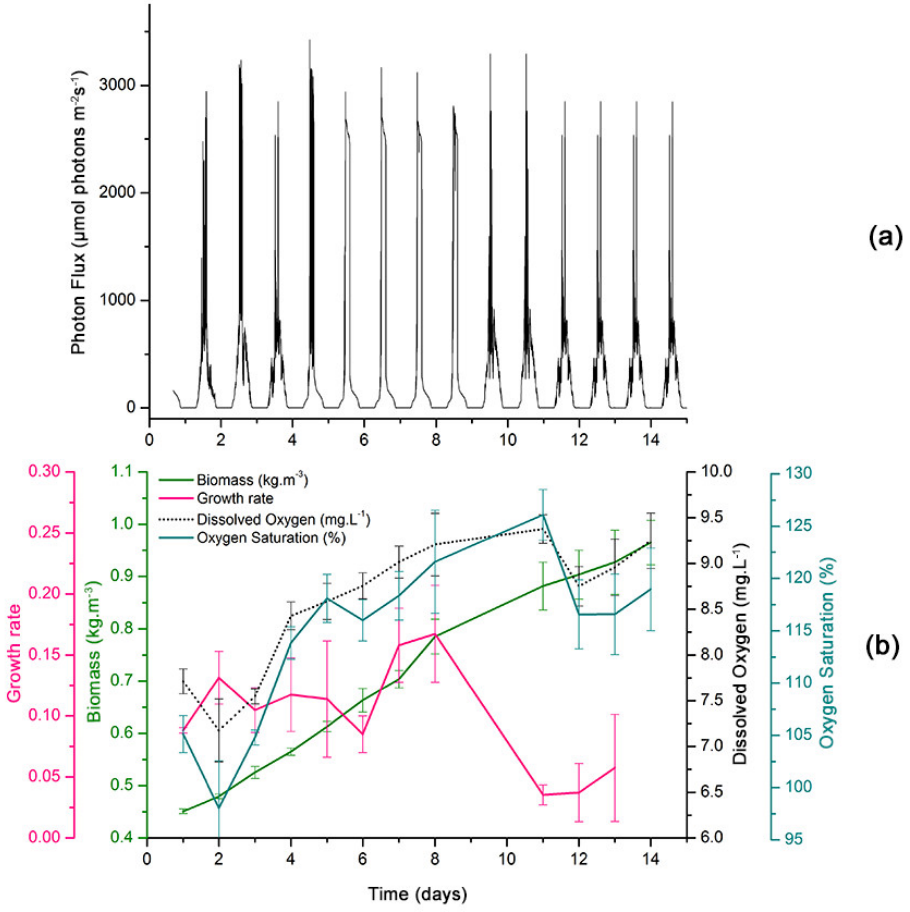


Figure 10: *P. tricornutum* biomass growth analyzed as a function of light intensity and dissolved oxygen variability during a batch culture regime. (a) PAR measured during the microalgae culture. (b) Biomass growth curve (green line), biomass growth rate (pink line), dissolved oxygen concentration in mg L^{-1} (black line) and oxygen saturation (dark green line) obtained during cultivation time. Error bars are standard deviation.

Otherwise, production in other outdoor PBR designs usually reveals higher biomass concentration, but lower areal and volumetric productivities when compared with bubble column PBR. The volumetric and areal productivities in an outdoor horizontal tubular reactor in Spain during summer have shown about 1.9 kg m^{-3} d^{-1} and 0.32 kg m^{-3} d^{-1} respectively, along with a mass concentration of *P. tricornutum* equivalent to 2.3 kg m^{-3} [89]. Using an outdoor cylindrical helical tubular reactor, Hall et al. [90] have obtained a volumetric productivity and biomass concentration of 1.4 kg m^{-3} d^{-1} and 3.0 kg m^{-3}, respectively, in a *P. tricornutum* culture in Spain. Sánchez et al. [91] have obtained a

volumetric productivity of *P. tricornutum* equivalent to 1.48 kg m^{-3} d^{-1}, cultured in an outdoor tubular reactor with a horizontal solar orientation.

Studies of *P. tricornutum* culture carried out by Sánchez et al. [74] in a fed-bath regime in outdoor conditions have achieved nearly 4 kg m^{-3} of biomass concentration and a maximum specific growth rate of 0.80 day^{-1} in a 60 L bubble column PBR after 15 days [74]. The study of Sánchez et al. [74] have suggested that for achieving higher biomass concentration using bubble column PBR it is needed to control the main culture parameters as temperature, pH and nutrient supplementation, even in outdoor conditions.

The OD_{680}/OD_{550} ratio for the experiments is shown in Figure 9. The ratio between the OD at 680 nm (OD_{680}) and 550 nm (OD_{550}) can be used as an indicator of chlorophyll content per cell, allowing a controlled parameter for cells bleaching due to photoinhibition. This ratio was maintained at around 1.03 during the entire cultivation period, seemingly unaffected by variations in natural light intensity, contrary to observations in other works.

Light intensity was measured during the entire cultivation period, the PAR value and its standard deviation was 870 ± 372 μmol photons m^{-2} s^{-1} (Figure 10a). The study conducted by Miron et al. [92] has revealed similar results with a PAR value of 900 μmol photons m^{-2} s^{-1} in a culture of *P. tricornutum* carried out in a bubble column PBR that have attained a biomass concentration of approximately 1 kg m^{-3} and a biomass productivity of 0.3 kg m^{-3} d^{-1}.

During the cultivation period, there was a large variation in natural light intensity. Besides this variability, the culture appeared to be unchanged by this factor. The apparent culture insensibility can be explained by the fluid flow in the bubble column PBR, where the cells did not experiment an extended period of darkness because of the high frequency of radial dark-light movement, from the central darker core of the reactor to the illuminated border of the column [74, 87]. Other studies have suggested that *P. tricornutum*'s growth does not change in accordance with light intensity [88]. In fact, studies of productivity in *P. tricornutum* cultures exposed to different light intensities, from 100 μmol photons m^{-2} d^{-1} onwards produces light saturation regardless of constant productivity which seems to be independent of increasing intensities [88, 93, 94]. However, cultures with limited light intensities are generally expected to increase the biomass productivity upon the enhancement of light intensities [95]. This fact can be explained by the photoinhibition process that occurs in cells under light intensities above 100 μmol photons m^{-2} d^{-1}. In addition, when the molecular machinery becomes inhibited no further inhibition occurs regardless the increase in irradiance [96].

Concerning the dissolved oxygen generated by photosynthesis during the cultivation period, it was observed a maximum of 9.38 ± 0.16 mg L^{-1} at day 8 of cultivation, period in which the culture also reached the highest areal productivity and growth rate (Figure 10b). The oxygen saturation ranged from 97.88 ± 4.50% to 125.90 ± 2.42% (Figure 10b). The

oxygen generated by photosynthesis during culture in bubble column PBR is generally freely removed because of the hydrodynamics and the good gas-liquid mass transfer characteristics of this type of reactors [97], hence the oxygen concentration does not exceed 126% of air saturation. On the other hand, similar results were achieved by other studies [98] using a membrane-based localized oxygen remover able to maintain dissolved oxygen at 120%, 32% lower than the conventional bubble column PBR. However, in this study no deoxygenation equipment was used to maintain the oxygen saturation below 126%.

High concentrations of dissolved oxygen in the culture can suppress the photosynthesis. Moreover, the combination of a high dissolved oxygen level and intense natural irradiance can lead to biomass damage by photo-oxidation [89]. Conventional tubular PBR usually enable oxygen accumulation above 400% [89], which leads to photo-oxidation and oxygen inhibition. On the other hand, in column bioreactors usually these processes do not occur because of the large diameter of the vertical column and the high gas-liquid mass transfer rates [74].

3.3.2 Total Lipid Measurement

After the harvesting and lyophilizing processes, the total lipids were extracted and quantified. Lipid extractions were carried out with Soxhlet extraction using hexane (at 60 °C) and the Bligh and Dyer method at room temperature and pressure. Table 1 shows crude lipid yields for the different extraction methods. As expected, the Bligh and Dyer method extracted a relatively large amount of lipids (9.08 ± 0.38 wt %) in 1 h, whereas Soxhlet extraction with hexane yielded only 3.99 ± 0.69 wt % lipids after 24 h, without previous cell disruption.

Table 1: Total lipids of *P. tricornutum* determined by the Soxhlet extraction method using hexane as solvent and by the Bligh and Dyer method, using methanol and chloroform as co-solvents.

Parameter	**Extracting solvent**	
	Hexane	Methanol/Chloroform
Total lipids (wt%)	3.99 ± 0.69	9.08 ± 0.38
Lipids productivity (g m^{-2} d^{-1})	10.37 ± 1.80	23.61 ± 0.99

Lipid productivity was calculated as the product of biomass productivity with lipid content. Lipid productivity obtained from hexane lipid extraction and the Bligh and Dyer method was 10.37 g m^{-2} d^{-1} and 23.61 g m^{-2} d^{-1}, respectively. Based on these results it can be concluded that the highest yield efficiency was achieved with the Bligh and Dyer method. Similar efficiency with hexane and methanol/chloroform solvent-based lipid extractions was observed in other studies [99, 100].

Previous studies have estimated lipid content in *P. tricornutum* between 5.4 wt% and 10.7 wt% after extractions carried out with chloroform/methanol solvents [101]. Chauton et al. [68] have also found a chloroform-extractable lipids content of 10% of biomass dry weight in *P. tricornutum* cultivated in a fed batch-culture regime. Wawrik and Harriman [102] have estimated 9.4 wt % of cellular lipid contents at the stationary phase for *P. tricornutum* cultivated indoor in batch regime.

Nevertheless, the lipid content in *P. tricornutum* was not as high as reported previously [21]. Lipid yield can vary as a result of light intensity, while exposed to higher light intensities the lipid content is lower than the lowest light intensity [88]. The cellular content of lipids also differs as a result of culture phases. Lower lipid content can be found in actively growing cells rather than in those in the stationary phase [68]. Indeed, in this study the cells were harvested when the culture just reached the stationary phase. Another possibility to improve lipid content in a microalgae culture is to consider the fed-batch cultivation system for providing a continuous production of high-value compounds and nitrogen starvation conditions. Naturally, this end point can be explained because the cells, cultivated under stressed conditions, usually respond by stimulating the production of energy reserves [103].

Despite the fact that the Bligh and Dyer method is normally used as a benchmark for quantifying the total lipid content from microalgae, it is not environmentally and economically feasible at an industrial scale, due to large quantities of hazardous waste solvents generated by the process. For this reason, some studies have looked for new or improved methods for enhancing the microalgae lipid extraction using hexane [99, 104]. Hexane is an organic, solvent mostly used in large scale lipid extraction because of its cost-effectiveness and recyclability. It is less toxic than chloroform and more environmentally friendly for biodiesel production [105, 106]. Although hexane has been reported to be less efficient than chloroform-based methods for microalgal lipid extractions, some studies have demonstrated that using hot compressed hexane could enhance its otherwise low efficiency [99]. Furthermore, other studies [104] have suggested that dividing the hexane/hydroalcoholic phase into two steps for improving lipid recovery would result in a reduction of toxic solvents and subsequently, reducing the total amount of solvents used.

The rapidly growing young cells generally lead to a lower content of storage lipids and more proteins than cells cultured at lower specific growth rates [107]. The variance observed in the total lipids quantification of microalgae biomass, when comparing different studies and culture conditions, can be explained by the large variability of environmental factors that affect the growth rate and productivities [108]. Also, lipid content is strongly influenced by environmental effects, such as nitrogen limitations, temperature and irradiance in *P. tricornutum* cultures [103].

3.3.3 Fatty Acids Compositions

Lipids are important components for energy storage for most organisms, including microalgae. The diatom *P. tricornutum* produces some fatty acids that are potentially significant raw material for biodiesel production and/or high-value compounds. The composition and relative abundance of each microalgae fatty acid was estimated based on the GC area signals (% area per sample) for the corresponding FAME as shown in Table 2 (see also Figure A.1 and Table A.1 in the Appendix A). The fatty acid profile obtained by gas chromatography analysis of *P. tricornutum*'s lipids showed that the alga contained more than 15 different fatty acids, of which five represent more than 8% of the total fatty acid content.

Table 2: Fatty acid compositional profiles of *Phaeodactylum tricornutum*.

Fatty Acids	**Chain**	**Mass fraction (%)**	**FAME yield (wt%)**[1]
Undecanoic acid	C11:0	0.07	0.01
Decanoic acid	C12:0	8.94	0.81
Pentadecanoic acid	C15:0	15.82	1.44
Pentadecenoic acid	C15:1	13.43	1.22
Palmitoleic acid	C16:1	4.89	0.44
Heptadecanoic acid	C17:0	12.06	1.10
Heptadecenoic acid	C17:1	1.12	0.10
Stearic acid	C18:0	3.18	0.29
Oleic acid	C18:1	2.47	0.22
Linoleic acid	C18:2	0.67	0.06
Henicosanoic acid	C21:0	2.27	0.21
Eicosadienoic acid	C20:2	0.51	0.05
Eicosatrienoic acid	C20:3	29.69	2.70
Arachidonic acid	C24:4	0.54	0.05
Docosadienoic acid	C22:2	0.61	0.06
Not identified	-	3.75	0.34
SFA	-	42.34	3.84
MUFA	-	21.91	1.99
PUFA	-	31.41	2.85

[1] Each FAME percentage in biomass calculated by the equation: FAME yield (wt%) = Crude lipid yield (wt%) × FAME content (%)/100

Analysis of FAME profile plays a crucial role when determining fuel properties. FAME consists mainly of saturated and unsaturated carbon chain lengths from C11 to C24. The most important fraction was the eicosatrienoic acid (C20:3), with a relative abundance of 29.69% or 2.70 wt% of the total microalgae dry biomass. In particular, the occurrence of C16-C18 fatty acids is considered as a good composition for biodiesel production [109]. *P. tricornutum* samples showed 24.39% of C16-C18 fatty acids, which can provide the most suitable relation between cold flow properties and oxidative stability [109]. Concerning the presence of saturated fatty acids (SFA) and monounsaturated fatty

acids (MUFA), the samples showed a proportion of 42.34% (3.84 wt%) and 21.91% (1.99 wt%) respectively (Table 2). Other studies [61] have shown a higher proportion of these fatty acids (SFA-50.16% and MUFA-48.79%), but this enhancement is explained by the use of different gas-liquid ratios. Yet, the relation between SFA and MUFA is similar to the values reported in this work. These findings suggest that biodiesel could achieve high cetane numbers and low iodine values, meeting European (EN 14214) and US (ASTM D6751) standard requirements [110, 111].

The FAME profile showed that *P. tricornutum* contained considerable amounts of PUFA (31.41% or 2.85 wt%) (Table 2). *P. tricornutum*'s PUFA production has been reported in outdoor cultures, and findings suggested that under nutrient-replete conditions the amount of PUFA can reach 60% [112].

However, as this study was conducted outdoors under nutrient replete conditions, the amounts of PUFA were lower than other studies, which could be explained by differences in temperature and light conditions. The European standard for biodiesel (EN 14214) requires less than 1% of highly polyunsaturated fatty acids ($\geq$ 4 double bonds), which could influence fuel properties of the resulting biodiesel. Although the high concentration of PUFA at *P. tricornutum*'s FAME analyses showed low values (0.54%) for arachidonic acid (C24:4), a highly polyunsaturated fatty acid present in this microalgae oil. The total amount of PUFA can be a problem when producing biodiesel that fulfills the standard criteria.

Considering FAME yields, SFA, MUFA and PUFA corresponded to 3.84 wt%, 1.99 wt% and 2.85 wt% of the total dried biomass (Table 2). It is known that the quality of fatty acids changes depending on the environmental culture conditions and the age of the culture. Yodsuwan et al. [113] have found that cultures with low nitrogen concentration result in higher levels of saturated fatty acids than those cultivated with higher nitrogen concentration in the exponential growth phase. Alonso et al. [114] have studied in continuous cultures the influence of age and nitrogen concentration in fatty acids distribution among different lipid classes of *P. tricornutum*. These authors have concluded that although age shows almost no influence on fatty acid content, both the saturated and monounsaturated fatty acids content increases when nitrogen concentration decreases. Oleic acid (C18:1) usually increases in older cultures of *P. tricornutum* at their late stationary phases [113]. Oleic acid is known to play an important role in the lipid metabolism of *P. tricornutum* as a precursor of PUFA during the exponential growth phase [69]. In fact, in this study, high contents of PUFA were found in the culture, harvested in the early stationary phase, when oleic content was lower and PUFA higher. Probably, in a later stationary stage of this culture it would be possible to find opposite results.

In this study, EPA, a common fatty acid present in *P. tricornutum* biomass, was not observed. The possible reason for absence of this fatty acid can be the fact that cell harvest was done immediately in the moment when the early stationary phase

started. EPA has been described as an important constituent of cell membranes and photosynthetic membrane lipids [88]. Hence, in this culture phase the cells were just finishing their replication process and using all the EPA produced in their metabolism. Also, Yongmanitchai and Ward [115] have reported that the optimum temperature conditions for producing EPA is in the range of 21.5–23.0 °C and pH at 7.6. In this work the temperature was lower, from 17 to 21 °C, and pH varied from 7 to 9, outside optimal conditions. Furthermore, some authors have reported that microalgae cultured outdoors, facing more environmental challenges such as irradiance, temperature variation and nutritional stresses, showed variations within their lipid composition and can produce energy-reservoir lipids instead of structural lipids [103, 116]. Acién Fernández et al. [91] have presented a model for predicting EPA productivity from *P. tricornutum* cultures in Spain, taking into account the existence of photo-limitation and photoinhibition under outdoor conditions. These authors have concluded that biomass productivity is limited by low light availability (e.g., during winter), but EPA content is higher under optimal light availability (e.g., during summer), especially when biomass productivity is higher. This could be caused by photoinhibition.

3.4 Conclusions

This study analyzed the growth and lipid productivity of *P. tricornutum*, cultivated in outdoor bubble columns PBR under natural conditions in Chile, and its potential for biodiesel production. Results showed a final dry weight biomass concentration of 0.96 kg m^{-3}, with a maximum volumetric productivity of 0.13 kg m^{-3} d^{-1} and total lipid content of 9.08 wt%. FAME profile was analyzed and the composition of these fatty acids showed a proportion of 42.34% of SFA, 21.91% of MUFA and 31.41% of PUFA. This profile meets the requirements of international biodiesel standards, showing that *P. tricornutum* can be a good alternative for biodiesel production. However, it is necessary to develop further studies to evaluate the lipid content and productivity of this microalga at different geographical locations, considering different climate and stress inducing conditions, to effectively determine its potential for biodiesel production and other applications, since the fatty acids profile and biochemical components change under diverse farming environments.

Chapter 4

Diatom-based Biosilica for Energy Applications

The use of living diatom-based biosilica for energy and nanoparticle application has recently shown great promise, mainly because the production of these materials by other means is costly and not environmentally friendly. This study aims to analyze the frustule composition of *Phaeodactylum tricornutum* dried biomass for determining the Si percentage. Frustules were purified by acid cleaning, using three different acids, in order to remove the organic and inorganic impurities. Frustules were analyzed by Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM/EDS) to observe their morphology and achieve the concentration of Si per weight of dry matter. The protocol using HCl as an organic matter acid cleaning agent proved to be the best alternative for purifying and quantifying biosilica in *P. tricornutum*.

4.1 Introduction

Diatoms are a group of unicellular microalgae spreading in a broad range of environments including freshwater, saline water, seawater and even wet soils [117]. Due to their interesting biochemical composition, their organic constituents, such as carbohydrates, lipids, proteins and vitamins, have been used in biotechnological purposes in a wide variety of fields of application, such as pharmaceutical, nutraceutical, chemical and bioenergy industries.

Furthermore, diatoms are characterized by possessing a highly silicified cell wall, known as the frustule, constituted by a siliceous skeleton that comprises a couple of valves connected by silica bands, girdled along the borders [118] (Figure 11). Frustules of diatoms vary in morphology, shape, size and silica content, and are usually used for the identification and classification of these organisms. Throughout geological periods and due to the abundance of these organisms in seawater, after diatom cells die and decompose, their silicified carapace sediments on the sea floor and forms an enormous amount of diatomaceous earth or diatomite [23]. Diatomite has been

applied extensively in industries as a sorbent, anti-caking agent, insulation material, filter material and abrasive agent [23]. Recently, diatom-based biosilica has been used by highly technological industries and considered for a wide range of applications, such as nanoparticles [23], electronic devices [119], drug delivery systems [120], biomolecule diagnostic devices [121], chemical sensors [122] and energy application [123].

However, diatomite is an expensive and non-renewable raw material. Its purification to a high level of purity for use in fine applications remains a huge challenge. Furthermore, the silicon industry usually applies high temperature, pressure and toxic reagents to manufacture this compound, leading to a highly energy-intensive process and the unviability of using silicon in a range of applications that require a high grade of purity. In this context, the cultivation of diatoms for these purposes or, alternatively, the usage of residual biomass deriving from a diatom-based biorefinery, represents a good alternative for both increasing the biorefinery eco-efficiency and producing a high purity grade and renewable silicon resource.

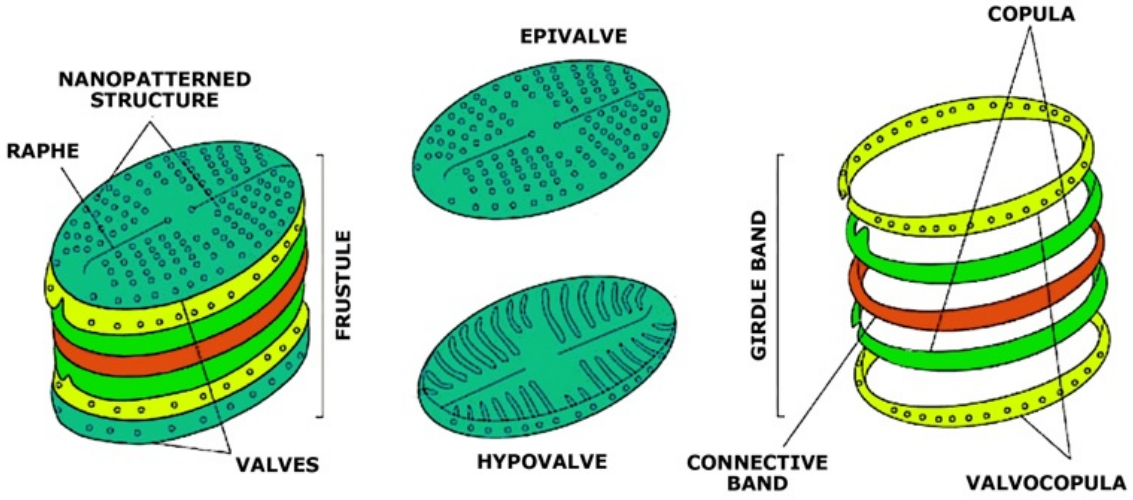


Figure 11: Schematic representation of the frustule. Green structures represent valves (hypovalve and epivalve) and the connective silica bands forming the girdle region are represented by yellow, light green and red rings.

Source: Adapted from Francius et al. [124]

Phaeodactylum tricornutum is a diatom mostly found in seawater, but it can be grown in saline and freshwater. Cells display a peculiar characteristic of a pennate diatom, and three different morphotypes have been recognized, the ovoid, fusiform and triradiate [25]. These different morphotypes can occur or prevail over one another, depending on the environmental conditions and culture stresses [125]. The cell wall of *P. tricornutum* is not highly silicified as in other diatomaceous species, but it shows different amounts of silica depending on the morphotype and culture conditions. Although all *P. tricornutum* morphotype possess silicified structures, only oval forms can synthesize a silica valve (epivalve and hypovalve) with pores and central raphe (Figure 11) [125], while in the

other two morphotypes the silica zone corresponds to the girdle-band region (Figure 11) [124]. Despite these structural differences, the same content of silicon has been reported in both oval and fusiform morphotypes [126].

The aim of this chapter was to perform frustule purification from dried biomass of *P. tricornutum*, in order to achieve the elemental composition and explore the opportunity of using biosilica from these purified frustules for energy and/or nanoparticle applications.

4.2 Materials and Methods

4.2.1 Diatom Strain

The strain of *P. tricornutum* (originating from Cañar Blanco, La Serena, IV Region, Chile) was cultivated outdoors under natural conditions in Chile, harvested and freeze-dried after 14 days of cultivation [127]. An amount of 250 mg of lyophilized *P. tricornutum* diatom was used to obtain frustules for elemental composition identification, silica quantification and morphology observation.

4.2.2 Biosilica Cleaning Procedure

The aim of this procedure was to remove the residual organic matter and traces of metal. In order to achieve the best acid treatment for frustule purification and a higher amount of biosilica, three different acid treatments were tested.

Biosilica Purification by HCl Treatment – Treatment 1

The purification process of diatom frustules was carried out according to the modified method described by Jiang et al. [128]. Samples of 250 mg dried biomass were used for acid cleaning in order to remove organic matter and metal. The samples were mixed with deionized water and 37% HCl in a ratio of 2:1 (v:v), respectively, and subsequently submitted to continuous magnetic stirring for 1 hour in a water bath at 100 °C, placed in a fume hood. The acid removal was then performed by washing samples with deionized water, followed by centrifuging. Thus, for the washing process, the samples were centrifuged at 4,000 rpm for 3 min (4000R Benchtop Refrigerated Laboratory Centrifuge, Centurion Scientific Ltd, West Sussex, UK) several times until the pH of the supernatant reached approximately 7.0. The remaining water was removed by placing diatoms frustules at 105 °C in a drying oven until complete water evaporation. The samples were subsequently heated in a furnace (Compact Muffle Furnace LE 6/11/B150 LE060K1BN, Nabertherm, Lilienthal, Germany) to 600 °C at a heating rate of 3 °C min^{-1} for 6 hours, to remove remaining organic matter.

Biosilica Purification by H_2SO_4 Treatment – Treatment 2

The treatment of diatoms using H_2SO_4 acid cleaning was carried out according to a modified protocol proposed by Bismuto et al. [122]. Briefly, 250 mg of freeze-dried samples were mixed with 25 mL of 97% sulphuric acid for 5 min at 60 °C. Subsequently, the acid was removed by washing with distilled water followed by centrifugation process at 3000 rpm for 10 min (4000R Benchtop Refrigerated Laboratory Centrifuge, Centurion Scientific Ltd, West Sussex, UK). The washing process was repeated until the supernatant became neutral. The cleaned frustules were placed on a drying oven at 105 °C until the water was fully evaporated.

Biosilica Purification by HNO_3 and H_2SO_4 Treatment – Treatment 3

The cleaning of frustules using HNO_3 and H_2SO_4 was performed using a modified method proposed by Desclés et al. [129]. An amount of 250 mg of dried biomass was oxidized by using potassium permanganate (final concentration 3%) in H_2SO_4. The oxidized material was eliminated by washing for 1 min with a mixture of 16% HNO_3 and 48% H_2SO_4 on 2:1 (v:v) ratio, respectively. Samples were neutralized by adding Tris-HCl buffer (1 M, pH 8), filtrated and washed with ethanol 95% using a glass membrane filter (0.47 μm). Filters containing cleaned frustules were placed in an oven at 105 °C until the water was fully evaporated.

4.2.3 Frustule Final Mass Estimation

Frustules treated under the same conditions, without acid cleaning, were used as control in all the experiments. The final mass of purified frustules was obtained by gravimetric weighing on an analytical scale. The percentage of purified frustules and biosilica was calculated by Equations 5 and 6.

$$\text{Frustules (wt\%)} = \frac{B_{frustule} \times 100}{B_{total}} \tag{5}$$

$$\text{Biosilica (wt\%)} = \frac{(B_{frustule} \times \%_{Si}) \times 100}{B_{total}} \tag{6}$$

Where, Frustules (wt%) is the percentage of frustules in the samples (wt%), B_{total} is the weight of total biomass before the treatment (g); $B_{frustule}$ is the weight of the treated biomass obtained after the cleaning process; Biosilica (wt%) is the percentage of biosilica in the samples; $\%_{Si}$ is the percentage of Si in the samples.

4.2.4 Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM/EDS) Analysis

The morphologies of the samples were characterized using a high resolution (Schottky) Environmental Scanning Electron Microscope (SEM) with X-Ray microanalysis and backscattered electron diffraction pattern analysis (Quanta 400 FEG ESEM/EDAX Genesis X4M, Thermo Scientific, Oregon, USA) operated at an acceleration voltage of 15 kV. Samples were coated with Au/Pd thin film by sputtering, using a SPI Module Sputter Coater. Corresponding energy-dispersive spectroscopy (EDS) was used to examine the elements of the frustules at 15 kV.

4.3 Results and Discussion

4.3.1 Yield of Frustule Purification

Biosilica utilization for most nanotechnological applications requires the removal of organic matter from the frustules [123]. In this study, diatom dried biomass was treated using three different methods to remove organic matter and metal, and to promote the increase in Si content per biomass dry weight. The efficiency of frustule purification was calculated for each treatment, and the content of biosilica per dry matter was also measured; the results are shown in Table 3. Considering the frustule recovery after purification process, treatment 2 showed the highest biosilica yields, followed by treatment 1 and treatment 3. Although treatment 2 achieved higher yields, EDS analysis (see Section 4.3.2) revealed a high content of carbon in the samples, indicating that most of the dry weight was in fact composed of organic matter, which leads to a higher amount of biomass, whereas biomass cleaned with treatment 1 showed a considerable amount of recovered frustules when the low carbon content in the samples was observed. On the other hand, treatment 3 reached fewer frustules recovered and, consequently, the lowest biosilica content (Table 3).

Table 3: Percentage of frustules and biosilica in purified *P. tricornutum* biomass per treatment.

Treatment	**Frustule (wt%)**	**Biosilica (wt%)**
1	13.78 ± 1.32	5.19 ± 0.53
2	20.86 ± 0.96	8.02 ± 0.25
3	6.35 ± 0.30	2.44 ± 0.12

Several methods for cleaning frustules have been described, such as high temperature ashing, hydrogen peroxide treatment, SDS/EDTA treatment and acid treatment. Acid cleaning is the most frequent method employed for this purpose [117, 126, 130]. The best

cleaning treatment to achieve high purity Si concentration after frustules purification is not yet established in the literature, because different diatom strains react differently to each method. Severe methods for cleaning frustules can cause damage to its structure, whereas milder methods might not completely remove the organic matter [131]. Consequently, it is necessary to analyze the behavior of each strain to establish the best alternative for frustule cleaning and harvesting.

4.3.2 SEM and EDS Analysis of Frustules

Diatom frustules were analyzed by SEM to observe morphologies and were scanned by EDS for elemental microanalysis, after each acid cleaning. Three different protocols were used to observe better conditions for biosilica purification. Comparing three treatments, differences in terms of Si content, morphology integrity and presence of remaining biomass were evidenced. All acid cleaning for *P. tricornutum* revelead to affect the native morphological configuration of frustules, which were collapsed completely by acid cleaning (Figure 12). Although the prior biomass freeze-dryer process led to stress damage in the cell structures, silica shells of *P. tricornutum* were restricted to oval forms, which was the only morphotype in which the silicification process occurred [132]. The oval morphotype was the least prevalent one in the diatom culture, performed in this study (see Chapter 5, Subsection 5.3.1, Figure 16). Although fusiform morphotypes have also silicic content, this process is restricted to the lateral bands (girdle bands) which surround the frustules and assume grid shapes, remaining dispersed in the treated material after frustule cleaning. Hence, it is not possible to identify the three-dimensional structure assumed by freshly cultivated diatoms (Figure 12). Consequently, it is important to note that the use of *P. tricornutum* biosilica should be applied only in conditions where the intact pore structures are not required, directing its use to Si content exploitation.

Furthermore, were a large amount of debris was observed surrounding the frustules in all the three acid cleaning treatments, which most probably consisted of organic matter, undissolved substances from the culture and organic matrix, mainly the mucilage secreted by the diatoms and extracellular polymeric substances (Figure 12).

In fact, Déscles et al. [129] described *P. tricornutum* as an unusual diatom because its three major morphotypes contain a delicate frustule composed of a low concentration of silica. Studies conducted by Willis et al. [133] demonstrated that *P. tricornutum's* ovoid morphotype secretes adhesive mucilage from the girdle band region as cell-substratum tethers, accumulating on the surface and forming a biofilm.

Depending on the treatment applied for frustule cleaning, organic matter is more or less removed from the final material; consequently, it could affect the 3D integrity of the frustules and final Si content [131]. Therefore, the final required material characteristics can be adjusted by using the appropriate pretreatment method, previously considering the

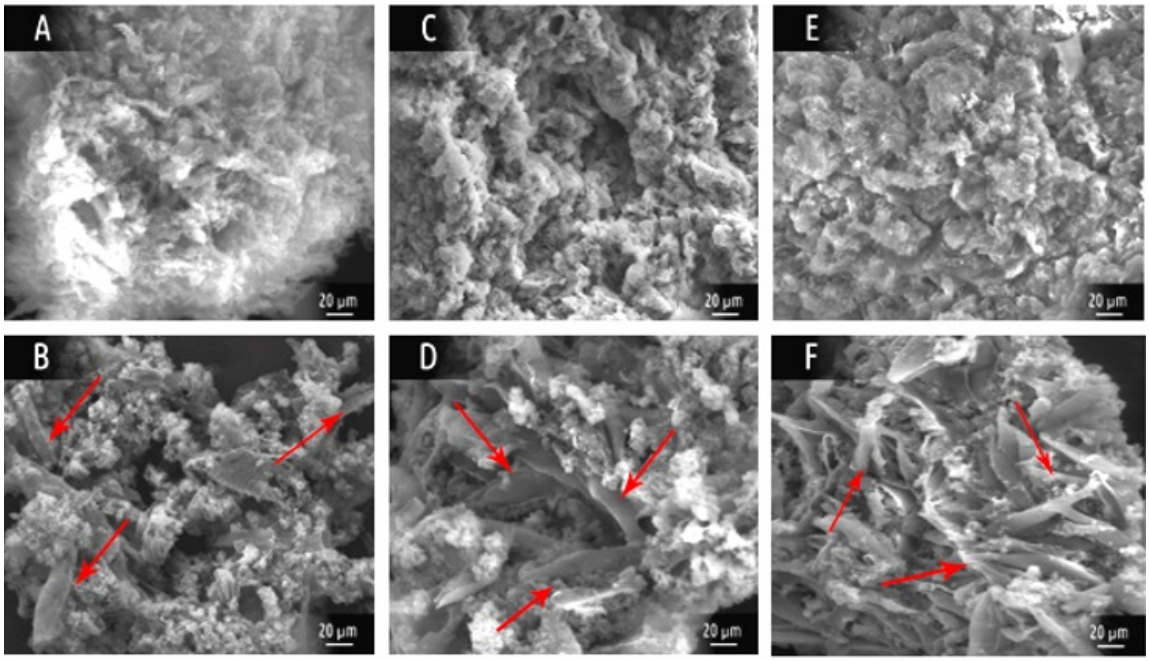


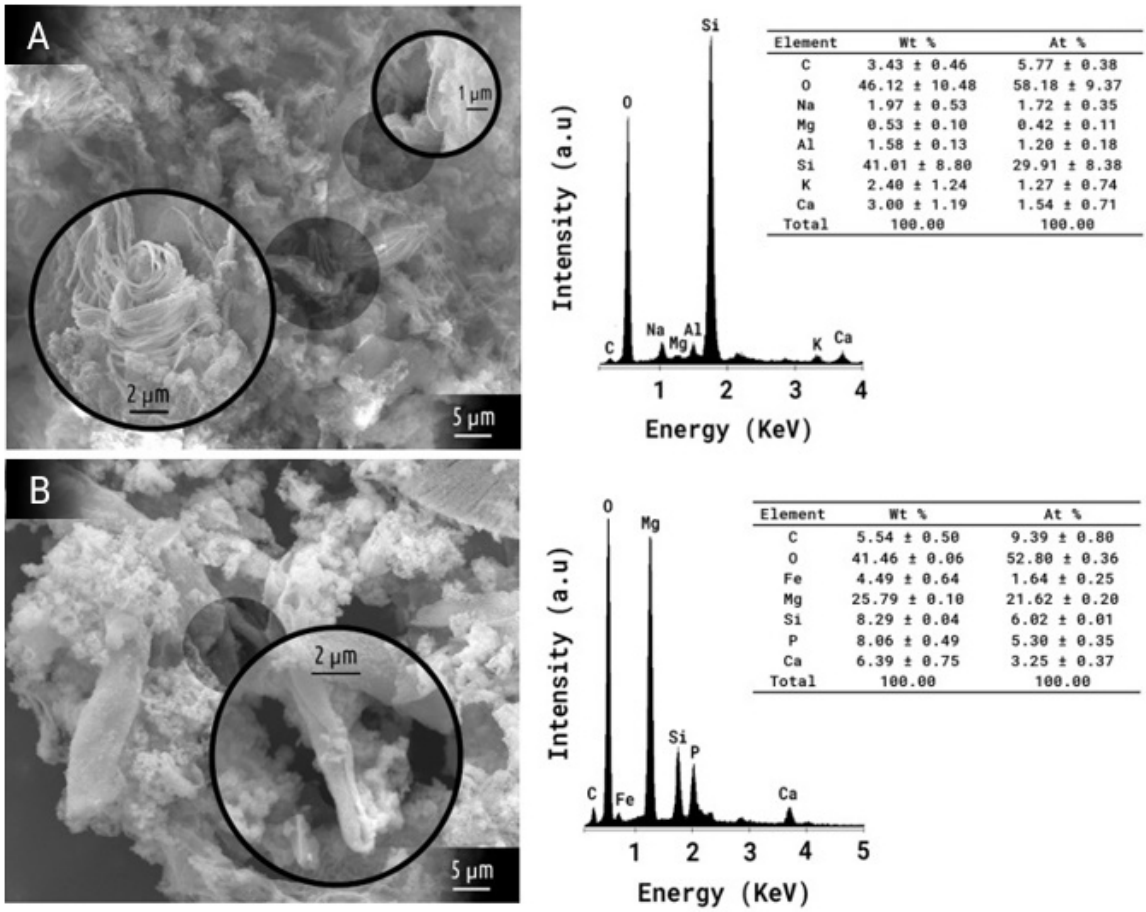
Figure 12: SEM micrographs of the *P. tricornutum* frustules with debris surrounding the frustules. (a) Frustules purified and cleaned with treatment 1; (b) Control frustules of the treatment 1; (c) Frustules purified and cleaned with treatment 2; (d) Control frustules from treatment 2; (e) Frustules purified and cleaned with treatment 3; (f) Control frustules from treatment 3. Arrows pointing to the frustules surrounded by cellular debris.

desired final use of the frustules.

Analysis of frustule morphologies and elemental composition obtained after each cleaning process are shown in 13, 14 and 15. Treatment 1, consisting of HCl acid cleaning followed by a baking process, revealed itself to be the most damaging to frustule structure while showing the highest Si content, which reached an amount of 41.01 ± 8.80 wt% (Figure 13). The baking process seemed to be the best method for organic matter cleaning from *P. tricornutum*, when the lowest content of carbon was observed in the acid cleaning samples (Figure 13a) with an amount of 3.43 ± 0.46 wt% and in untreated acid samples (Figure 13b) with an amount of 5.54 ± 0.50 wt%. It is important to mention that the untreated acid samples were not submitted to acid cleaning. However, these samples were calcined together with the treated acid cleaning samples.

Treatment 2 was performed with concentrated H_2SO_4 that demonstrated to be equally damaging to the cell morphology, a relative concentration of Si content of 28.64 ± 1.91 wt% (Figure 14a) against 8.29 ± 0.04 wt% of untreated samples (Figure 14b). However, EDS analysis showed that the lowest Si content was observed in frustules cleaned with treatment 2.

Treatment 3 was carried out with a combination of HNO_3 and H_2SO_4, which was evidenced to be the best alternative to purify the frustules, concerning the maintenance of the frustules' morphology (Figure 15a). Biosilica content revealed a concentration of



Element	Wt %	At %
C	3.43 ± 0.46	5.77 ± 0.38
O	46.12 ± 10.48	58.18 ± 9.37
Na	1.97 ± 0.53	1.72 ± 0.35
Mg	0.53 ± 0.10	0.42 ± 0.11
Al	1.58 ± 0.13	1.20 ± 0.18
Si	41.01 ± 8.80	29.91 ± 8.38
K	2.40 ± 1.24	1.27 ± 0.74
Ca	3.00 ± 1.19	1.54 ± 0.71
Total	100.00	100.00

Element	Wt %	At %
C	5.54 ± 0.50	9.39 ± 0.80
O	41.46 ± 0.06	52.80 ± 0.36
Fe	4.49 ± 0.64	1.64 ± 0.25
Mg	25.79 ± 0.10	21.62 ± 0.20
Si	8.29 ± 0.04	6.02 ± 0.01
P	8.06 ± 0.49	5.30 ± 0.35
Ca	6.39 ± 0.75	3.25 ± 0.37
Total	100.00	100.00

Figure 13: SEM micrographs of the *P. tricornutum* frustules and EDS analysis purified by Treatment 1. (a) Treated biomass (b) Untreated samples. Graphs contain the samples elemental composition obtained by EDS analysis. Wt% indicates the relative concentration of the element and At% indicates the atomic weight percent of the element.

35.15 ± 5.42 wt% of Si in the samples.

Indeed, EDS analysis showed that the Si content improved when the cleaning process was performed with treatment 1 (Figure 13), followed by treatment 3 (Figure 15) and the lowest Si content was observed in frustules cleaned with treatment 2 (Figure 14).

On the other hand, the control samples of all experiments showed that the baking process used in treatment 1 seemed to play an important role in improving the Si content both in acid cleaning samples and untreated samples. This finding was supported by the the higher values of the Si content found in untreated samples of treatment 1, 8.29 ± 0.04 wt%, compared with those untreated samples of the other two treatments applied, whose values were 3.78 ± 0.28 wt% for treatment 2 and 4.54 ± 0.07 wt% for treatment 3 (Figure 14 and 15).

Diatoms are bioaccumulative organisms that incorporate some trace levels of metals in their silica shells [131]. In the culture medium of *P. tricornutum* some elements are available for their metabolism to function. However, some of them were observed to be incorporated into the silica cell walls, as shown in Figures 13, 14 and 15, showing

the relative concentration of silicium, iron, magnesium, potassium, calcium, manganese, aluminium and sulphur in different amounts for frustules as measured with EDS analysis.

Furthermore, all cleaning processes promoted the removal of Mg content, which was the highest element found in the untreated samples (Figures 13, 14 and 15). A possible explanation for high Mg proportion in the untreated samples can be the high chlorophyll pigment concentration in the *P. tricornutum* biomass, since the Mg element is part of the molecular structure of this substance. Comparing EDS analysis, the baking process seemed to contribute significantly to reducing the C content of the frustules, which can be observed by the differences between the C percentage in untreated samples of treatment 1 and untreated samples of the other two treatments. The EDS analysis showed the lowest values for C content in untreated samples from treatment 1, compared with higher values for C content in untreated samples of treatments 2 and 3 (Figure 13, 14 and 15). This difference is mainly due to the clear decrease in the organic matter that the baking process achieved. Treatment 1 significantly decreased the weight percentage of C, P and Mg, whereas metal content increased notably. Meanwhile, treatment 2 decreased the weight

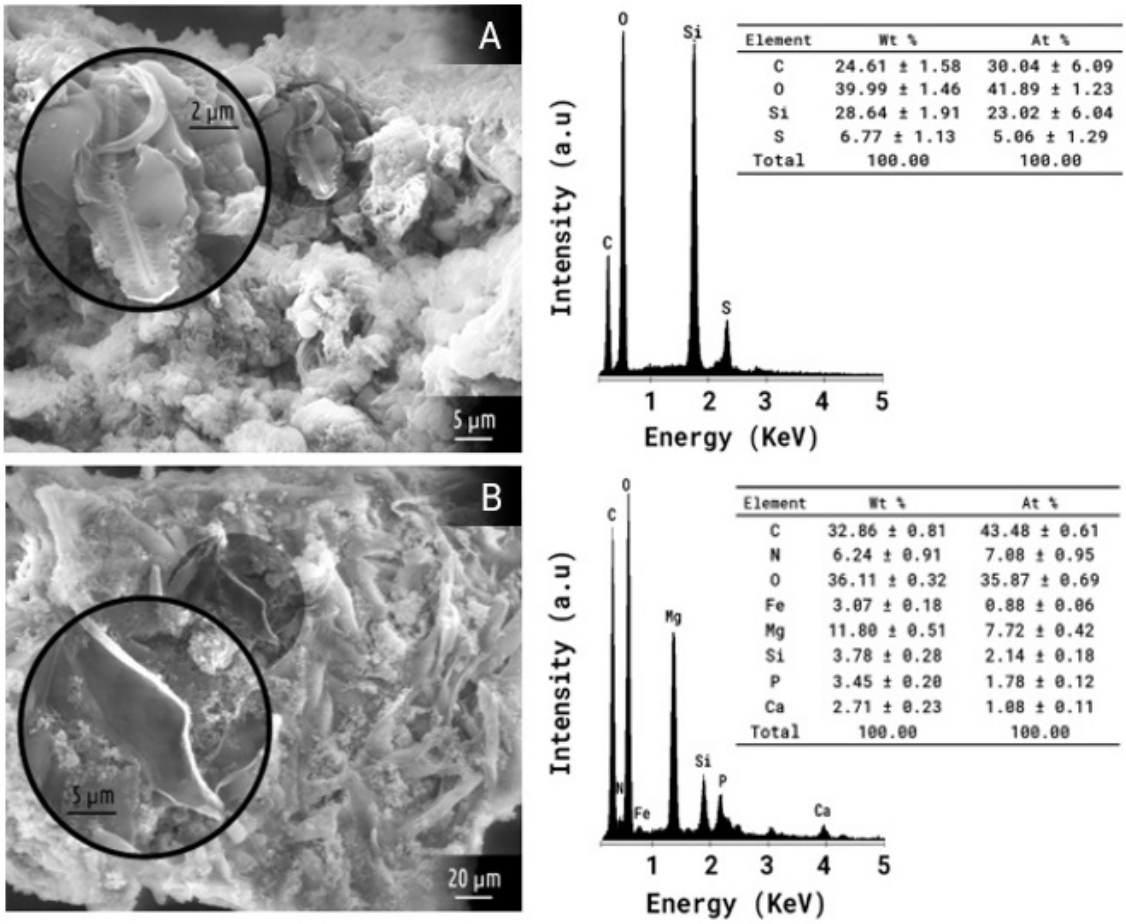


Figure 14: SEM micrographs of the *P. tricornutum* frustules and EDS analysis purified by Treatment 2. (a) Treated biomass; (b) Control samples. Graphs contain the samples' elemental composition obtained by EDS analysis. Wt% indicates the relative concentration of the element and At% indicates the atomic weight percent of the element.

percentage of C, P, Mg, Ca and increased the content of S. Finally, treatment 3 removed the content of C, P, Mg, Ca and S, but the content of Mn was higher (Figure 13, 14 and 15). The removal of elements present in the silica cell walls can be achieved by different cleaning treatments, so it is necessary to apply the correct treatment in order to allow the chemical mechanism to promote the elimination of these components.

Although the production and commercialization of diatom-based nanodevices have still not been carried out, the potential of this approach has been studied by several groups, reported in the literature and been the subject of patent applications. One of the possible proposals for diatom-based biosilica application is energy production and storage, such as photovoltaic devices and batteries. Dye-sensitized solar cells (DSSCs), proposed by O'regan and Grätzel [134], are based on the light-scattering properties of a diatom frustule-TiO_2 composite material. This system has demonstrated significant potential as inexpensive and high efficiency photovoltaic devices, being a good alternative raw material for this technology. Furthermore, Jeffryes et al. [123] have extensively discussed the use of diatom-based silica for solar cells, batteries, and electroluminescent

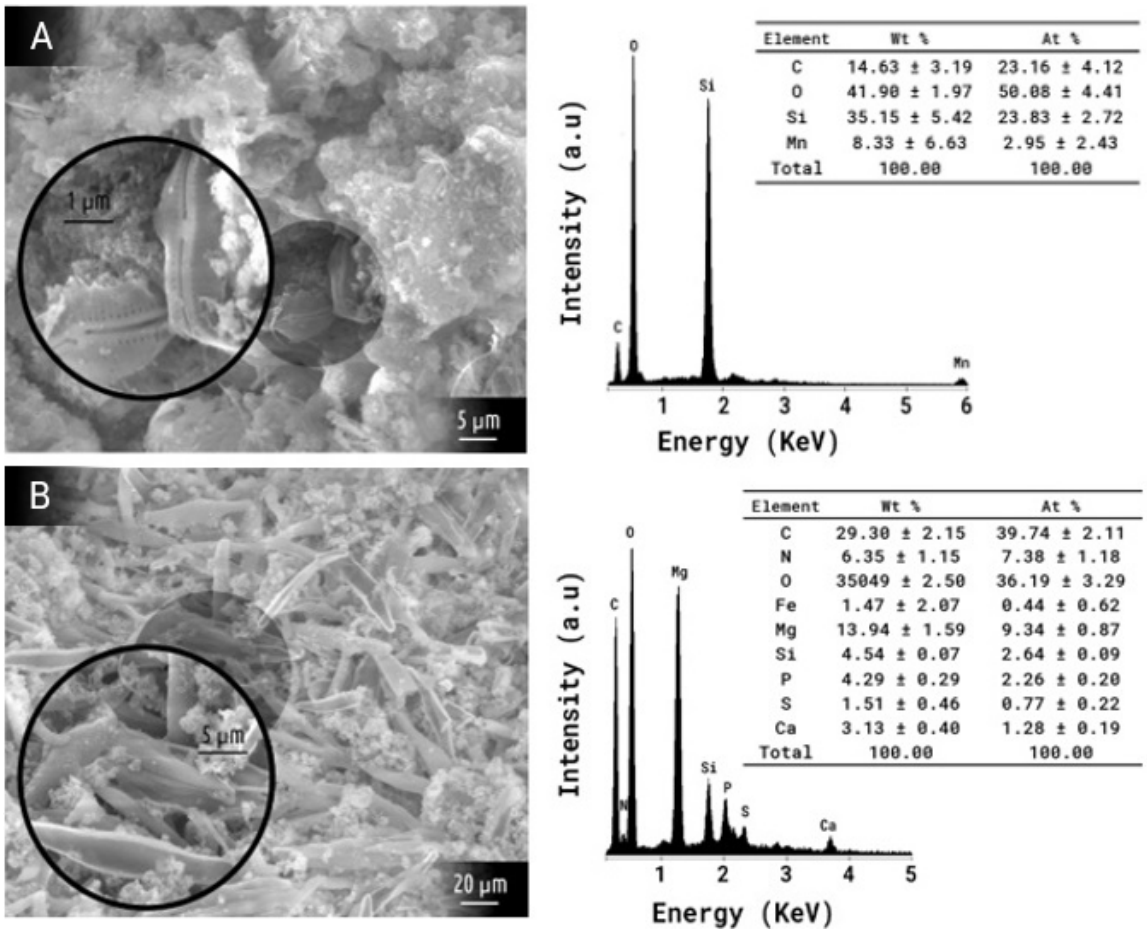


Figure 15: SEM micrographs of the *P. tricornutum* frustules and EDS analysis purified by Treatment 3. (a) Treated biomass; (b) Control samples. Graphs contain the samples' elemental composition obtained by EDS analysis. Wt% indicates the relative concentration of the element and At% indicates the atomic weight percent of the element.

devices by insertion of metal oxide, such as titanium or germanium dioxide, into the nanostructure of the diatom frustule.

Diatom-based biosilica has been recently applied to nanotechnology industries in a broad range of sectors. The main reason for the significantly growing use of these organisms is the relative ease with which some noble elements can be obtained, at a low expected cost, from diatom cultivation, and the renewable nature of these compounds. In this context, biosilica from *P. tricornutum* can be obtained directly by freshly culturing or after diatom biomass production for biofuels and/or high-value compounds by valorization of residual biomass, which will be extensively discussed in Chapter 6.

4.4 Conclusions

This work characterized the biosilica from diatoms after the application of three different acid cleaning treatments. It was observed that the elemental composition of *P. tricornutum* changes depending on the cleaning method applied. The removal of the organic matter can be more or less efficient depending on the treatment method applied, which influences the value of the Si content of the cleaned frustules. Several methods can be applied to obtain cleaned frustules, containing different elemental composition. The selection of the correct method depends on the intended final application of the frustules.

Chapter 5

Biotechnological Approach for Diatom-based Biorefinery

Microalgae biorefineries have been proposed as an important strategy for enhancing the economic profitability of bioproducts. The biorefinery concept applied to microalgae also implies valorization of residual biomass, converting it into biofuels and/or high value compounds, in an integrated way. For biotechnological proposes, it is important to determine biochemical composition of microalgae biomass, for correct addressing of the high-value compounds produced and enhancing the economic and environmental feasibility of the culture process. This study aimed to analyze the biochemical composition of *P. tricornutum* cultivated in an outdoor pilot-scale bubble column photobioreactor under natural conditions in Chile, as raw material for the production of biofuel and high-value compounds in order to propose a *P. tricornutum* biorefinery approach. The *P. tricornutum* biomass concentration was 0.96 kg m^{-3} d^{-1} with volumetric productivity of 0.13 kg m^{-3} d^{-1}. The samples showed a proportion of 7.85 wt% of carbohydrates, 38.40 wt% of proteins, 9.08 wt% of lipids, 0.86 wt% of fucoxanthin and 5.19 wt% of biosilica. The *P. tricornutum* biomass was proposed for biofuels production, associated with high-value compounds as by-products, under a biorefinery approach.

5.1 Introduction

One of the major challenges of today's society is finding sustainable ways to obtain bio-products from renewable resources, with a significant emphasis on the biorefinery concept, which aims to maximize the development and exploitation of biomass in a variety of products [135]. Among the several biomass sources for supplying biorefineries, microalgae emerged as a potentially sustainable feedstock for numerous bio-products with several advantages over conventional crops [21]. This is justified by their ability to thrive in both fresh and marine water, and unlike terrestrial crops, algae do not require fertile soil or arable land for cultivation [136]. In addition, microalgae have a high photosynthetic response, high product accumulation and high biomass production rates compared to other terrestrial crops, being among the most productive biological systems for biomass

generation and carbon capture [137].

In a biorefinery, through the application of complex technologies for biomass processing, it is possible, depending on the raw material and its composition, to separate a broad spectrum of marketable products [138]. In general terms, microalgae biomass contains valuable lipids, proteins, and carbohydrates [139]. More specifically, we can extract from microalgae high value-added compounds such as polyunsaturated fatty acids (PUFAs, e.g. omega 3 fatty acids), polysaccharides, proteins, peptides, carotenoids (e.g. β-carotenes, astaxanthins and xanthophylls), chlorophylls, terpenes and lectins, which are the base raw-material in various industrial sectors such as pharmaceuticals, biotechnology, nanotechnology, food and feed, colorants, bioplastics and energy [140].

Since microalgae accumulate different compounds of interest, in order to recover and separate several of these components it is necessary to use a variety of cascade extraction techniques [141]. First, one must determine the biochemical composition of microalgae biomass to correctly select the most adequate fractionation techniques to separate the high-value compounds produced and to enhance the economic feasibility of the culture process. Second, it becomes necessary to optimize a series of integrated biomass processing techniques, such as pretreatment, extraction and purification of target components [142], starting with the most valuable ones and ending in the least valuable ones. Making these processes efficient and economical is currently one of the main limitations and challenges of the process in microalgae biorefineries, having been the main objective of several research studies [13]. On the other hand, by recovering multiple algae compounds it is possible to valorize the complete biomass and improve the economics of the value chain.

It is possible to find different descriptions of microalgae biorefineries in the literature [143]. However, their development has been essentially at laboratory-scale. More studies are needed, in particular to obtain more experimental data, not only on a small scale but especially on a larger scale, in order to demonstrate the feasibility of a potential microalgae product's value chains and to test applications aiming to develop their market [144].

Marine diatoms are one the most productive and environmentally adaptable microalgae in the world, responsible for 20% of global carbon fixation and the dominant primary producers in the ocean. They are very abundant, highly diversified, and with high variability, estimating that there are more than 100,000 species. Its most distinctive cellular feature is the cell wall made of nanostructured silica, which is reproduced faithfully through generations by genetically controlled assembly processes [29].

Diatoms have been exploited on a commercial scale for decades due to their interesting biochemical composition, lipid productivity equivalent or greater than other classes of microalgae and to their capacity to produce other high-added-value compounds. Their main application has been for aquaculture, due to their high growth rates and lipid yields,

tolerance of harsh environmental conditions, good performance in large-scale cultures and for not competing with other sources for freshwater uses [107]. Particularly, diatoms have capacity of inducing the production and accumulation of triacylglycerol (TAG) under Si limitation, avoiding damages on photosynthesis apparatus, gene and protein expression, mainly associated with N limitation [29].

Among diatoms, *P. tricornutum* normally lives in marine habitats and is one of the most thoroughly studied species. Beardall and Morris [145] performed experiments on the adaptation of *P. tricornutum* to different light intensity, having observed an increase in chlorophyll content of cells grown in batch culture at reduced light intensities (at about 0.7 klux) in comparison to those grown at 12 klux, suggesting that growth at a suboptimal light intensity enhances the ability to utilize lower light levels. Grima et al. [146] cultivated *P. tricornutum* in an outdoor tubular photobioreactor and determined its specific growth rate in the exponential phase of 0.254 day^{-1}, having obtained a maximum biomass concentration of 25 g L^{-1} by the end of the cultivation period.

Fernández et al. [79] modeled the eicosapentaenoic acid (EPA) productivity of *P. tricornutum* in outdoor cultures, taking into account photolimitation and photoinhibition. Alonso and Belarbi [114] studied the influence of culture age and nitrogen concentration on the distribution of fatty acids among the different acyl lipid classes of *P. tricornutum*, concluding that culture age had almost no influence on the fatty acid content of around 11% of dry weight, but had a greater impact on the fatty acid profile, together with the nitrogen content, in which the content of polar lipids decreased with culture age. Fernández et al. [93] studied the operational conditions of an outdoor helical reactor for the production of microalga *P. tricornutum*, obtaining biomass productivities up to 1.5 g L^{-1} per day and a photosynthetic efficiency up to 14%. Burrows et al. [147] proposed a strategy for optimizing the lipid production in *P. tricornutum* under nitrate deprivation, showing that about 60% of lipid is synthesized de novo during 3 days of total NO_3 deprivation and that these lipids are primarily TAGs.

Benavides et al. [66] compared biomass productivity of *P. tricornutum* grown outdoors in open ponds and photobioreactors (PBRs), having obtained the optimal biomass concentration of respectively 0.6 g L^{-1} and 1.0 g L^{-1}. Chauton et al. [68] studied the effect of nitrogen or phosphorus limitation on cultures of *P. tricornutum*, showing that P limitation led to a higher carbon content per cell than N limitation. In N limited cells a large fraction of the carbon was stored in carbohydrates, and a lipid content of around 10% of dry weight was found independently of the treatment. Ak et al. [148] studied the effects of N deficiency on *P. tricornutum* cultures, showing that it caused the decrease of protein content and biomass amount while the lipid content increased. Fajardo et al. [104] studied the extraction of lipids from *P. tricornutum* biomass using ethanol as a solvent with a recovery of over 90% of the saponifiable lipids in the biomass. Gao et al. [70] studied cultures of *P. tricornutum* in a flat-plate photobioreactor, and have obtained

the highest volumetric productivities of lipids, EPA, chrysolaminarin, and fucoxanthin of, respectively, 9.6, 93.6, and 4.7 mg $L^{-1}d^{-1}$, during the exponential phase (day 6), and concluding that extractable amounts of these components can be obtained by regulating the culture conditions.

German-Báez et al. [149] performed a characterization of the under-utilized *P. tricornutum* biomass, showing a relatively high protein and carbohydrate content, of respectively 36.67% and 46.78%, and 45.57% of total dietary fiber. Haro et al. [65] studied the effect of culture conditions on the quantity and quality of lipid production of a Chilean strain of *P. tricornutum*. They have concluded that growth and PUFA proportion (20-25% of total fatty acids) were stimulated at the highest nitrate and phosphate concentrations, but not total lipid accumulation (of about 15% lipids per biomass dry weight).

McClure et al. [150] examined the impact of culture conditions on fucoxanthin production of *P. tricornutum*, concluding that at low light intensities the specific fucoxanthin concentration was greater than at a higher intensity. The nitrate enriched medium led to a significant increase in the specific fucoxanthin concentration of 59.2 ± 22.8 mg g^{-1}, volumetric concentration of 20.5 mg L^{-1} and bioreactor productivity of 2.3 mg L^{-1} day^{-1}. Mirón et al. [92] performed an elemental composition of the *P. tricornutum* biomass cultivated outdoors in bubble column and airlift photobioreactors, obtaining average values of: 49.2% C, 6.3 % H, 0.8% N, and 1.3% S. These authors also observed that low irradiance favored accumulation of the light capture pigments, while increasing daily irradiance led to accumulation of carbohydrates. Eicosapentaenoic acid (EPA, 20:5n3) constituted between 27 and 30% of the total fatty acids present or 2.6-3.1% of the dry biomass. Morais et al. [151] studied the effect of salinity, carbon source (glycerol and glucose) and photoperiod in *P. tricornutum* cultures, concluding that the addition of a carbon source to the culture medium increases lipid production, 15% salinity resulted in the highest microalgae growth, and glycerol (of 0.1 M concentration) was the carbon source best assimilated by microalgae, providing cultures with up to 1.3 g L^{-1} of biomass. These authors also concluded that mixotrophic cultivation, at 24 h of light photoperiod, in the presence of glycerol, resulted in 338.97 mg L^{-1} of lipids that was about 80% higher than the autotrophic.

Pérez et al. [71] developed a kinetic model to estimate the specific growth rate of *P. tricornutum* in lab scale batch cultures, achieving optimum pH of 7.8 and specific growth rate of 0.064 h^{-1}, and optimum temperature at 20.4 °C in aerated cultures and at 22.3 °C in non-aerated cultures. Remmers et al. [88] quantified the impact of different incident light intensities on the TAG and EPA yield in nitrogen starved batch cultures of *P. tricornutum*, and found that their maximum content is independent of the applied light intensity. Rodolfi et al. [112] analyzed how changes in nitrogen availability affect productivity, oil yield, and fatty acid (FA) composition of *P. tricornutum*. The highest

areal productivity of biomass (about 18 g m^{-2} d^{-1}) and EPA (about 0.35 g m^{-2} d^{-1}), was achieved in nitrogen replete cultures, while the highest FA productivity (about 2.6 g m^{-2} d^{-1}) was achieved in nitrogen-starved cultures.

Santos-Ballardo et al. [103] assessed the importance of outdoor culture conditions for enhancing the efficiency and the energy balance of *P. tricornutum* for biodiesel production, concluding that outdoors cultures performed better than lab cultures in terms of the lipid content and fatty acid profiles. Song et al. [61] demonstrated that gas flow rate plays an important role in *P. tricornutum* growth and lipid production, observing a linear relationship between specific growth rate, lipid content, FAME content and gas liquid ratio. Vandamme et al. [152] studied the impact of harvesting using either alum or alkaline flocculation, or centrifugation of *P. tricornutum* biomass, concluding that alkaline flocculation can be used as the primary harvesting method without impacting the lipid extraction efficiency. Wishkerman and Arad [67] examined the formation of silver nanoparticles by *P. tricornutum* cultivated at 25 °C for a period of 8 days, suggesting as potential applications for silver nanoparticles biosensors, cosmetics, medicines and inks due to their unique optical, conductive and antibacterial properties.

Xu et al. [153] investigated the effects of salicylic acid on the fatty acid accumulation of *P. tricornutum*, showing that 40 μM salicylic acid increased the total fatty acid content accumulation 1.3-fold of the control after 4 days of exposure. Yodsuwan et al. [113] monitored the lipid accumulation in *P. tricornutum* showing that lower nitrogen concentration favoured a higher lipid content, and that under nitrogen-deficient conditions, a large amounts of saturated fatty acids were produced, mainly palmitic acid (C16:0), while EPA was produced in large amounts when there was sufficient nitrogen. Yongmanitchai and Ward [115] studied the growth and omega-3 fatty acid production of *P. tricornutum*, determining the optimum culture temperature (21.5 °C to 23 °C) and initial pH (7.6) for EPA production. The authors obtained EPA yields of up to 133 mg L^{-1} of culture, in which EPA constituted up to 30 to 40% of total fatty acids, increasing with nitrate and urea concentrations in the culture medium.

Therefore, this study aims to analyze the biochemical composition of *P. tricornutum* biomass, obtained in an outdoor pilot-scale bubble column photobioreactor, under natural conditions in Chile, for producing biofuel and high-value compounds in a biorefinery.

5.2 Materials and Methods

5.2.1 Microalga Cultivation – Inoculum Preparation

The diatom cultures were performed using an autochthonous strain of *P. tricornutum* Bohlin, originated from Cañar Blanco, La Serena, IV Region, Chile. The initial lab cultures were stored in 250 mL Erlenmeyer flasks, transferred into 1 L Erlenmeyer

flasks for 7 days of sub-cultivation and finally, scaled-up into 20 L plastic carboys for approximately 14 days. The cultures were exposed to a 200 μmol photons m^{-2} s^{-1} artificial light in 16:8 (light:dark) cycles, at 23 ± 1 °C and aerated with atmospheric air, supplied from an air blower (LA-120A, Nitto Kohki Co Ltd., Tokyo, Japan). The cultures were grown in Walne medium [80] prepared with natural seawater filtered and autoclaved for 45 min at 121 °C and 20 psi and supplemented with vitamins and silicate [127].

5.2.2 Outdoor Growth and Kinetics Parameter Monitoring

Cultures were grown outdoor according on a batch mode in 4 bubble column photobioreactors (PBRs), with working volume of 200 L each, totalizing 800 L in all, as previously described by Branco-Vieira et al. [127]. It was kept the same conditions of sub-cultivation in 20 L plastic carboys containing *P. tricornutum* culture, which were utilized as an inoculum for each 200 L PBR. For the outdoor culture a commercial modified Guillard's f/2 formulation [81] was used with silicate (Pentair, Aquatic Eco-Systems, Minneapolis, USA) and the cultures were submitted to natural conditions during the Chilean summer, in the Concepción city (36°50'02.1"S;73°01'49.3"W).

The cultures were monitored every day until stationary growth phase. Optical density at 750 nm (OD_{750}) was used for the dry biomass concentration indirect calculation, by linear regression B = 0.89 x OD_{750}+0.38 (R^2 = 0.97, biomass (g L^{-1}) = B)), previously determined. Growth dynamics of the microalgae was quantified by the growth rate (Equation 2) and the progressive sum of daily growth rate (Equation 3).

5.2.3 Microalgae Elemental Analysis

Elemental analysis of carbon, hydrogen, nitrogen and sulfur (CHNS) content of microalgae biomass was performed using an Organic Elemental Analyzer (TruSpec Micro 630-200-200, Leco Corporation, Saint Joseph, USA). The furnace temperature was set to 1,075 °C with the oven temperature maintained at 850 °C.

5.2.4 Carbohydrate Analysis

The analyses of carbohydrates were performed using a modified phenol-sulfuric method [154]. In previous experiments we constructed a calibration curve using D+ glucose as a standard. Approximately, 5 mg of dried biomass was used to perform the analysis using 1 mL of H_2SO_4 1.0 M. The mixed biomass was sonicated for 5 minutes and then 4 mL of H_2SO_4 1.0 M was added to the biomass. The samples were heated at 100 °C for 1 hour and centrifuged at 4,000 rpm at 10 °C for 15 minutes. One milliliter of phenol 5% was added to the resulting acid supernatant and after 40 minutes, 5 mL of H_2SO_4 18.4 M was added to the mixture. The absorbance in the range of 485 nm was measured in a spectrophotometer

Dynamica HALO SB-10 (Dynamica Scientific Ltd, Newport Pagnell, UK) and used to estimate biomass carbohydrate concentration to mg L^{-1} using a glucose standard curve by the following relationship: C = 152.19 x OD_{485} (R^2 = 0.98, carbohydrates concentration (mg L^{-1}) = C)), previously determined. Finally, the carbohydrate content of the biomass was calculated using Equation 7:

$$\text{Carbohydrate } \left(\%, \frac{w}{w}\right) = \frac{\left[\left(\frac{C}{Vm}\right) \times Ve\right]}{m} \times 100 \tag{7}$$

Where C is the carbohydrate concentration (mg mL^{-1}) obtained from the calibration curve; Vm is the volume (L) of the samples used; Ve is the volume (mL) of the acid extract used in the treatment; m is the amount of dried biomass (mg).

5.2.5 Protein Analysis

Protein extraction from *P. tricornutum* was carried out by adapting the procedure described by Lowry et al. [155]. Briefly, 5 mg of dried microalgal biomass was hydrolyzed in 5 mL of 0.1 M NaOH for 20 min at 100 °C. The biomass was centrifuged at 3,000 rpm for 15 min at 10 °C. The supernatant was saved in another tube to use a volume of 250, 500 and 750 mL of this supernatant to complete to 1 mL using 0.1 M NaOH. Lowry's reagent C (5 mL) was added to the tube. The tube was vortexed and after 15 min, 0.5 mL of Folin reagent was added and the mixture were vortexed. After 40 min reaction in the dark reaction the samples' absorbance was measured at a wavelength of 750 nm in a spectrophotometer (BioTek, Synergy™ HT Multi-Detection Microplate Reader, BioTek Instruments, Vermont, USA). A spectrophotometer blank was prepared similarly, without the microalga biomass. The protein concentration was obtained using a BSA calibration curve, previously established, by the following relationship: P = 611.69 x OD_{750} (R^2 = 0.98, protein concentration (mg L^{-1}) = P). Finally, the protein content of the biomass was calculated using Equation 8:

$$\text{Protein } \left(\%, \frac{w}{w}\right) = \frac{\left[\left(\frac{P}{Vm}\right) \times Ve\right]}{m} \times 100 \tag{8}$$

Where P is the protein concentration (mg mL^{-1}) obtained from the calibration curve; Vm is the volume (L) of the samples used to complete 1 mL of 0.1 M NaOH after hydrolysis; Ve is the volume (mL) of the alkaline extract used in the treatment; m is the amount of dried biomass (mg).

5.2.6 Fucoxanthin Analysis

Fucoxanthin analysis was carried out using a slightly modified spectrophotometric method described by Wang et al. [156]. The extraction process was performed using 100% ethanol, and the pigments were extracted from 10 mg of freeze-dried biomass. The extraction process was performed with 10 mL of solvent for 2 hours in the dark, using a magnetic stirrer for constant agitation. After extraction, the mixture was centrifuged at 3,000 rpm for 15 minutes and supernatant was collected for pigment analysis.

The extracts were measured in a spectrophotometer in order to estimate the fucoxanthin content, according to Wang et al. [156] by Equation 9.

$$\text{Fucoxanthin (mg g}^{-1}) = \frac{(6.39\times A_{445} - 5.18 \times A_{663}) \times V}{(1000 \times W)} \tag{9}$$

Where Fucoxanthin mg g^{-1} is fucoxanthin content in mg g^{-1}; A_{470} is absorbance at 470 nm; A_{628} is absorbance at 628 nm; A_{580} is absorbance at 580 nm; A_{661} is absorbance at 661 nm; A_{480} is absorbance at 480 nm; A_{631} is absorbance at 631 nm; A_{582} is absorbance at 582 nm; A_{665} is absorbance at 665 nm; A_{445} is absorbance at 445 nm; A_{663} is absorbance at 663 nm; V is the total volume of the pigment extract; W is the weight of the sample used for extraction.

5.2.7 Organic Matter and Ash Contents

Approximately 100 mg of dried biomass was used to estimate organic matter and ash content. Samples were dried for 48 hours at 105 °C and combusted in a muffle furnace at 575 °C until reaching a constant weight, in order to obtain the residual inorganic ash, according to Wychen and Laurens [157]. The difference between dry biomass and remaining ash weight was used to calculate the organic matter content of the samples.

5.2.8 Biosilica Estimation

The purification process of diatom frustules was carried out according to a modified method described by Jiang et al. [128]. Samples of 250 mg dried biomass were used for acid cleaning in order to remove the organic matter and metal. The samples were mixed with a mixture of deionized water and 37% HCl at 2:1 (v:v) ratio, respectively, and subsequently submitted to continuous stirring for 1 hour, at 100 °C, in a water bath placed in a fume hood. The acid removal was performed by washing samples with deionized water. For the washing process, the samples were centrifuged at 4,000 rpm for 3 min (4000R Benchtop Refrigerated Laboratory Centrifuge, Centurion Scientific Ltd, West Sussex, UK) several times until the pH of the supernatant reached approximately 7.0. The organic matter was removed by placing diatom frustules at 105 °C in a drying oven

until water completely evaporated. The samples were subsequently heated to 600 °C in a furnace (Compact Muffle Furnace LE 6/11/B150 LE060K1BN, Nabertherm, Lilienthal, Germany) at a heating rate of 3 °C min^{-1} for 6 hours. Frustules treated under the same conditions but without acid cleaning were used as control of the experiment.

5.2.9 Lipid Analysis and GC Chromatrography

Total lipids of *P. tricornutum* were quantified according to the Bligh and Dyer [84] method described by Branco-Vieira et al. [127]. Briefly, an amount of 300 mg of dried biomass was used to obtain total lipids by a two-step extraction method using chloroform, methanol and distilled water, as solvents. In the first step, a ratio of 1:2:0.8 (v/v/v) of chloroform, methanol and distilled water were added to the samples. The samples were sonicated for 30 min, and a second round of extraction was performed by adding chloroform, methanol and distilled water at a ratio of 2:2:1.8 (v/v/v), respectively, and then subsequently sonicated for another 30 min. After centrifugation at 3,000 rpm for 15 min, the lower layer was carefully recovered and the extracted lipids were gravimetrically weighed, after chloroform evaporation at room temperature, in order to estimate the total microalgae lipids content.

Total lipids were transesterified according to the method described in Branco-Vieira et al. [127], and the products were analyzed by a gas chromatograph-mass spectrometer (GC 6000 Vega Series 2, model 6300-03b, Carlo Erba Instruments Ltd, Wigan, UK), equipped with a HP-FFAP cross-linked FFAP (25 m length, 0.32 mm diameter, 0.52 μm film thickness) column. The detailed procedure of GC chromatrography has also been described by Branco-Vieira et al. [127].

5.2.10 Scaling-up Scenarios

In order to improve the valorization of the compounds and to estimate the microalga biorefinery in a realistic way, the cultivation of *P. tricornutum* was proposed under a scaling-up scenario based on the baseline scenario results.

1. **Baseline (BL):** the cultivation was performed in one modular 0.8 m^3 PBR, occupying an area of 1.27 m^2. The baseline scenario was based on the experimental results obtained by the authors.

2. **Scaled-up (SL):** the cultivation was scaled-up to a 1,270 m^2 pilot-plant, containing 1,000 modules of 0.8 m^3 PBR, totalizing 800 m^3 of culture.

The *P. tricornutum* plant scenarios were based on the methodology described by Spruijt et al. [158] to calculate the potential microalgae biomass production. The cultivation system assumes a 24 hours of PBR operation, for 12 months a year. For

quantification of *P. tricornutum* biomass, the biomass glucose content and its conversion into potential dry matter biomass per month were considered. To achieve the potential glucose production per month the Equation 10 was used.

$$GP\ (kg) = GRAD \times PBR_{surf}\ \times E_{glucose} \times PE\ \times\ T_f\ \times\ CO_2 \tag{10}$$

Where *GP (kg)* is the potential amount of glucose production, measured in kg per month; *GRAD* is the monthly global radiation in MJ m^{-2}; PBR_{surf} is the surface area occupied by the PBR in m^{-2}; $E_{glucose}$ is the energy content of glucose in MJ kg^{-1} (assumed to be 15.63 MJ kg^{-1} [159]); *PE* is the photosynthesis efficiency (calculated according to Fernández et al. [93]); T_f is the calculation for growth inhibiting factors like sub-optimal temperatures (obtained by Equation 11); and CO_2 is the availability of CO_2 for microalgae cultivation.

The temperature factor was based on the growth model described by James and Boriah [160], and it is calculated as an exponential limitation caused by suboptimal temperature. The optimal temperature for *P. tricornutum* cultivation in Chile was achieved by experimental results and was set to 20 °C. The temperature factor is calculated by Equation 11.

$$T_f = e^{-K(T-Topt)^2} \tag{11}$$

Where T is the actual temperature; $Topt$ is the species specific optimal temperature and K is an empirical constant. In this study the value of 0.004 was adopted for the empirical constant.

The average of carbon fixation was calculated by Equation 12.

$$Carbon\ capture =\ Carbon\ \times P \times \frac{CO_{2MW}}{Carbon_{MW}} \tag{12}$$

Where *Carbon capture* is the carbon content of microalga biomass (in % w/w); *Carbon* is the mass fraction of carbon in grams of carbon per gram of biomass (g g^{-1}); P is the biomass productivity; and CO_{2MW} is the molecular weight of CO_2 (in g mol^{-1}) of CO_2; $Carbon_{MW}$ is the molecular weight of carbon (in g mol^{-1}).

Finally, the potential of microalga biomass production was calculated using the estimated glucose content converted into dry matter, measured kg per month. In order to estimate the potential biomass production, the reciprocal production values described by Vertregt and De Vries [159] where used through Equation 13.

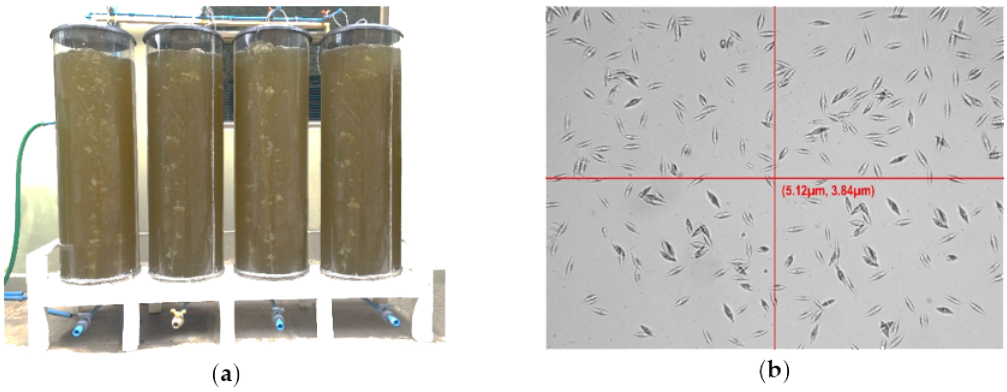
$$B_{pot} = \frac{GP}{(1.211 \times\ \%_{carb}) + \left(3.030 \times\ \%_{Lip}\right) + (1.793 \times\ \%_{Prot}) + (0.906 \times\ \%_{Ash})} \tag{13}$$

Where B_{pot} is potential biomass production in kg; GP is obtained by Equation 10; $\%_{carb}$, $\%_{Lip}$, $\%_{Prot}$, $\%_{Ash}$ are the percentage of carbohydrate, lipids, proteins and ashes of the *P. tricornutum* biomass.

5.3 Results and Discussion

5.3.1 Biomass Production

The *P. tricornutum* biomass was obtained from outdoor batch cultures under natural conditions in Chile over a period of 14 days (Figure 16a). The culture and cells were daily monitored (Figure 16b) and the stationary phase began after 8 days of cultivation, when the accumulated growth rate was 0.97 day^{-1}. The biomass concentration was about 0.96 ± 0.04 kg m^{-3} and the maximum volumetric productivity was 0.13 kg m^{-3} d^{-1} according to data published by Branco-Vieira et al. [127].



(a) (b)

Figure 16: Cultivation of *P. tricornutum* biomass under natural conditions. (a) *P. tricornutum* batch culture regime in an outdoor bubble column PBR; (b) Microscopic photos of *P. tricornutum* cultivated outdoor in a bubble column PBR (obtained from Motic BA210 Binocular Microscopy 40x and Motic Images Plus 2.0 ML software).

The microalga biomass was obtained under BL scenario, utilizing a Chilean autochthonous strain of *P. tricornutum* in one module of PBR. The cultivation system was carried out with minimum interventions during the experiments, applying commercial and replete culture medium nutrients and atmospheric CO_2. This approach was used to both reduce the costs of production and elucidate the biochemical composition of this strain in their natural environment, in order to address the best alternatives for customizing the biomass.

5.3.2 Biochemical Characterization

Biochemical characterization was carried out through the measurement of the total lipids and fatty acid profile, carbohydrate, protein, fucoxanthin and bio-silica contents.

Lipids constitute an important macromolecule for energy storage and structurally functions in a variety of organisms. Lipid composition of the diatom *P. tricornutum* has been interesting as a raw material for producing biodiesel and/or high-value compounds. The lipid composition achieved for *P. tricornutum* was performed by the Bligh and Dyer method at room temperature and pressure [127], and the values were 9.08 ± 0.38 wt% and 14.25 ± 1.01 ash-free dry weight (AFDW%) (Table 4). Lipid contents can vary as function of different factors, such as light intensity, culture phase, culture system, nutrient availability and stress conditions. The *P. tricornutum* biomass had reached higher lipid concentration (43%) when cultivated under controlled parameters in an indoor laboratory environment and in small volumes [152]. Furthermore, the *P. tricornutum* biomass produced a high lipid content (53.04 ± 3.26 wt%) under photoautotrophically nitrogen-deficient cultivation, whereas a similar amount of lipid content observed in this study was obtained when cultivated in nitrogen-sufficient conditions (9.61 ± 3.89 wt%) [113].

The *P. tricornutum* fatty acids composition was estimated based on the GC area signals (% area per sample) for the corresponding Fatty Acid Methyl Esters (FAME) [127] . The *P. tricornutum* samples showed a proportion of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of 42.34%, 21.91% and 31.41%, respectively. The total amount of FAME in microalgae dry biomass was 3.84 wt% for SFA, 1.99 wt% for MUFA and 2.85 wt% for PUFA. Concerning biodiesel production from this microalga, the analyses of FAME play an important role in determining the fuel properties and oxidative stability, predominantly, the incidence of C16-C18 fatty acids, which represent in this study an amount of 24.39%. Moreover, to be considered a good raw material for biodiesel production, the percentage of highly polyunsaturated fatty acids (≥ 4 double bonds) must be less than 1%, according to European standards for biodiesel (EN 14214).

The carbohydrates were measured by a modified phenol-sulfuric method. The carbohydrates are a result of the microalgae photosynthesis activity and it is the first compound to be assembled. Biomass of *P. tricornutum* showed 7.85 ± 0.22 wt% and 17.31 ± 0.48 AFDW% of total carbohydrates (Table 4). Similar results have been reported in other studies [74] when *P. tricornutum* was cultivated under higher outdoor irradiance, which clearly showed that in the absence of light limitation the carbohydrate contents were independent of the biomass concentration; besides, in the present work the carbohydrate concentration was less than 10 wt%. However, *P. tricornutum* has been reported to show more than 40 wt% of carbohydrates, when it was grown under low indoor irradiance (72 μmol photons m^{-2} s^{-1}) [107]. Cultivation systems conducted under nutrient limitations have been reported to contain different amounts of carbohydrate concentrations in *P. tricornutum*. Cultures using nitrogen limited cells have shown almost 60 wt% of carbohydrates, while those with phosphorus limited cells have shown an amount of 29-39 wt% of carbohydrates [68].

The total proteins were quantified by a modified Lowry method. The total protein content was about 38.40 ± 2.46 wt% and 64.00 ± 4.10 AFDW%. Fernández-Reiriz et al. [161] measured the protein contents in different culture growth phases and observed that protein levels increased in the later phases of diatom cultures, reaching about 2.58 wt%, 13.38 wt.% and 17.51 wt% of proteins in the exponential phase, early stationary phase and late stationary phase of *P. tricornutum* culture, respectively. Chauton et al. [68] found a percentage of 16 to 26 wt% of protein content in cells of *P. tricornutum* cultivated under nitrogen starvation, while those cells cultivated with limited phosphorus showed about 19 to 23 wt% of proteins. Miron et al. [92] obtained an average of 50 wt% of protein in *P. tricornutum* cultivated in a bubble column photobioreactor. Chrismadha & Borowitzka [162] reported 30-70% of protein content in *P. tricornutum* biomass grown semi-continuously in a helical tubular photobioreactor. Protein content corresponds to the most abundant cell biomolecule, constituting approximately 30-60% of the total cell biomass under nutrient-replete conditions [94]. The high content of proteins and low amounts of lipids can be explained by the rapid growth of the cells which generally leads to a lower amount of energy stored in the form of lipids [107].

Fucoxanthin is characterized as a carotenoid type pigment belonging to the xanthophyll class, widely found in brown algae and diatoms. This carotenoid has been demonstrated to be a safe and effective dietary supplement for human nutrition [156]. Recent studies have shown the effectiveness of this pigment's activity as anti-inflammatory, anti-tumor, anti-obesity, anti-diabetes, antimalarial, and against other disorders [163–165]. Industrial production of this pigment has focused on macroalgae and little has been found in the literature about the usage and extraction of fucoxanthin for commercial applications [166], while *P. tricornutum* has been reported to contain fucoxanthin as its main carotenoid [70, 166, 167].

Table 4: Biochemical characterization of *P. tricornutum*.

Biochemical composition	**Total Quantity (wt%)**	**Total Quantity AFDW (wt%)**
Lipids	9.08 ± 0.38	14.25 ± 1.01
Carbohydrates	7.85 ± 0.22	17.31 ± 0.48
Protein	38.40 ± 2.46	64.00 ± 4.10
Fucoxanthin	0.86 ± 0.06	8.29 ± 0.55
Bio-silica	5.19 ± 0.56	10.06 ± 0.71
Ashes and others	38.67 ± 1.50	–

In this study, the fucoxanthin analysis revealed an amount of 0.86 ± 0.06 wt% and 8.29 ± 0.55 AFDW% (Table 4), corresponding to 8.55 ± 0.56 mg g^{-1}. Other studies using the same solvent for fucoxanthin extraction have achieved a percentage of 0.46 wt% of fucoxanthin [168].

Studies conducted by Kim et al. [166] have shown a range from 15.42 to 16.51 mg g^{-1} of fucoxanthin extracted, using ethanol as the extraction solvent, followed by its chromatography purification. However, Wang et al. [156] obtained an amount of 9.24 mg m^{-1} of fucoxanthin extracted with ethanol from fresh cultures in a late stage of cultivation. In the present study, was achieved an equivalent concentration of 8.21 ± 0.54 mg L^{-1} of fucoxanthin.

Finally, diatoms are capable of producing a nanostructured rigid cell wall composed by amorphous hydrated silica, know as frustules. The evolutionary property of this silica wall is to promote nutrient uptake and provide protection against adverse environmental conditions, by secreting cellular metabolites on the frustules surface [119]. In this study the biosilica analysis revealed an amount of 5.19 ± 0.56 wt% and 10.06 ± 0.71 AFDW% after cleaning by acid treatment and baking (Table 4). In the literature, there is a lack of information about the biosilica percentage on the total dried biomass of *P. tricornutum* samples. Studies conducted by Tesson et al. [126] have shown a percentage of biosilica of about 1.96 wt%, corresponding to the surface chemical composition of silica and silicate from *P. tricornutum* samples.

5.3.3 Biorefinery Proposal for *P. tricornutum*

Phaeodactylum tricornutum biomass has an interesting biochemical composition for use in an integrated biorefinery approach, mainly in order to optimize the environmental and economic feasibility of the process. The biorefinery concept for microalgae also implies valorization of residual biomass, converting it into biofuels and/or high value compounds. Furthermore, biorefining the biomass into biofuels and high value-added products comprises more than knowledge about the biochemical composition of the microalgae, but above all the applicability of existing technologies and the emergence of new ones to make these transformations feasible.

Insights about new industrial plants and the development of biomass transformation processes as a function of the local microalgae biodiversity composition leads to regional self-sufficiency and the assessment of technologies for sustainable processing at local level.

In order to conduct the analysis on a realistic way, the biomass production was inferred from BL scenario to SL scenario, thought the growth model described in the methods section. The BL scenario was based on the experiments conducted by the authors, in a natural Chilean environment, using an autochthonous strain of *P. tricornutum*, as described in the methods section and in Branco-Vieira et al. [127].

The cultivation system assumed 1,000 modules of PBR with 24 hours of operation, over 12 months a year. The parameters used to calculate the biomass production were based on the equations described in the methods and shown in Table 5 and 6.

Table 5: Parameters used to calculate biomass production for SL scenario.

Parameters	**Abbreviation**	**Value**	**Unit**	**Source**
Average temperature	T	Table 6	°C	[169]
Biomass concentration	B	0.96	kg m^{-3}	Calculated
Carbon	C	0.54	g g^{-1}	Measured
Carbon capture	–	52	%	Calculated
CO_2 availability	CO_2	0.04	%	Atmospheric
Glucose energy	$E_{glucose}$	15.63	kJ g^{-1}	[159]
Hydrogen	H	0.22	g g^{-1}	Measured
Monthly global radiation	$GRAD$	Table 6	MJ kg^{-1}	[170]
Nitrogen	N	0.22	g g^{-1}	Measured
Potential biomass production	B_{pot}	Figure 17	kg	Calculated
Potential glucose production	GP	Table 6	ton	Calculated
Optimal temp. (biomass)	T_{opt}	20	°C	Measured
PBR surface	PBR_{surf}	1270	m^2	Calculated
Photosynthesis efficiency	PE	5.00	%	Calculated
Temperature factor	T_f	Table 6	–	Calculated
Total Pigments	X_p	2.13	%	Calculated
Volumetric biomass product.	P	0.13	kg $m^{-3}d^{-1}$	Calculated

Table 6: Monthly parameters used to calculate biomass production for SL scenario.

Par.	**JAN**	**FEV**	**MAR**	**APR**	**MAY**	**JUN**	**JUL**	**AUG**	**SEP**	**OCT**	**NOV**	**DEC**
T	18.4	17.1	15.2	12.3	12.9	8.6	9.3	10.4	12.1	12.6	15.1	16.3
GRAD	735	775	663	501	320	449	308	416	598	604	740	621
T_f	0.98	1.00	0.96	0.87	0.87	0.67	0.72	0.77	0.87	0.87	0.96	0.98
GP	2.94	3.78	3.66	3.28	3.28	2.54	2.74	2.93	3.28	3.28	3.66	3.73

The SL scenario had a capacity to produce 23 ton of dried biomass (B_{pot}) a year in 1,000 m^2, with the lowest amount of biomass produced in June, July and August because of the low daylight intensity during winter in Chile (Figure 17). Considering this production, the water and energy used for biomass culturing was about 24,782 m^3 water $year^{-1}$ and 33,291 kWh $year^{-1}$, respectively. Since in the model the wastewater from the culture is recycled back to the PBR to minimize the water use. Thus, it was considered a percentage of 90% of water returning to the PBR, leading to an amount of wastewater produced of 2,465 m^3 $_{water}$ $year^{-1}$; this percentage includes the wastewater from the PBR culture medium, resulting from the biomass centrifugation process that does not return to the PBR; the water remaining in the alga paste after biomass harvesting and water loss by crashes or PBR cleaning. Concerning the CO_2 uptake from the atmospheric air to the biomass production, an amount of 46,361 $kgCO_2$ $year^{-1}$ was used by the microalgae.

Considering that average temperature in Concepción, Chile, is 13 °C, while the optimum temperature for the growth of alga strain is around 18 °C, it was proposed to implement a heating system to mitigate the loses of biomass production during the

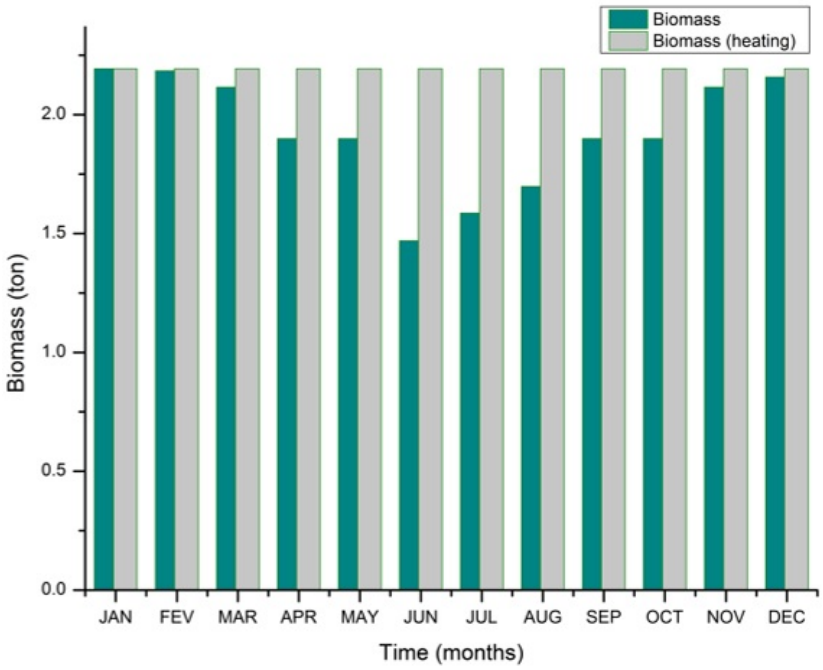


Figure 17: *P. tricornutum* biomass production per month for a scaling-up (SL) scenario in Chile. Green bars are the biomass produced under natural conditions; gray bars are the biomass production when heating system was applied.

winter, and the fraction of biomass produced under this condition was evaluated. It was observed that by heating the system it is reached an amount of 25 ton of dried biomass a year in 1,000 m^2, corresponding to 12.16% of the biomass produced without a heating system (Figure 17). Furthermore, the water and energy uses increased to 28,288 m^3 water $year^{-1}$ and 391,191 kWh $year^{-1}$, respectively, which corresponds to 14.04% more water consumption and 91.49% more energy consumption. Consequently, because the heating system is highly energy intensive, the difference percentage between biomass production without heating does not justify its use.

Considering the biomass production in this model, it is possible to achieve an annual productivity of biomolecules characterized in *P. tricornutum* as shown in Table 7, considering productivity per hectare.

Table 7: Annual productivity of products from *P. tricornutum* for SL scenario.

Products	**Volumetric Productivity (ton ha^{-1})**	**Areal productivity (ton ha^{-1})**
Algae Dry Biomass	182.0	157.2
Carbohydrates	14.3	12.3
Proteins	69.9	60.4
Lipids	16.5	14.3
Fucoxanthin	1.8	1.6
Biosilica	9.4	8.1

After biomass characterization and analysis of potential biomass production under SL scenario, three different biorefinery processes were considered for *P. tricornutum* biomass. In the first one, was addressed to biofuel production, such as biodiesel, bioethanol and biomethane. In the second one, the biomass uses were considered for the production of high value compounds targeted at nutraceuticals and pharmaceuticals, such as protein for feed, fucoxanthin and bio-silica. Furthermore, the third integrated biorefinery proposed was based on the production of fucoxanthin as a main product, addressing the co-products of biofuels and other high-value compounds.

Biofuel production from microalgae is both a promising and challenging issue that has been studied over the last decade by a huge number of authors. It is a promising alternative because microalgae offer diverse advantages over traditional crops for biofuels production, besides their high productivity, combined with the energy and food security provided by the cultivation of these organisms. However, the major impact on implementing this concept for biofuel production from microalgae is related to the sustainability and economic feasibility of the process.

In recent years, much work has focused on studying the production of microalgal biomass targeting the manufacture of just one type of biofuel, such as biodiesel for example. These studies have been demonstrating the economic unviability of this approach, mainly due to the high final cost of microalga biomass production [112, 171, 172].

Considering this question, it has been proposed to apply a biorefinery approach to address the production of biofuel combined with the valorization of co-products. Therefore, a biofuel biorefinery was analyzed in this work which had a downstream process targeting the production of biodiesel, bioethanol and biomethane.

In this first approach, the *P. tricornutum* biorefinery for biofuel production, it is considered the biodiesel as the main product, simultaneously produced from microalga lipid, the extraction and processing of the microalga paste into others biofuels. After biomass production and lipid extraction, the alga paste still contains carbohydrates that can be extracted and used for bioethanol production, while the residual biomass of these processes can be used to feed an anaerobic digester for biomethane production.

The flowsheet for this biorefinery proposal is shown in Figure 18, consisting of four major processes: (1) microalga cultivation in PBR; (2) harvesting of microalga biomass through centrifugation process and pre-treatment step including biomass drying; (3) biochemical characterization of the biomass, lipids and carbohydrate extraction; (4) conversion of lipids into biodiesel by transesterification process, production of bioethanol by fermentation of carbohydrates and conversion of the residual biomass into biomethane.

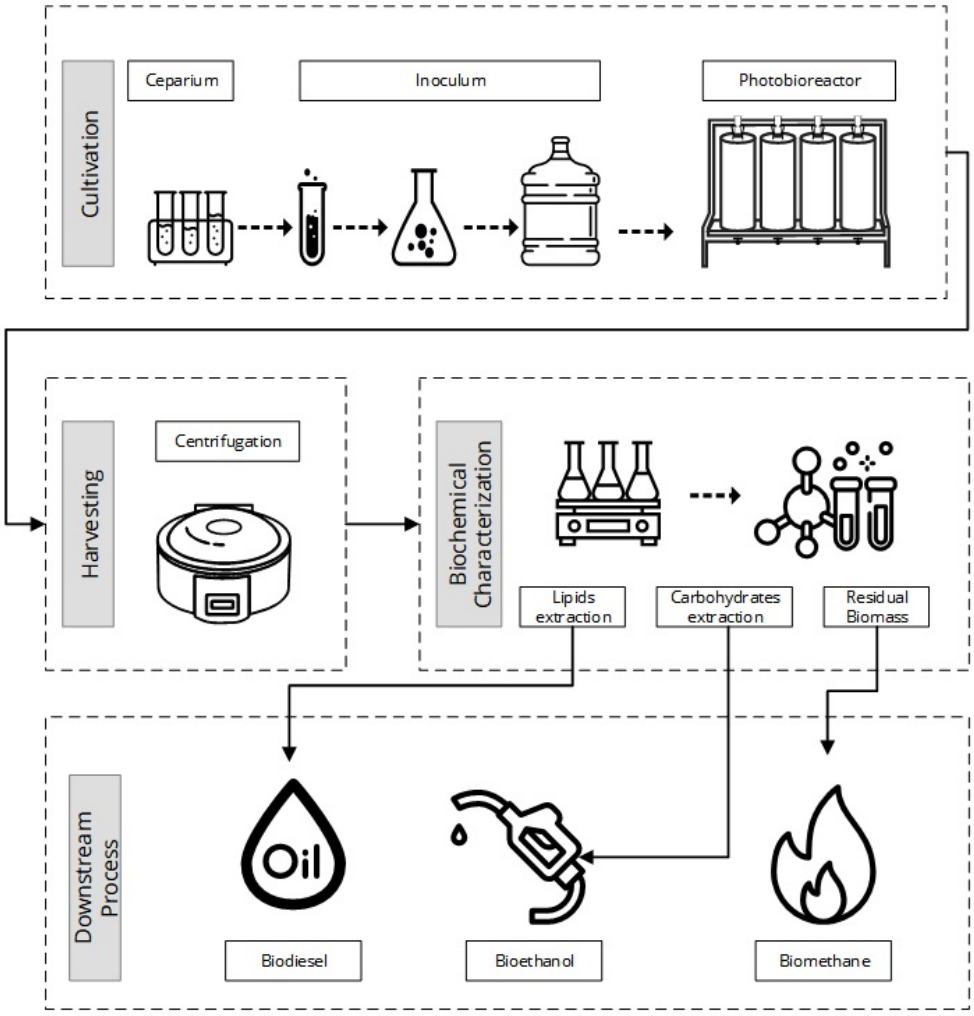


Figure 18: *P. tricornutum* biorefinery for biofuels production, targeting biodiesel, bioethanol and biomethane. Rectangles show the overview of each upstream and downstream process considered in this study.

At each process stage, a number of technological alternatives are available. Hence, in this work, the production of biomass is accomplished under the SL scenario, described above. For harvesting the biomass, the use of a centrifuge was considered with an output of alga paste containing about 15% of dry matter.

After the biochemical characterization, the cells were disrupted by using a dry milling method. The lipids were recovered using a combination of polar and non-polar solvents (hexane and Isopropyl Alcohol).

The transesterification process was carried out using methanol and a catalyst for subsequent conversion to FAME, the final product of biodiesel.

The remaining biomass, known as alga paste, is mainly containing protein and carbohydrate can be used for other uses. The sequence of this biorefinery approach is to extract carbohydrates from the alga paste and convert them into bioethanol. For this purpose, the biomass was rehydrated, followed by enzymatic hydrolysis of the released

polymeric carbohydrates into monomeric carbohydrates in order to be fermented by yeasts. After fermentation, the resulting ethanol can be concentrated by distillation.

Finally, the last step in this first biorefinery proposal is to employ the remaining biomass in biomethane production. This step is characterized by a fermentation process under anaerobic conditions, made by microorganisms capable of reducing the organic matter to methane (CH_4) and CO_2, i.e. to biogas.

Considering this scenario and using a methodology proposed by Spruijt et al. [158] for calculating the annual production of biofuels (biodiesel, bioethanol and biomethane), utilizing the process data obtained through applying the experimental case and model applied in this study, it was possible to obtain the productivity of biofuels shown in Table 8.

Table 8: Annual production of *P. tricornutum* biofuels for SL scenario.

Biofuel	**Annual Production (m^3 $year^{-1}$)**
Biodiesel	5.07
Bioethanol	1.68
Biomethane	1739

However, the technological alternatives available nowadays for microalga-based biofuel production have been demonstrated not to be profitable, because of the high cost of microalgae biomass production. Hence, new insights need to emerge to contribute to the feasibility of this approach.

The alga paste, resulting after the extraction of lipids and carbohydrates, accounted for the high percentage of other high-value compounds, such as protein, pigments and biosilica and, so there is the potential for this paste to serve for a number of further applications [173].

Considering this fact, a second biorefinery approach was proposed, which took into account the use of alga paste to produce high-value compounds, such as fucoxanthin, protein and biosilica from this diatom (Figure 19). In order to choose the best methodological alternative to achieve these biomolecules, it is necessary to consider the final use of the product. For nutraceutical and pharmaceutical uses of fucoxanthin and protein, it is important to maintain the integrity of these substances, avoiding spoilage. Otherwise, the uses of these biomolecules for this purpose are compromised due to the contamination and denaturation of the compounds.

The biosilica purification of the remaining biomass is usually performed with solvents and processes that cause damage to the native structures, but this fact is not important when the main proposed use of biosilica is the Si content. The remaining biomass contains the diatom carapace, or frustules, which are constituted of a highly silicified cell wall, comprising a pair of valves connected by silica bands girdled along the margins [118].

The flowsheet for this biorefinery approach is quite similar to the first biorefinery scenario proposed. The processes shown in Figure 19 consist of: (1) microalga cultivation in PBR; (2) harvesting of microalga biomass through centrifugation and pre-treatment including biomass drying; (3) biochemical characterization of the biomass; and fucoxanthin and/or protein extraction; (4) uses of fucoxanthin, protein and biosilica.

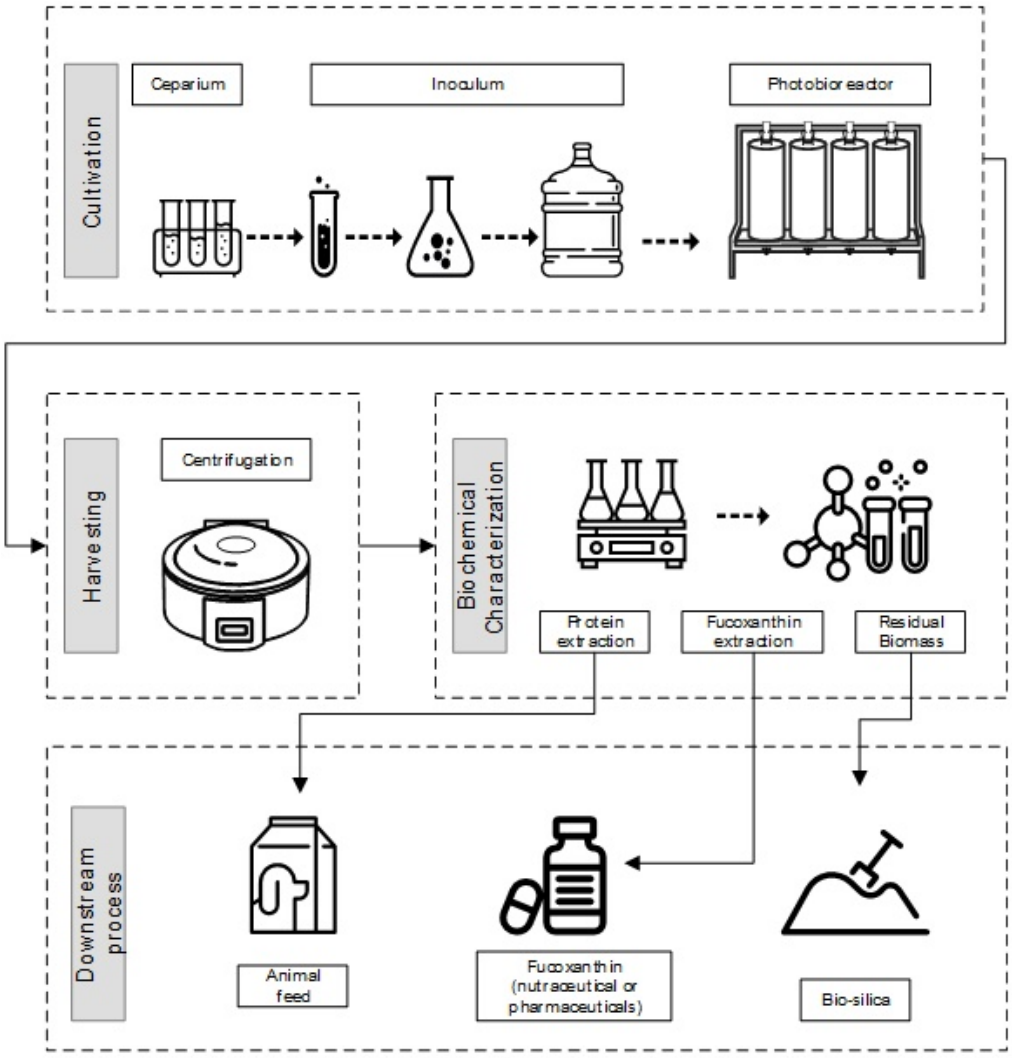


Figure 19: *P. tricornutum* biorefinery for high-value compounds production, targeting fucoxanthin, protein and biosilica. Rectangles show the overview of each upstream and downstream process considered in this study.

The industrial large-scale production of microalgae pigments has gained significant interest with respect to manufacturing a commercially viable raw material for health, nutrition, and cosmetic industries. The step of fucoxanthin extraction can be done by using conventional extraction methods, based on organic solvents, such as acetone, ethanol, and ethyl acetate [167]. Although these methods offer a simple approach to extract the microalga pigments, they may compromise the structure and availability of other high-value compounds present in the biomass, leading to low efficiency in the

extraction yield, high solvent consumption and low purity of the final product [174].

Recent alternatives and more sophisticated techniques such as pulsed electric fields, liquid pressurization, subcritical fluids, microwaves, ultrasounds, high-pressure homogenization and supercritical fluids [174] have been applied in recent years. Among supercritical fluid techniques, supercritical carbon dioxide (SC-CO_2) has been considered as a good alternative to organic solvents, since it is less toxic, environmentally friendly and preserves the bioactivity of fucoxanthin. Therefore, in this study the use of SC-CO_2 was proposed for fucoxanthin extraction. Fucoxanthin is mainly used in the nutraceutical and pharmaceutical industries, being submitted to a rigorous regulations for its quality and purity. Thus, by using SC-CO_2 extraction, a solvent free pure extract is obtained.

After fucoxanthin extraction, the remaining biomass can be used for protein extraction. This scenario can to be applied to the sale of remaining protein-rich biomass, immediately after fucoxanthin extraction, for feed proposes. If protein extraction is considered, its extraction can be accomplished through several methods such as organic solvents, chromatography methods, ion-exchange, size exclusion, affinity and hydrophobic interaction chromatography to achieve these bioactive peptides. However, the protein extraction remains one major issue in an industrial scale operation and more research and development are needed in this field to establish a feasible protein based manufacturing process [175].

The biosilica utilization can be considered after the protein extraction from the protein-rich biomass, or part of the protein-rich biomass can be destined for biosilica purification without a prior protein extraction step. In this case, a percentage of the protein-rich biomass needs to be defined for the feed uses and another part for the biosilica purification. Biosilica purification can be done by using acid cleaning, which depends on the purity level required of the biomass related to the Si content. In this study it was proposed to use the HCl treatment followed by baked cleaning for biosilica purification, because this treatment was revealed to be the most efficient method of biosilica cleaning with regard to the Si content in the final product, obtained by the authors.

Considering this scenario for calculating the annual production of high-value compounds (fucoxanthin, protein and biosilica), utilizing the process data obtained through the experimental case and model applied in this study, it can be possible to obtain the amount of the bioactive compounds shown in Table 9.

Table 9: Annual production of *P. tricornutum* high-value compounds for SL scenario.

Compound	**Annual Production (ton year^{-1})**
Fucoxanthin	0.23
Protein	1.18
Biosilica	8.88

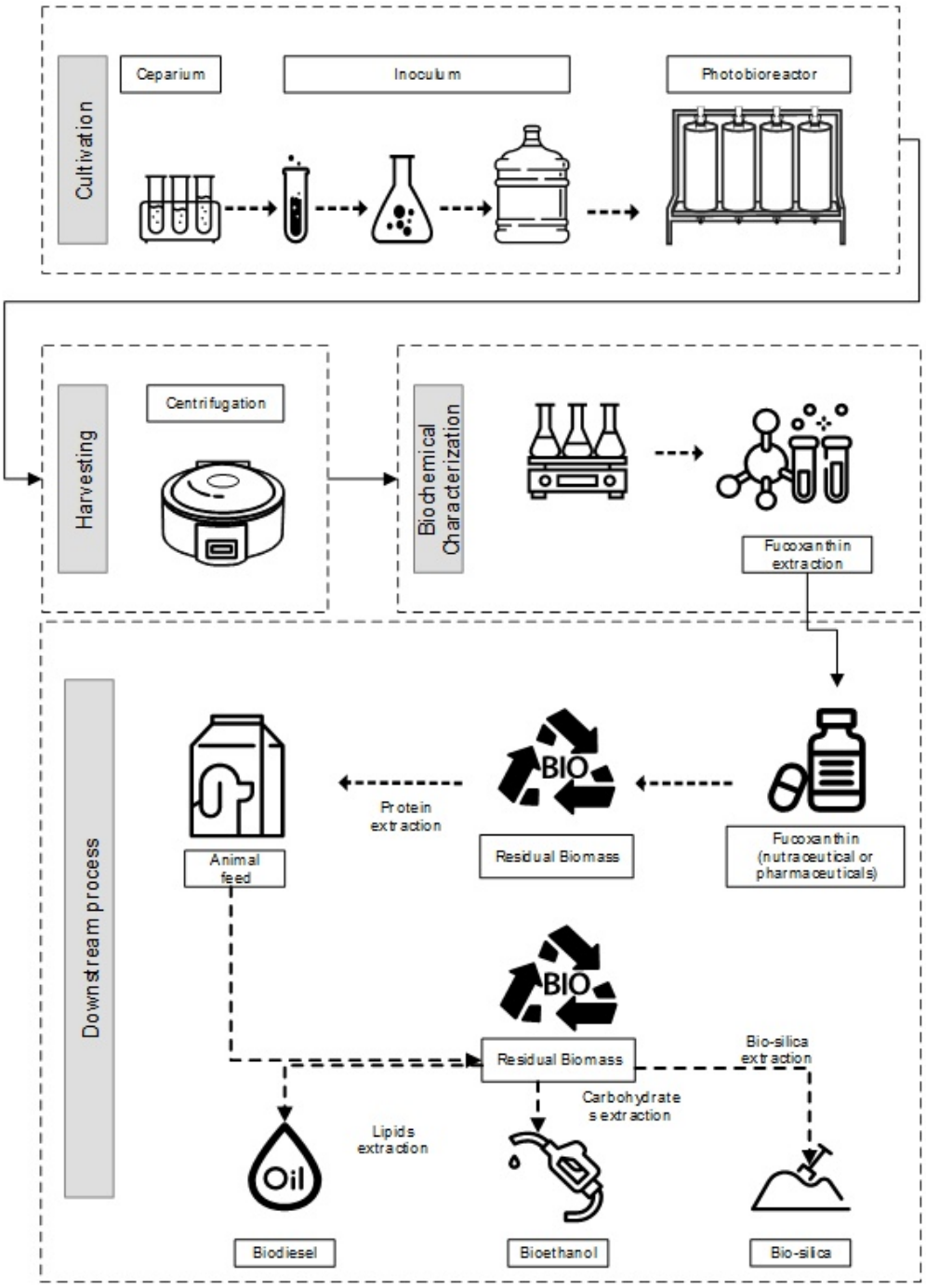


Figure 20: *P. tricornutum* biorefinery for high-value compounds production, targeting fucoxanthin as main product, protein extraction and valorization of residual biomass for the production of biofuels and biosilica. Rectangles show the overview of each upstream and downstream process considered in this study.

The third biorefinery proposal in this study considers the integration of biofuels and high-value compounds in one biorefinery, in order to increase the feasibility of the microalgae biofuels production and to maximize the use of the waste from the processes. It is important to consider for implementing the biorefinery concept, the sustainable use of local biomass resources, leading to self-sufficiency based on raw materials availability

at local level. Also, the most accessible technologies can be used for the sustainable extraction of the target compounds, bearing in mind the intrinsic characteristics of this particular biomass.

The third biorefinery flowsheet integrates the other two biorefinery approaches, differing mainly in the downstream process. The processes system shown in Figure 20 consists of: (1) microalgae cultivation in PBR; (2) harvesting of microalgal biomass through a centrifugation process and pre-treatment step including biomass drying; (3) biochemical characterization of the biomass and fucoxanthin; (4) destination uses of fucoxanthin, protein extraction from the fucoxanthin residual biomass, extraction of lipids and carbohydrates from the protein residual biomass, conversion of lipids into biodiesel by transesterification process, production of bioethanol by fermentation of carbohydrates and biosilica purification from the carbohydrates' residual biomass.

It is important to note that for this biorefinery concept the fucoxanthin and protein are bioactive compounds targeting nutraceutical and/or pharmaceutical industries, and for this reason, it is necessary to extract these molecules before other treatments, to avoid the contamination of biomass with toxic substances and any damage to their structure, as well. For fucoxanthin extraction the use of SC-CO_2 or other recent and non-toxic methods is recommended for application, taking into account the same procedure for protein extraction. Since the residual biomass achieved after protein extraction, the use of organic solvents or other possible contaminating methods to treat the biomass is no longer a major concern regarding the final products. The production of biodiesel and bioethanol, as well biosilica, can be done using the same methods proposed earlier.

A biorefinery approach offers a great opportunity for processing microalga biomass under a sustainable perspective, using all the potential of each strain, maximizing the uses of raw materials, reducing wastes and energy input in order to increase the economic and environmental feasibility of the process.

5.4 Conclusions

A wide range of products has been obtained from microalgae using the biorefinery concept. In this study the biochemical composition of *P. tricornutum* samples cultivated under natural conditions in Chile was investigated, and an amount of 7.85 wt% of carbohydrates, 38.40 wt% of proteins, 9.08 wt% of lipids, 0.86 wt% of fucoxanthin and 5.19 wt% of biosilica was obtained. Three different biorefinery approaches were proposed by applying a *P. tricornutum* biomass production using a scaled-up scenario for the production of biofuels and high-value compounds. The biorefinery concept is a useful tool for making sound and profitable decisions regarding microalga biofuel production, together with other high-value products, exploiting the total biochemical potential of the local strains and available resources.

Chapter 6

Techno-Economic Analysis for *Phaeodactylum tricornutum*-based Biorefinery

Industrial production and commercialization of microalgae bioproducts have become a good alternative to traditional raw materials, due to their high growth rate, high CO_2 sequestration and the ease with which they can be cultivated in non-arable soil, freshwater and seawater. Marine diatoms have been especially exploited because of their high variability, interesting biochemical composition and lack of competition with other crops for freshwater and land uses. This study aims to analyze the technical and economic feasibility of a biorefinery based on *Phaeodactylum tricornutum* cultivated in an outdoor pilot-scale bubble-columns photobioreactor under natural conditions in Chile, for production of biofuels and high-value compounds. The production of microalgae-based biofuels was analyzed, directed at using algae biomass and at taking advantage of the biomass by co-product commercialization. The cost of microalgae-based biofuels remains higher compared to conventional fuels and, if greater technological maturity cannot make this approach cheaper it will be economically unfeasible to consider it in the short term. Nevertheless, the integration of high-value compounds with biofuel production under a biorefinery concept may diversify the income sources, making the process viable.

6.1 Introduction

Microalgae bioproducts are considered to be potential raw materials for a diverse number of industrial applications. Recently, a considerable amount of information has become available on the public and private investments in the research and development of microalgae-based compounds for a range of industrial sectors, such as food, feed, nutraceuticals, pharmaceuticals and biofuels, among others [138, 143, 150]. The gamble on these microorganisms can be explained by their intrinsic characteristics, which make them good candidates for a range of industries. Microalgae show a fast growth potential and do not require arable land; some species can develop in wastewater, saline water and

seawater, and so they offer a good alternative in mitigating some environmental problems and contribute to energy security [176].

Despite the potential of these organisms, there are some hurdles and challenges to overcome in order to establish strategies to expand the production and commercialization of these bioproducts. The commercialization of different microalgae components under an economic and environment feasibility, involves a biomass biorefining approach, which embrace a wide range of technologies able to extract the monomeric components of the biomass and transformed them into value-added products [177].

The biorefinery concept encompasses the multidisciplinary application of knowledge for the sustainable production and marketing of bioproducts along the entire process chain. Development of sustainable biorefinery demands the integration of innovative technologies with technical and economic feasibility in order to make progress beyond the "business as usual" scenario [178].

Microalgae-based biofuels still remain mostly at pilot scale around the world. The production and commercialization of these products have been restricted to a few countries, mainly the United States of America, Australia and some European countries, and they represent less than 5% of energy produced overall [178].

There are several techno-economic analyses concerning microalgae biomass production and specific bioproducts available in the literature. However, most of them have focused on theoretical data or laboratory scales, or have even been carried out in open systems [179, 180]. However, few analyses have been focused on real data processes using closed photobioreactors for biomass production. The gap in theoretical studies mainly concerns the difficulty of reproducing the model in a specific region, due to the diverse environmental and social conditions. The unrealistic photosynthetic efficiency, when theoretical or controlled laboratory data are extrapolated to natural solar radiation and temperature of a specific region, is another concern in the limitations of these analyses, which strongly affects productivity and influences costs of the project [181]. Nevertheless, when techno-economic analyses have focused on specific products and are based on consistent input data, they become very useful tools for decision makers in strategic planning and evaluating economic viability and/or focus on further improvements in order to achieve feasibility.

This Chapter focuses on analyzing the potential of microalgae-based biorefinery technology for the production of biofuels and high-value compounds and commercialization on the industrial scale, and investigating the competitiveness to replace other conventional fuel production in the Chilean situation.

6.2 Biorefinery Process Description

6.2.1 Location Site and Cultivation System

The present study proposed the construction of an industrial microalgae plant facility at Concepción, Chile. The town of Concepción is located in the Bío-Bío Region, Chilean VIII Region, geographical coordinates 36° 50' S and 73° 03' W, with average elevation of 12 m. According to Köpper classification, Concepción shows a Mediterranean climate with oceanic influence [182]. The last Chilean census, carried out in 2017, indicated that the town has approximately 223,574 habitants over a total area of km^2 [183].

The model was developed based on experimental data obtained in this work from a pilot-plant, and the biomass production was scaled up to perform the techno-economic evaluation. The microalgae cultivation system was performed in a photobioreactor (PBR) described by Branco-Vieira et al. [127] under natural conditions at Concepción, using natural seawater and a Chilean *P. tricornutum* strain, cultivated during summer-autumn. Concepción is an interesting site for microalgae cultivation due to: (1) the temperate climate; (2) the proximity to the coast, which can provide the water for the culture medium and other sea resources, if required; (3) the presence of an industrial conglomerate, which can supply the CO_2, required for microalgae cultivation, thus contributing to mitigating industrial emissions, as well; (4) the fact that Concepción has low population density, which enables the use of land; (5) the town's Central-Southern localization in the country, which facilitates supply and transport of products.

The average monthly environmental parameters observed in Concepción in 2016 are shown in Table 10. The 2016 annual mean temperature observed in the region of Concepción is 13.4 °C, with minimum of 9.3 °C and maximum of 18.7 °C. The annual mean relative humidity (RH) is 79.2%, mean precipitation is 51.3 mm, mean evaporation is 61.2 mm or 0.1 kg m^{-2}, mean horizontal global radiation is 56,076.2 cm^{-2} month, mean hours of sunshine are 216.2 hours $month^{-1}$ and mean pressure is 1,017.7 hPa. It is important to note that in 2016, when the biomass production was done, Concepción had a dry winter, which could be observed by the very low rate of precipitation in June.

6.2.2 Process Flowsheet of Microalgae Industrial Plant Facility

Microalgae cultivation can be performed in open or closed bioreactors. A number of factors must be considered to choose the best alternative for producing microalgae-based bioproducts. Both types of bioreactors show advantages for industrial applications; however, the final product needs to be taken into account in order to define what kind of system will be better for each case. Generally, open systems are less expensive and easier to operate than closed systems. On the other hand, closed systems provide higher biomass productivities, better management of contamination and higher environmental controls.

Table 10: Monthly values for environmental parameters from Concepción, Chile.

Months	Days	Temperature (°C)			RH (%)	Precipitation	Evaporation		Global Radiation	Sunshine	Pressure
		Min	Mean	Max		(mm)	(mm)	(kg m^{-2})	(J cm^{-2} month)	(hours $month^{-1}$)	(hPa)
January	31	13.5	18.4	23.9	71.7	1.8	105	0.23	73,47	285.5	1,014.8
February	28	11.6	17.1	23.6	70.2	0.0	107	0.27	77,466	317.8	1,014.5
March	31	10.5	15.2	21.6	77.2	14.2	75	0.19	66,275	241.1	1,015.8
April	30	8.3	12.3	18.0	81.2	67.2	49	0.14	50,132	181.2	1,016.6
May	31	10.4	12.9	16.8	90.0	117.6	17	0.05	31,984	98.5	1,017.3
June	30	5.0	8.6	14.2	85.8	3.4	18	0.06	44,89	174.5	1,021.3
July	31	6.8	9.3	13.1	87.7	189.4	13	0.04	30,762	108.9	1,018.5
August	31	7.2	10.4	14.8	85.5	54.0	35	0.10	41,621	162.6	1,021.4
September	30	8.0	12.1	17.9	77.2	49.8	49	0.13	59,777	218.5	1,019.6
October	31	8.3	12.6	17.5	80.1	71.8	46	0.11	60,417	229.4	1,018.6
November	30	10	15.1	20.8	72.2	14.4	104	0.24	74,001	290.5	1,017.5
December	31	11.5	16.3	21.9	71.7	32.4	114	0.24	62,12	285.8	1,016.3

In this study, data was used from a bubble-column PBR, a type of closed PBR that is cheaper than those with different shapes. The microalgae biomass produced was proposed for use under a biorefinery concept, for which it is important to consider the utilization of the products and waste generated by each process in a circular economy approach, and to evaluate the biotechnological potential of the strain.

The proposed industrial microalgae production flowsheet is shown in Figure 21. The microalgae cultivation process started by (1) pumping of the seawater to the PBR, (2) mixing the seawater with the nutrients required by *P. tricornutum* growth, (3) supply of atmospheric air to the microalgae culture medium with an air blower, in order to supplement the culture with atmospheric CO_2 concentration and promote the culture mixing. After microalgae cultivation the (4) culture medium is pumped to the reservoir tank for (5) harvesting through centrifugation process and the recovered biomass, containing 15% of dry matter, which can be used for the downstream process. The remaining wastewater from the culture medium after biomass centrifugation is (6) filtered and returns to the seawater tank in order to be reused in another culture batch.

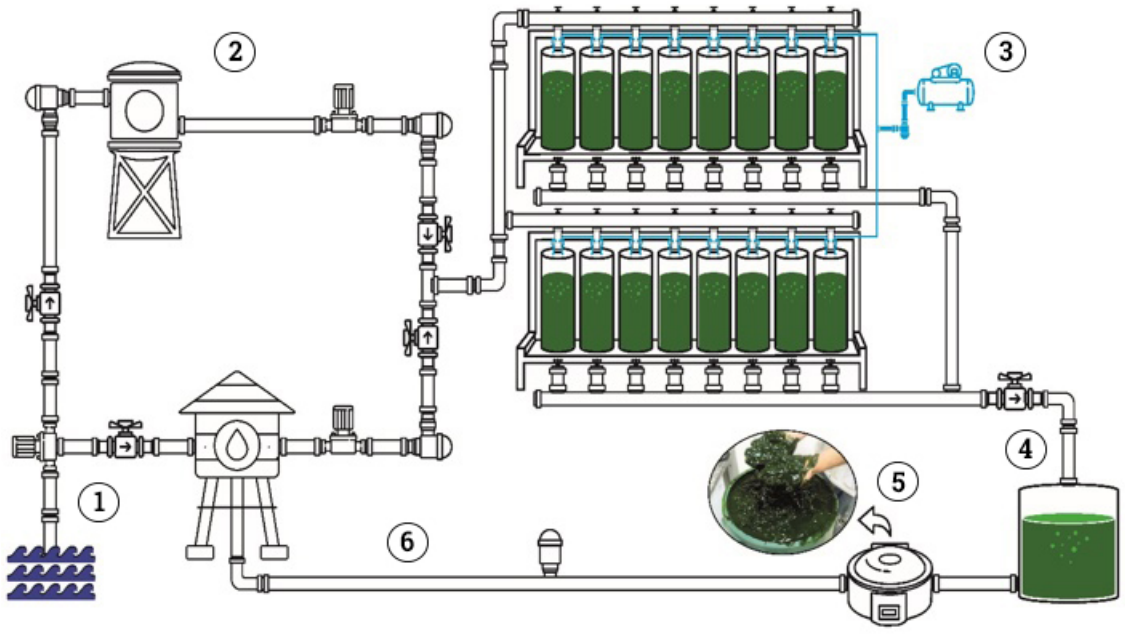


Figure 21: *P. tricornutum* biomass production flowsheet.

In order to estimate the microalgae biomass production a model proposed by Spruijt et al. [158] and applied by Branco-Vieira et al. [127] was used for the scenario analyzed in this study. The biomass production was considered under three different sizes of microalgae industrial facility, as described below:

1. **Scaling-up Scenario 1 (SL1)** - the cultivation was scaled up to 1.3 ha pilot-plant, containing 1,000 modules of 0.8 m^3 PBR, totalizing 8,000 m^3 of cultivation.

2. **Scaling-up Scenario 2 (SL2)** - the cultivation was scaled up to 12.7 ha pilot-plant, containing 10,000 modules of 0.8 m^3 PBR, totalizing 80,000 m^3 of cultivation.

3. **Scaling-up Scenario 3 (SL3)** - the cultivation was scaled up to 127 ha pilot-plant, containing 100,000 modules of 0.8 m^3 PBR, totalizing 800,000 m^3 of cultivation.

It was assumed that the industrial facilities works in 24 hours of PBR operation, for 12 months a year. The annual potential of *P. tricornutum* biomass obtained by the applied model showed a production of 231 tons of microalgae dried matter by the SL1 scenario, an amount of 2,312 tons by SL2 scenario and 23,115 tons by SL3 scenario plant facilities. The biochemical composition, the total biomass and high-value compounds produced under each industrial plant size of this microalga is shown in Table 11.

Table 11: Annual quantity of biomass and bioproducts produced by *P. tricornutum* under each industrial plant size.

Bioproducts	**Total Quantity (wt%)**	**SL1 (ton)**	**SL2 (ton)**	**SL3 (ton)**
Microalgae biomass	–	231	2,312	23,115
Lipids	9.08 ± 0.38	20.97	209.93	2,098.84
Carbohydrates	7.85 ± 0.22	18.13	181.49	1,814.53
Protein	38.40 ± 2.46	88.76	887.62	8,876.17
Fucoxanthin	0.86 ± 0.06	2.33	23.31	233.09
Bio-silica	5.19 ± 0.56	11.88	118.81	1188,11
Ashes and others	38.67 ± 1.50	–	–	–

The capital cost and equipment needed for industrial plant facilities were divided by three different steps for microalgae production: supplies, PBR investment and harvesting investment. Culture crashes were considered for PBR cleaning and maintenance over the year, and this parameter was taken into account for evaluation of potential biomass production. For this purpose, 4 days $year^{-1}$ were considered for reactor cleaning; 7 days $year^{-1}$ for culture downtime per cleaning event and 14 days $year^{-1}$ for production downtime per cleaning event. The industrial plant requires low quality (LQ) and high quality (HQ) labor, which is based on daily operations and on extra labor needed in case of cleaning or PBR crash; in this case just LQ is required. The amount of labor requirement is directly dependent on the number of PBRs and the industrial plant size.

The capital cost of investment was based on the construction of a SL1 scenario plant, and a scaling factor was assumed for capital cost calculations of the SL2 and SL3 scenarios. The scaling factor depends on the number of PBR units installed and the capital investment cost of installing one PBR unit. In this study, the basic capital cost investment was calculated for 1,000 modules of PBR in 1.27 ha of land (SL1 scenario). The capital costs for the SL2 and SL3 scenarios were calculated based on the methodology proposed by Sinnott and Towler [184], expressed by Equation 14.

$$C_2 = C_1 \times \left(\frac{S_2}{S_1}\right)^n \tag{14}$$

Where, C_2 is the capital cost of the project with capacity S_2, C_1 is the capital cost of the project with capacity S_1 and n is the scaling factor, equal to 0.6 and well-known as six-tenths rule.

The prices assumed per unit of variable costs inputted, needed for industrial biomass production, are listed in Table 12, as well as the equipment for PBR operability in Table 13 and for the biomass harvesting process in Table 14. The equipment price was obtained directly from local and international suppliers. Consumables, labor costs and other inputs were obtained directly from the Chilean government and companies. Infrastructure was calculated by information from the Ministry of Housing and Urban Planning of Chile, relating to category A2 construction (storehouse type) [185].

Table 12: Prices of supplies necessary for microalgae industrial plant.

Variable Cost	Price (€)	Unit	Source
Water	0.593	€ m^{-3}	[186]
Electricity	0.121	€ kWh^{-1}	[187]
Labor LQ	8.88	€ hr^{-1}	[188]
Labor HQ	20.65	€ hr^{-1}	[188]
Fertilizer (N)	0.12	€ kg^{-1}	[189]
Fertilizer (P)	0.62	€ kg^{-1}	[189]

Table 13: Capital goods investment for PBR and biomass production.

Investment of capital goods	Price (€)	Life Span	Source
Reactor construction	119,731	10	Calculated
Circulation pump	10,000	8	[158]
Heating & cooling equipment	55,000	10	[158]
Process control	17,514	15	[158]
Infrastructure	65,619	15	Calculated
Total	**267,864**		

Table 14: Capital goods investment for biomass harvesting.

Investment of capital goods	Price (€)	Life Span	Source
Centrifuge	294,021	10	[158]
Infrastructure	16,564	15	[158]
Total	**310,585**		

6.2.3 Downstream Process

After obtaining the microalgae biomass, it was addressed to biofuel (biodiesel, bioethanol and biomethane) production and high-value compound commercialization, targeting protein, fucoxanthin and biosilica. The production of biofuels was performed according to the method proposed by Spruijt et al, modified in this study. [158]. The economic evaluation was performed under two different approaches: (B) for biofuel production directly from microalgae biomass (Figure 22) and (CP) to biofuel production after valorization of microalgae biomass co-products (Figure 23).

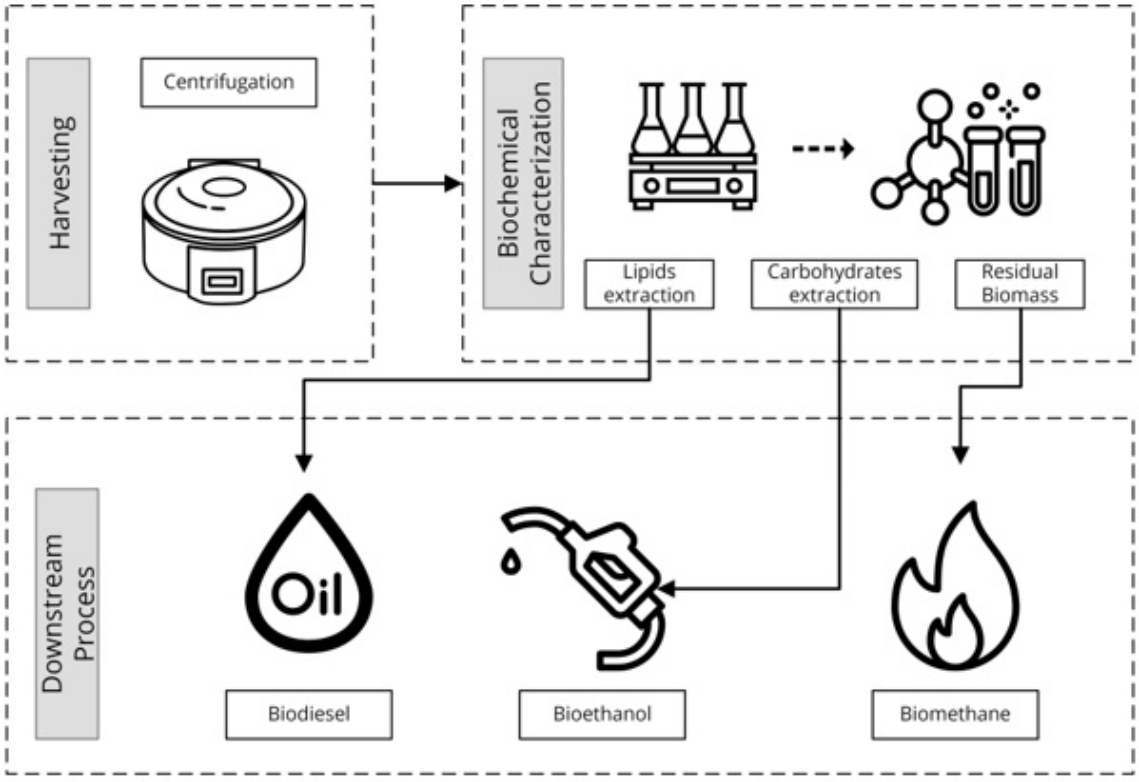


Figure 22: *P. tricornutum* biorefinery for biofuel production (biodiesel, bioethanol and biomethane) immediately after microalgae biomass production. Rectangles show the overview of downstream process considered this study.

The selling prices assumed per unit of outputted microalga biomass co-products are listed in Table 15.

Table 15: Selling prices of microalga biomass co-products.

Variable Cost	Price (€)	Unit	Source
Fucoxanthin	600	€ kg^{-1}	[190]
Biosilica	10	€ kg^{-1}	[191]
Protein	10	€ kg^{-1}	[192]

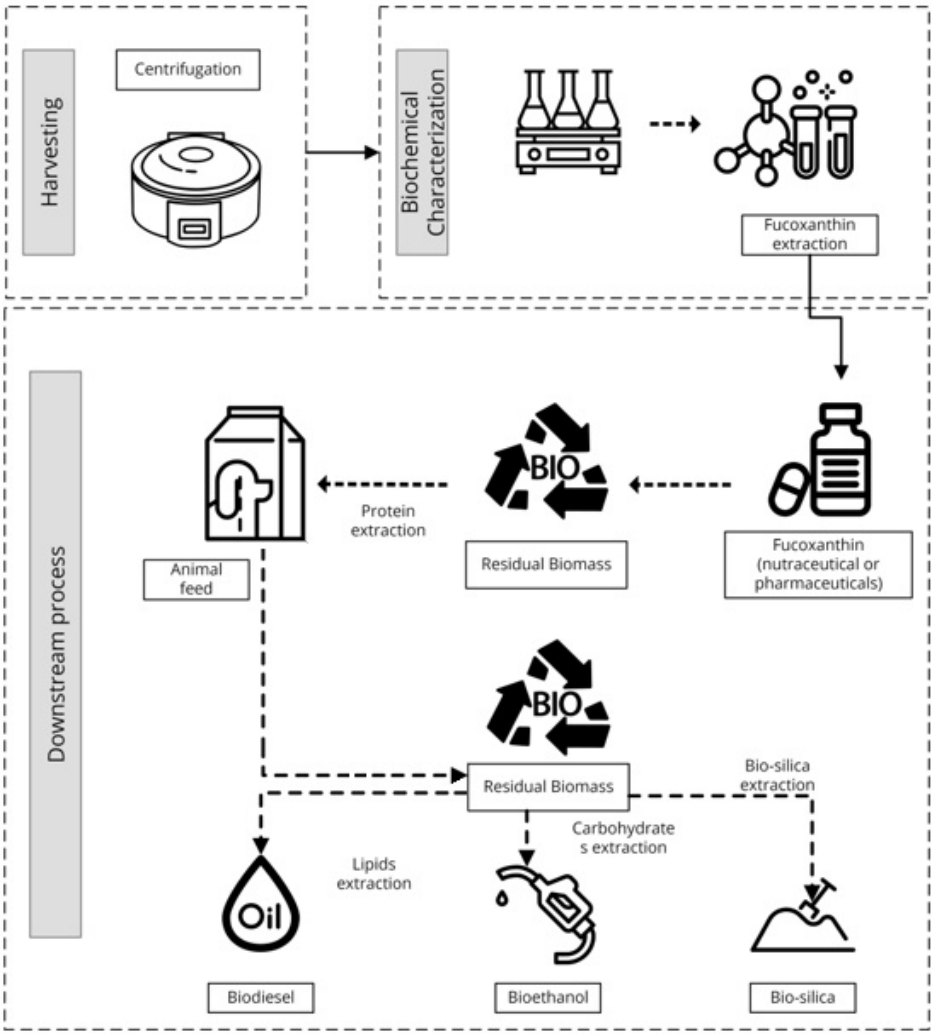


Figure 23: *P. tricornutum* for production of high-value compounds and biofuels. High-value compounds targeted fucoxanthin, protein extraction and valorization of residual biomass for biosilica and biofuel production. Biofuels focused on biodiesel and bioethanol. Rectangles show the overview of downstream processes considered this study.

The production of biofuels after biomass co-product valorization was carried out just for biodiesel and bioethanol production, because the residual biomass utilization after co-product valorization was supposed to prioritize the production of biosilica over biomethane.

6.2.4 Biodiesel

Biodiesel production was carried out using a supercritical carbon dioxide (SC-CO_2) for lipid extraction. To achieve this purpose five steps were considered for biodiesel economic evaluation: (1) biomass drying; (2) disruption of cell walls by milling process; (3) SC-CO_2 lipid extraction; (4) lipids refining prior to transesterification; (5) transesterification process.

The cost of this process was calculated based on the SL1 scenario, e.g. the production

of 231 tons of microalgae biomass, and the other SL2 and SL3 scenarios were estimated using the scaling up methodology earlier described (Subsection 6.2.2). Accordingly to the modified method proposed by Spruijt et al. [158] the downstream process for biodiesel production required the input parameters shown in Table A.2 for biomass drying process; Table A.3 for the disruption of the cell walls by milling process; Table A.4 for SC-CO_2 lipid extraction; Table A.5 for lipids refining prior to transesterification, and Table A.6 for transesterification process.

Cost of capital goods for each process were calculated based on parameters listed in Table 16. The costs of the transesterification process were based on Hass et al. [193].

Table 16: Capital goods and supplies investment for biodiesel production.

Investment of capital goods	**Price (€)**	**Life Span**	**Source**
Ball Mill	10,000	15	[158]
SC-CO_2 Extractor	120,000	20	[158]
Refining	14,432	20	[158]
Transesterifying Equip.	22,520	20	[158]
Process Control	15,695	5	[158]
Infrastructure	30,000	20	[158]
Total	**212,647**		
Chemicals	0.2	–	[158]
Methanol	0.282	–	[158]
Carbon dioxide	0.142	–	[158]
Total	**0.624**		
Total Costs	**213,271**		

6.2.5 Bioethanol

The remaining biomass, called the alga paste, is mainly characterized by protein and carbohydrate contents and this material can have other applications. The sequence of this biorefinery approach involves extracting carbohydrate from the alga paste and converting it into bioethanol. For this purpose, to the biomass was rehydrated, followed by enzymatic hydrolysis of the released polymeric carbohydrates into monomeric carbohydrates, in order to be fermented by yeasts. After fermentation the resulting ethanol can be concentrated by distillation process. For producing bioethanol, the following steps were considered for the economic evaluation: (1) cell disruption by wet milling process; (2) fermentation of the carbohydrates by yeasts and; (3) distillation process of the bioethanol.

According to the procedure adopted for biodiesel production, the cost of each step was calculated based on the SL1 scenario, and for the other two scenarios they were estimated using the scaling up methodology. According to modified method proposed by Spruijt et al. [158] the downstream process for bioethanol production required the input parameters

shown in Table A.7 for the cell disruption process; Table A.8 for the distillation of bioethanol. Capital goods for each process were calculated based on parameters listed in Table 17.

Table 17: Capital goods and supplies investment for bioethanol production.

Investment of capital goods	Price (€)	Life Span	Source
Ball Mill	76,000	10	[158]
Fermentation	24,189	20	[158]
Distillation	24,189	20	[158]
Process Control	12,438	5	[158]
Infrastructure	30,000	20	[158]
Total	**166,816**		
Enzyme	6	–	[158]
Yeast	9	–	[158]
Carbon Dioxide	0.142	–	[158]
Total	**15.142**		
Total Costs	**166,831**		

6.2.6 Biomethane

Biomethane production was proposed from the remaining biomass after lipid and carbohydrate extraction. The production of biogas is made by microorganisms capable of reducing the organic matter to methane (CH_4) and CO_2, through a fermentation process. The annual biomethane production was calculated based on methodology proposed by Spruijt et al. [158]. Similarly to biodiesel and bioethanol production, the biomethane economic calculations were carried out for the SL1 scenario and results were scaled-up for the SL2 and SL3 scenarios. The downstream process for biomethane production required the input parameters shown in Table A.10. Cost of capital goods for biomethane production were calculated based on parameters listed in Table 18.

Table 18: Capital goods investment for biomethane production.

Investment of capital goods	Price (€)	Life Span	Source
Digester	17,000	10	[158]
Green Gas Processer	440	20	[158]
Total	**17,440**		

The minimum capacity of a digester is 1 million m^3 of biogas per year, and so the economic analysis was based on these values. Differently from the biodiesel and bioethanol analysis, the maintenance and labor costs were calculated as 2.0% and 2.5% of the total investment, respectively.

6.3 Economic Assessment Parameters

The economic analysis of the industrial process considered in this study was based on the investment of fixed capital, annual production costs and annual revenues. For economic analysis € 1 was considered equivalent to CLP 724 and US$ 605 based on the quotation on January 19, 2018.

6.3.1 Total Return and Total Cost

The total return is the product of the yearly amount of microalgae biomass at its selling price, while the total cost of the plant facility was obtained by the sum of the variable costs, capital goods cost and cost for land use. In order to calculate the yearly capital goods cost, the depreciation, maintenance and discount rate were considered for these fixed capitals. Depreciation was estimated based on 10% of the cost price of each item as its resale value, multiplied by its life span. The percentage maintenance costs were also estimated per item [158]. The discount rate for capital goods was assumed to be 12% [194], with a factor of 0.55 for residual value after the end of the lifetime of each item. Land use costs were calculated per year, based on 1,470 m^2, which is 1,270 m^2 for PBR construction and another 200 m^2 for infrastructure and equipment. The price per m^2 was based on information from the Ministry of Housing and Urban Planning of Chile, relating to the A2 construction category (storehouse type) [185].

6.3.2 Return On Investment (ROI)

In order to obtain a perspective about the viability of the proposed project and to make it possible for the decision makers to plan improvements in the current approach, the return on investment (ROI) was used as a financial parameter of this study, which was calculated by Equation 15.

$$\text{ROI} = \frac{Total\ return - Total\ cost}{Total\ investment} \tag{15}$$

Where the total return and total cost of capital goods were explained in the earlier section, and total investment is the total investment on capital goods, without considering the depreciation and interests for this fixed capital.

6.3.3 Pay-back Time

In this study pay-back time was used to analyze the necessary period, in years, for obtaining the return on investment. In order to calculate this parameter, Equation 16 was used.

$$\text{Pay} - \text{back} = \frac{Total\ investment}{Total\ return - Total\ cost} \tag{16}$$

6.4 Techno-Economic Evaluation for *P. tricornutum*-based Biorefinery

6.4.1 Biomass Production

The proposed industrial algae facility was composed of three different sizes of plants. The SL1 scenario included a total of 1,000 similar modules of 0.8 m^3 PBR, totalizing 8000 m^3 of cultivation, with a total area of 1.3 ha. This scenario was used to calculate the total investment of the project, using data obtained directly from one module of PBR, localized at the same site as the proposed facility and using the same strain *P. tricornutum*. The industrial plant was assumed to operate daily for 24 hours, where employees work only during diurnal periods. Crashes resulting from PBR cleaning and maintenance were also done in diurnal periods. Photosynthetic efficiency achieved by *P. tricornutum* in this site was about 5%, average biomass concentration of 0.96 kg m^{-3} and volumetric productivity of 0.13 kg m^{-3} d^{-1}. The water input is obtained direct from the sea and it was used an amount of 26,501 m^3 of water per year for culture medium. The CO_2 required for microalgae growth was supplied from atmospheric air by air blower and contributes to mitigating about 891,558 kg of CO_2 annually. The same system that supplied CO_2 to the culture medium promoted the mixing of the culture, which corresponds to energy consumption of 16,094 kw h^{-1} $year^{-1}$, while the centrifugation process required 316,818 kw h^{-1} $year^{-1}$. The major nutrients for the microalgae culture medium are nitrogen and phosphorus, which represent a consumption of 16,721 kg and 1,128 kg, respectively. Freshwater is necessary for the cooling system when the temperature is higher than that tolerated by *P. tricornutum*. In this case, the annual consumption of cooling water was 8,837,829 m^3. The energy supplied to the culture system was about 111,968 GJ per year and labor requirements were about 19,682 hours and 2,460 hours of LQ and HQ, respectively. Labor considered for system crashes resulting from cleaning and maintenance were about 255 hours per year. The volume of biomass culture addressed to the harvesting process was estimated at 240,781 m^3, which corresponds to 231,150 kg of dried biomass and the potential for producing an amount of 20,970 kg of lipids, 18,130 kg of carbohydrates, 88,768 kg of protein, 2,331 kg of fucoxanthin, 11,881 kg of biosilica.

After biomass harvesting, an amount of 226,722 m^3 of wastewater returned to the PBR recycling tank for feeding another microalgae culture batch, leaving an amount of residual wastewater of 25,191 m^3, containing 2,519 kg of nitrogen and 504 kg of phosphorus. It is important to note that part of this residual wastewater remained in the alga paste, because the centrifugation process resulted in a concentrated biomass containing 15% of dry matter. Lost biomass represented by culture crashes corresponded to 41,891 kg per year (Figure 24).

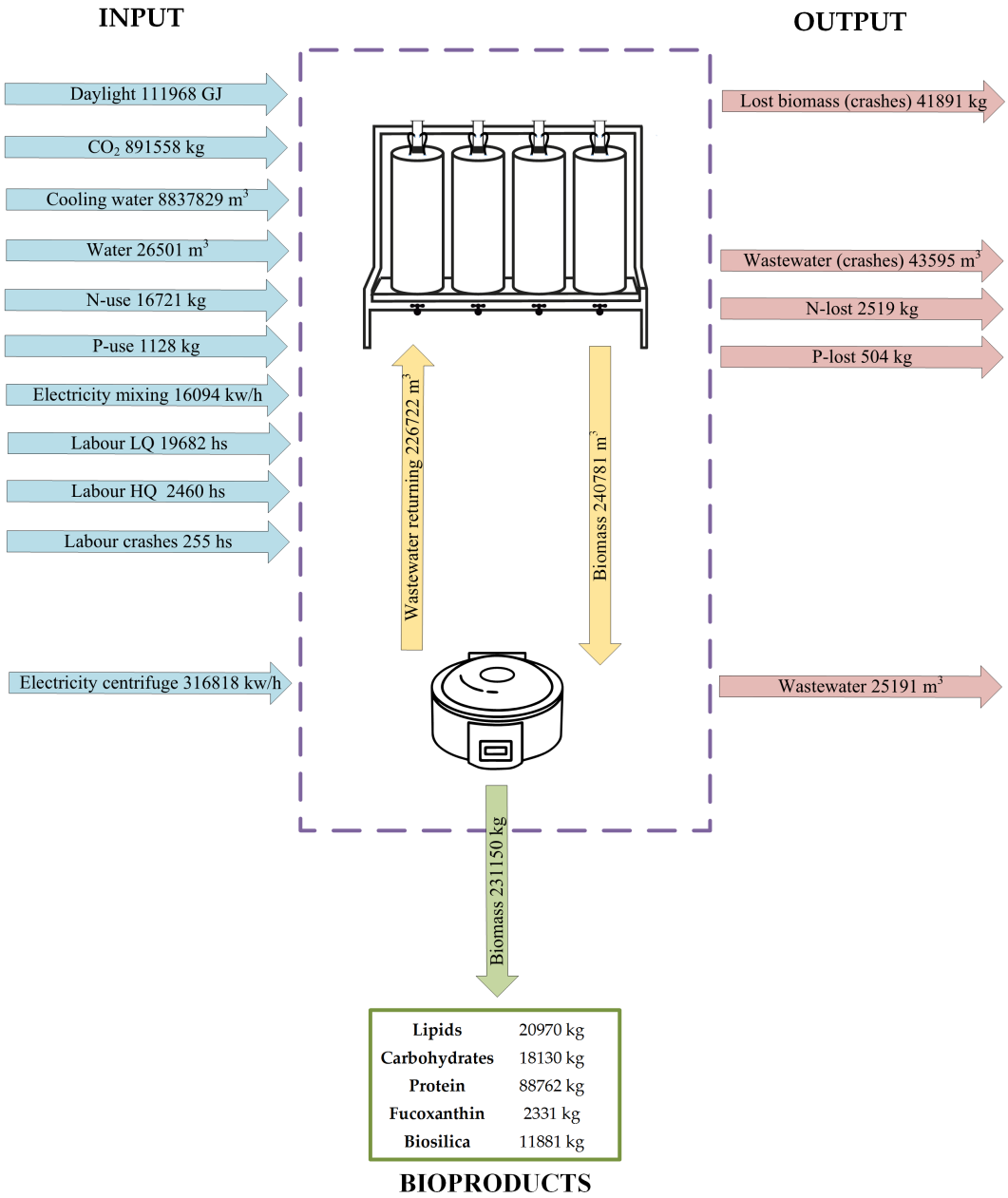


Figure 24: Input and output currents in the *P. tricornutum* biomass production in the SL1 scenario and the potential of compounds extracted by the biomass. Blue arrows indicate the input parameters, red arrows indicate the output parameters and green arrow indicates the bioproduct potential. The input and output product consumption was estimated per year.

The investment costs were made scalable for another two scenarios used in this study, SL2 which represented an amount of 10,000 similar modules of 0.8 m^3 PBR, totalizing 80,000 m^3 in a total area of 12.7 ha, and the SL3 scenario, containing 100,000 similar modules of 0.8 m^3 PBR, totalizing 800,000 m^3 of cultivation and a total area of 127 ha. The scalable method was performed by using the known investment for the SL1 scenario and the equation that allows costs to be different scale levels (Equation 14).

Costs related to biomass production are shown in Table 19. The production of biomass was analyzed under SL1, SL2 and SL3 scenarios, and the associated costs of biomass without co-product valorization (B) and with co-product valorization (CP). Variables costs include all necessary supplies for biomass production, such as electricity, fertilizers, labor hours, water use and wastewater treatment (Table 19). The variable costs were also included the selling price of co-products (fucoxanthin, protein and biosilica), and in this case these prices were inputted with negative values, indicating that the production and commercialization of these items impacted positively on the reduction of the project total cost.

Table 19: Cost of variables and capital goods for each scaling scenario for biomass production.

Costs	**SL1 (€)**		**SL2 (€)**		**SL3 (€)**	
	B(*)	**CP(**)**	**B(*)**	**CP(**)**	**B(*)**	**CP(**)**
Variable costs						
Water use	15,717	15,717	157,169	157,169	1,571,695	1,571,695
Electricity	40,429	40,429	404,294	404,294	4,042,943	4,042,943
Fucoxanthin	0	- 1,398,532	0	- 13,985,324	0	- 139,853,235
Biosilica	0	-118,811	0	- 1,188,112	0	- 11,881,120
Labor LQ	177,042	177,042	704,818	704,818	2,805,933	2,805,933
Labor HQ	50,805	50,805	202,259	202,259	805,209	805,209
Protein	0	-887,617	0	- 8,876,168	0	- 88,761,676
Fertilizer (N)	2,062	2,062	20,624	20,624	206,237	206,237
Fertilizer (P)	696	696	6,956	6,956	69,56	69,56
Wastewater	19,468	19,468	194,676	194,676	1,946,760	1,946,760
Total	306,22	- 2,098,741	1,690,797	- 22,358,806	11,448,336	- 229,047,696
Capital goods						
Depreciation	196,474	196,474	782,177	782,177	3,113,902	3,113,902
Interest	152,24	152,24	606,077	606,077	2,412,838	2,412,838
Maintenance	120,586	120,586	480,063	480,063	1,911,165	1,911,165
Total	469,3	469,3	1,868,317	1,868,317	7,437,904	7,437,904
Land costs						
Land	9,587	9,587	92,467	92,467	911,116	911,116
Total Costs	**785,107**	**- 1,619,854**	**3,651,581**	**- 20,398,022**	**19,797,356**	**- 220,698,676**

(*)B – represents the production of biomass without co-product valorization.

(**)CP – represents the production of biomass and valorization of co-products.

All analyzed scenarios demonstrated that the total costs of biomass production were compensated when co-products were commercialized (Table 19). Furthermore, in all three analyzed scenarios the total costs indicated that commercialization of co-products contributed considerably to reducing the costs, showed by negative values assumed by

CP analysis of the scenarios. However, the total results of all scenarios analyzed were positive, indicating that the production of microalgae biomass is profitable under the considered conditions, with or without co-product valorization (Table 20). The pay-back time was lower when considering co-product valorization and scale-up, indicating that the economies of scale are an important factor for determining the cost price of microalgae biomass production. The ROI was positive for all analyzed scenarios with higher values for the SL3 scenario, and also proportional to the economy of scale.

Table 20: Financial parameters of the project for each scaling scenario for biomass production.

Parameters	**SL1**		**SL2**		**SL3**	
	B(*)	**CP(**)**	**B(*)**	**CP(**)**	**B(*)**	**CP(**)**
Total return (€)	8,090,257	8,090,257	80,902,569	80,902,569	809,025,692	809,025,692
Total costs (€)	785,107	- 1,619,854	3,651,581	- 20,398,022	19,797,356	- 220,698,676
Total results (€)	7,305,150	9,710,111	77,250,988	101,300,591	789,228,336	1,029,724,368
Cost price (€)	3.40	- 7.01	1.58	- 8.82	0.86	- 9.55
Total goods (€)	2,306,663	2,306,663	9,182,992	9,182,992	36,558,148	36,558,148
ROI (%)	332	436	856	1118	2174	2832
Pay-back (year)	0.3	0.2	0.1	0.1	0.0	0.0

(*)B – represents the production of biomass without co-product valorization.

(**)CP – represents the production of biomass and valorization of co-products.

Concerning the final cost of biomass, in this study was obtained a price of biomass of about 3.40 € kg^{-1}, 1.58 € kg^{-1} and 0.86 € kg^{-1} for B analysis of SL1, SL2 and SL3 scenarios were obtained, respectively. On the other hand, all CP analyzed scenarios showed negative values for biomass production, corresponding to biomass price of about -7.01 € kg^{-1}, -8.82 € kg^{-1} and -9.55 € kg^{-1} for CP analysis of SL1, SL2 and SL3 scenarios, respectively (Table 21).

Table 21: Prices assumed for each scaling scenario for biomass production.

	SL1		**SL2**		**SL3**	
	B(*)	**CP(**)**	**B(*)**	**CP(**)**	**B(*)**	**CP(**)**
Fucoxanthin	0.00	-6.05	0.00	-6.05	0.00	-6.05
Bio-silica	0.00	-0.51	0.00	-0.51	0.00	-0.51
Protein	0.00	-3.84	0.00	-3.84	0.00	-3.84
Fertilizer (N)	0.01	0.01	0.01	0.01	0.01	0.01
Fertilizer (P)	0.00	0.00	0.00	0.00	0.00	0.00
Water	0.07	0.07	0.07	0.07	0.07	0.07
Electricity	0.17	0.17	0.17	0.17	0.17	0.17
Labor LQ	0.77	0.77	0.30	0.30	0.12	0.12
Labor HQ	0.22	0.22	0.09	0.09	0.03	0.03
Wastewater	0.08	0.08	0.08	0.08	0.08	0.08
Capital goods	2.03	2.03	0.81	0.81	0.32	0.32
Land	0.04	0.04	0.04	0.04	0.04	0.04
Total	**3.40**	**-7.01**	**1.58**	**-8.82**	**0.86**	**-9.55**

(*)B – represents the production of biomass without co-product valorization.

(**)CP – represents the production of biomass and valorization of co-products.

Regarding the cost analysis of each process step for biomass production, it was observed that the biomass price is mainly determined by capital goods costs, followed by labor and electricity for B analysis of all three scenarios (Figure 25a). In contrast, other studies have demonstrated that fertilizers contributed as major factors for increasing microalgae biomass prices [195]. However, in this study, these elements contributed less than 1% in all scenarios. These data are in agreement with other studies in the literature [181, 196], suggesting that the economic aspects of the culture systems (PBR or open ponds) remain the central concern in making the microalga biorefinery a reality [178]. Nevertheless, the CP analysis showed that commercialization of fucoxanthin and protein substantially improved the price of biomass (Figure 25b).

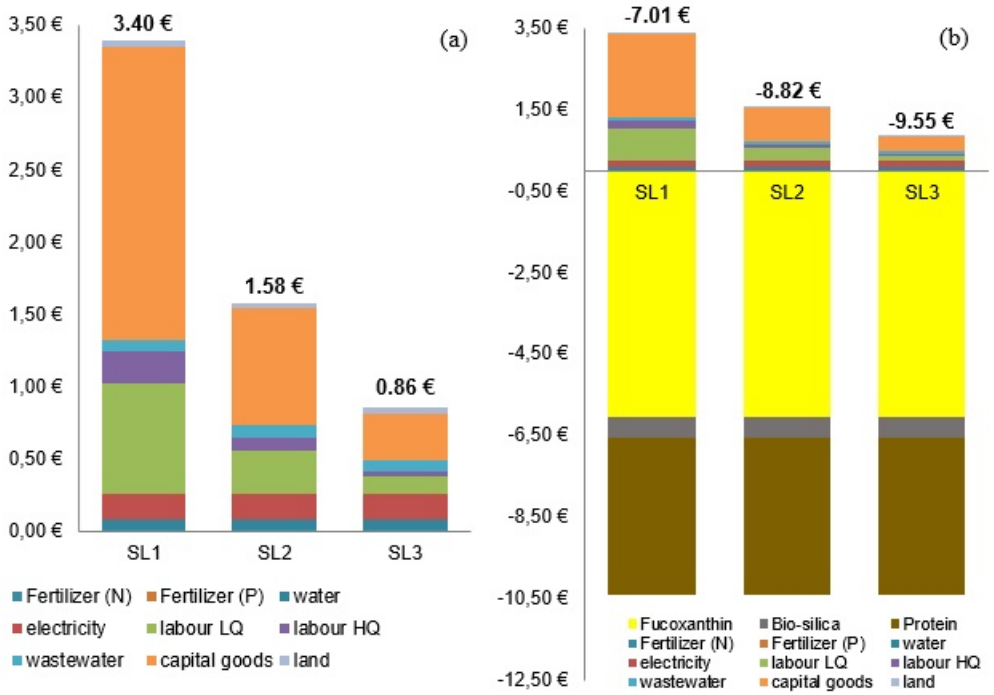


Figure 25: Prices obtained for *P. tricornutum* biomass production in SL1, SL2 and SL3 scenarios and contribution of each step to the final cost of biomass. (a) Biomass production without co-product valorization (B analysis); (b) Biomass production with co-product valorization (CP analysis).

6.4.2 Biofuel Production

During recent decades, microalgae have been investigated as good candidates for biofuel production [20, 21, 62]. Microalgae biomass contains lipids and carbohydrates that can be extracted for biofuel production. Algae oil can be extracted from biomass by solvent extraction, oil press and expeller and supercritical fluid extraction [197]. Further treatments are necessary to refine the microalgae oil into biodiesel in order to meet the international standards for biodiesel commercialization. On the other hand, microalgae

carbohydrates can be hydrolyzed by enzymes to produce simple sugars which can be fermented to bioethanol, using yeasts [198]. Finally, the microalgae biomass can be converted through direct synthesis into biogas by anaerobic digestion [199].

In this study, the economic profitability of biodiesel, bioethanol and biomethane production was analyzed directly after the microalgal harvesting and after biomass co-product valorization.

Economic evaluation of microalgae-based biodiesel under scenarios SL1, SL2 and SL3 of biomass production, as well as the associated costs of biodiesel production without biomass co-product valorization (B) and with biomass co-product valorization (CP) can be observed in Table 22. Variable costs included all necessary supplies for biodiesel production, such as algae biomass produced in an earlier step (upstream process), electricity, chemicals, methanol, carbon dioxide, labor hours, water use and wastewater treatment (Table 22). It is important to observe that algae biomass in all CP analysis showed negative values, due to the production and commercialization of co-products (fucoxanthin, protein and biosilica), impacting positively on the reduction of the project's total cost. In comparison, in the B analysis, the biomass was achieved without co-product commercialization. In Table 22 the cost prices of capital goods and the associated costs with depreciation, interest and maintenance of this fixed capital can be seen.

Table 22: Investment of variable cost and capital goods for each scaling scenario for biodiesel production.

Costs	**SL1 (€)**		**SL2 (€)**		**SL3 (€)**	
	B(*)	**CP(**)**	**B(*)**	**CP(**)**	**B(*)**	**CP(**)**
Variable costs						
Algae biomass	785,911	- 1,620,363	3,652,173	- 20,387,447	19,878,917	- 220,748,439
Water use	22	22	883	883	35,147	35,147
Electricity	1,158,734	1,158,734	46,397,404	46,397,404	1,873,849,857	1,873,849,857
Chemicals	525	525	23,012	23,012	1,140,487	1,140,487
MeOH	7,884	7,884	313,86	313,86	12,495,005	12,495,005
Carbon dioxide	474,271	474,271	47,420,256	47,420,256	4,741,956,010	4,741,956,010
Wastewater	31	31	1,236	1,236	49,19	49,19
Labor LQ	727,535	727,535	27,288,037	27,288,037	1,079,685,133	1,079,685,133
Labor HQ	8,155	8,155	32,464	32,464	129,243	129,243
Total	3,163,068	756,794	125,129,325	101,089,704	7,729,218,989	7,488,591,633
Capital goods						
Depreciation	79,395	79,395	316,076	316,076	1,258,321	1,258,321
Interest	43,333	43,333	172,51	172,51	686,776	686,776
Maintenance	27,342	27,342	108,851	108,851	433,344	433,344
Total	150,069	150,069	597,437	597,437	2,378,441	2,378,441
Total costs	**3,313,137**	**906,864**	**125,726,762**	**101,687,141**	**7,731,597,430**	**7,490,970,075**

(*)B – represents the production of biomass without co-product valorization.

(**)CP – represents the production of biomass and valorization of co-products.

Table 23: Financial parameters of the project for each scaling scenario for biodiesel production.

Parameters	SL1		SL2		SL3	
	B(*)	CP(**)	B(*)	CP(**)	B(*)	CP(**)
Total return (€)	379,891	379,891	37,989,062	37,989,062	3,798,906,181	3,798,906,181
Total costs (€)	3,313,137	906,864	125,726,762	101,687,141	7,731,597,430	7,490,970,075
Total results (€)	- 2,933,247	-526,973	- 87,737,700	- 63,698,080	- 3,932,691,249	- 3,692,063,893
Selling price (€)	0.75	0.75	0.75	0.75	0.75	0.75
Cost price (€)	6.54	1.79	2.48	2.01	1.53	1.48
Total goods (€)	1,432,485	1,432,485	5,702,824	5,702,824	22,703,353	22,703,353
ROI (%)	-196	-28	-1530	-1108	-17314	-16254
Pay-back (year)	-0.5	-3.5	-0.1	-0.1	0.0	0.0

(*)B – represents the production of biomass without co-product valorization.

(**)CP – represents the production of biomass and valorization of co-products.

The analysis of the three scenarios showed that the total cost of biodiesel was lower when microalgae biomass co-products were commercialized (CP), when compared to B analysis (Table 23). Moreover, total costs indicated that commercialization of biomass co-products contributed considerably to diminishing the cost price, for all analyzed scenarios. Nevertheless, unlike what was observed for the production of biomass, the total results, ROI and pay-back time of all scenarios analyzed for biodiesel production were negative, indicating that the production of microalgae-based biofuel is not profitable under the considered conditions (Table 23). Prices obtained for biodiesel production indicated that the economies of scale are important only when small levels of production were considered, while in higher levels of scaling the algae biomass price did not impact on the biodiesel cost, even if the algae biomass assumed negative values obtained after co-products valorization (Figure 26).

The fact that the price of algae does not influence on the biodiesel cost under higher levels of production, could be explained by the higher cost of technology used in this study for biodiesel production. The biomass drying process, dry ball milling for cell disruption and SC-CO_2 applied to lipid extraction are higher energy-intensive methodologies that lead to high electricity consumption (Figure 26 and 27).

Improvements in these technologies in order to use cheaper electricity sources, such as solar or wind microgeneration energy, can be an alternative to increase the profitability of using these methods, because from the environmental point of view these sources represent better alternatives than those commonly used in the "business as usual" scenario. Concerning the final cost of biodiesel, in this study this was about 6.54 € kg^{-1}, 2.48 € kg^{-1} and 1.53 € kg^{-1} for B analysis of SL1, SL2 and SL3 scenarios, respectively (Figure 26a and 27a). On the other hands, all CP analyzed scenarios showed values corresponding to 1.79 € kg^{-1}, 2.01 € kg^{-1} and 1.48 € kg^{-1} for CP analysis of SL1, SL2 and SL3 scenarios, respectively (Figures 26b and 27b).

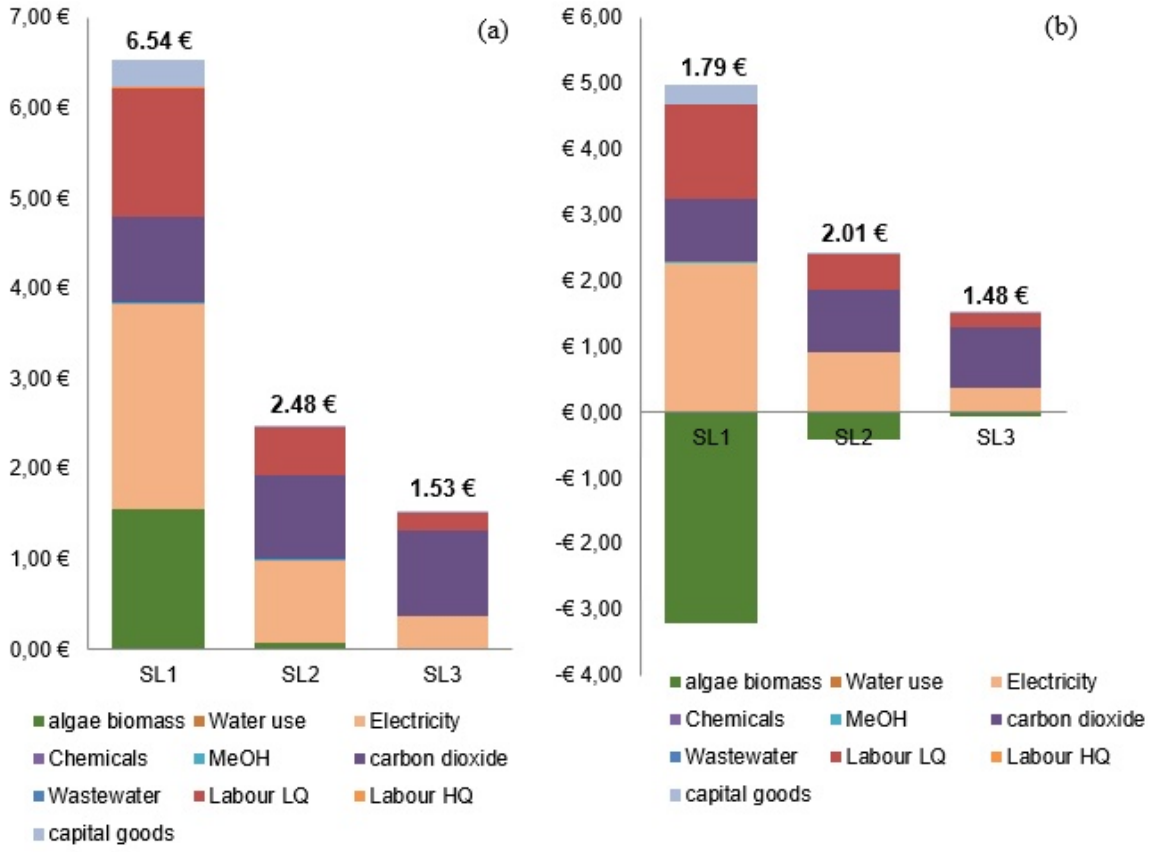


Figure 26: Prices obtained by *P. tricornutum* biodiesel production in the SL1, SL2 and SL3 scenarios. (a) Biodiesel production without co-product valorization (B analysis); (b) Biodiesel production with co-product valorization (CP analysis).

Analysis of bioethanol production from microalgae biomass can be observed in Table 24. Economic assessment of microalgae-based bioethanol under scenarios SL1, SL2 and SL3, as well as the costs of bioethanol production without biomass co-products valorization (B) and with biomass co-products valorization (CP) revealed that this approach is only profitable on a small scale, with biomass co-products valorization (Table 24).

Cost of capital goods and variable costs for bioethanol production, such as algae biomass, water use, electricity, enzymes for carbohydrates hydrolysis, yeasts for monomeric carbohydrates fermentation, labor hours and wastewater treatment are also shown per item in Table 24. Similar to results for biodiesel production, algae biomass in all CP analysis showed negative values, due to the production and commercialization of co-products (fucoxanthin, protein and biosilica). The impact of the selling price of bioethanol on CP analysis revealed that negative values of algae biomass reduce the production cost of bioethanol; however, bioethanol achieved a competitive value only in SL1 CP, showing that there is a tenuous threshold between profitable bioethanol cost proportional to the values of algae biomass.

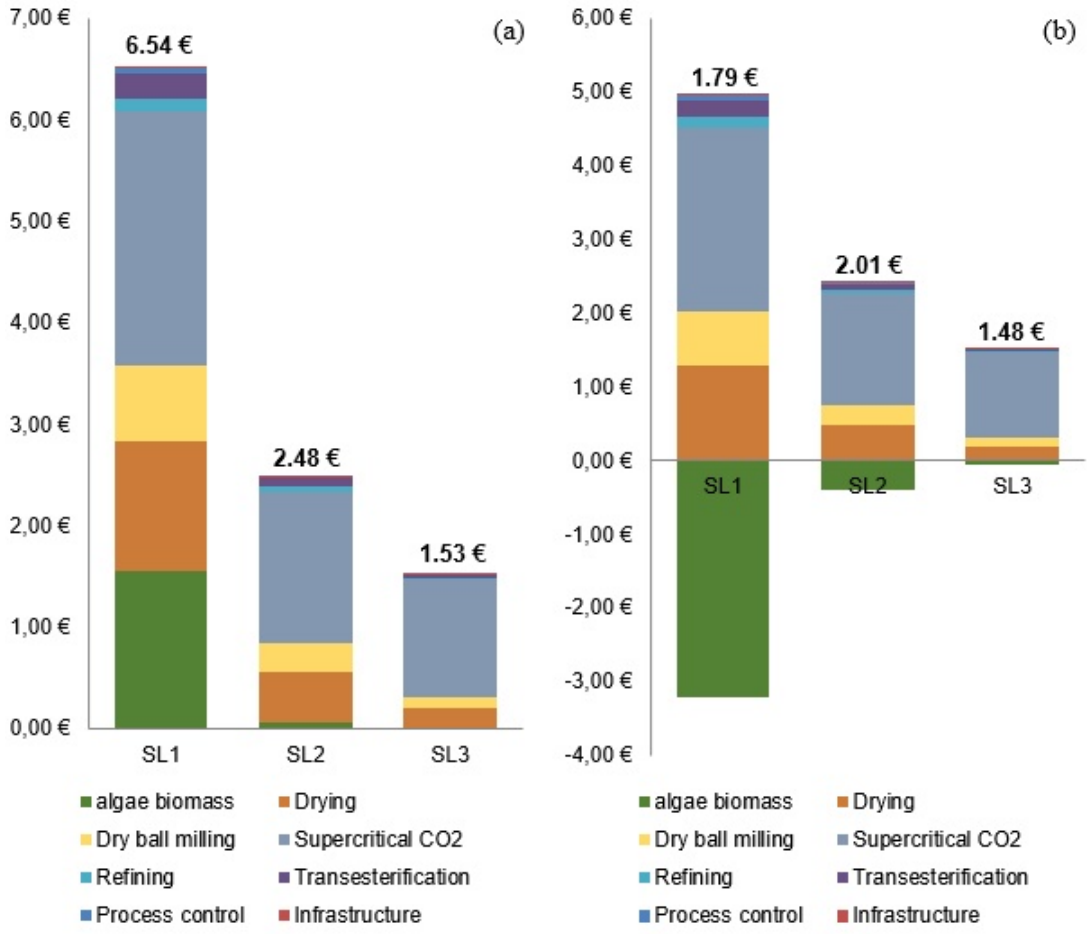


Figure 27: Prices obtained for *P. tricornutum* biodiesel production in the SL1, SL2 and SL3 scenarios and contribution of each step of the process to the final cost of biodiesel. (a) Biodiesel production without co-products valorization (B analysis); (b) Biodiesel production with co-products valorization (CP analysis).

The total results, ROI and pay-back time of all scenarios analyzed for bioethanol production were negative, indicating that the production of microalgae-based bioethanol is not profitable under the considered conditions (Table 25). Exception is observed in values obtained in the SL1 CP scenario; in this case the total results, ROI and pay-back time were positive (Table 25). The economy of scale, similar to biodiesel production, was significant for determining profitable prices only when a small scale of production are considered, while in scaled-up scenarios the microalgae biomass price did not influence the bioethanol production costs (Figure 28).

Technologies used in this study to model bioethanol production showed a high percentage of influence on the final cost of bioethanol. Enzyme and energy consumption were the variable costs that most contributed to the high price of bioethanol in this study (Figure 28). In contrast, the SL1 B analysis showed that algae biomass represented about 5.94 € kg^{-1} of produced bioethanol without co-product valorization, while in SL1 CP analysis the price of biomass per kg of bioethanol decreased to -12.94 € kg^{-1}.

Table 24: Investment of variables cost and capital goods for each scaling scenario to bioethanol production.

Costs	SL1 (€)		SL2 (€)		SL3 (€)	
	B(*)	CP(**)	B(*)	CP(**)	B(*)	CP(**)
Variable costs						
Algae biomass	785,911	- 1,620,363	3,652,173	- 20,387,447	19,878,917	- 220,748,439
Water use	36,438	36,438	145,061	145,061	577,5	577,5
Electricity	269,801	269,801	20,027,973	20,027,973	1,975,120,326	1,975,120,326
Enzyme	427,443	427,443	42,744,331	42,744,331	4,274,433,113	4,274,433,113
Yeast	528	528	52,821	52,821	5,282,059	5,282,059
Wastewater	54,16	54,16	454,32	454,32	11,311,650	11,311,650
Labor LQ	85,72	85,72	341,257	341,257	1,358,567	1,358,567
Labor HQ	4,893	4,893	19,479	19,479	77,546	77,546
Total	1,664,894	-741,379	67,437,415	43,397,794	6,288,039,677	6,047,412,321
Capital goods						
Depreciation	82,968	82,968	1,049,042	1,049,042	32,789,955	32,789,955
Interest	72,464	72,464	1,116,747	1,116,747	37,419,668	37,419,668
Maintenance	19,256	19,256	247,788	247,788	7,799,234	7,799,234
Total	174,687	174,687	2,413,578	2,413,578	78,008,857	78,008,857
Total costs	**1,839,581**	**-566,692**	**69,850,993**	**45,811,372**	**6,366,048,535**	**6,125,421,179**

(*)B – represents the production of biomass without co-products valorization.

(**)CP – represents the biomass production and valorization of co-products.

The fermentation process followed by distillation were the costliest processes in bioethanol production. However, in the SL1 B and CP scenarios the cost of wet ball milling was also considerable (Figure 29).

The final costs of bioethanol, were about 13.90 € kg^{-1}, 5.28 € kg^{-1} and 4.81 € kg^{-1} for B analysis of SL1, SL2 and SL3 scenarios, respectively (Figure 28a and 29a). Moreover, all CP analyzed scenarios showed values corresponding to -4.28 € kg^{-1}, 3.46 € kg^{-1} and 4.63 € kg^{-1} for CP analysis of SL1, SL2 and SL3 scenarios (Fig. 28b; 29b).

Table 25: Financial parameters of the project for each scaling scenario to bioethanol production.

Parameters	SL1		SL2		SL3	
	B(*)	CP(**)	B(*)	CP(**)	B(*)	CP(**)
Total return (€)	54,109	54,109	5,410,896	5,410,896	541,089,619	541,089,619
Total costs (€)	1,839,581	- 566,692	69,850,993	45,811,372	6,366,048,535	6,125,421,179
Total results (€)	- 1,785,472	620,801	- 64,440,097	- 40,400,476	- 5,824,958,915	- 5,584,331,559
Selling price (€)	0.41	0.41	0.41	0.41	0.41	0.41
Cost price (€)	13.90	- 4.28	5.28	3.46	4.81	4.63
Total goods (€)	1,097,932	1,097,932	16,920,414	16,920,414	566,964,663	566,964,663
ROI (%)	-148%	71%	-368%	-226%	-1015%	-973%
Pay-back (year)	-0.7	1.4	-0.3	-0.4	-0.1	-0.1

(*)B – represents the production of biomass without co-products valorization.

(**)CP – represents the biomass production and valorization of co-products.

Regarding the analysis of costs related to each biomass production step, it is possible to confirm that the main bottlenecks are the fermentation and distillation processes, because the cost of enzymes use is still not competitive on the market and because of the high energy consumption during the distillation process. Therefore, further improvements are necessary for a breakthrough in order to allow microalgae-based bioethanol to be a viable alternative to conventional ethanol sources. Biomethane economic assessments were performed for the SL1, SL2 and SL3 scenarios; however, only the B analysis was carried out, because the residual matter remaining after algae biomass co-products valorization was addressed towards silica purification.

The total costs associated with biomethane production can be observed in Table 26. Variable costs are represented by algae biomass and labor costs; while fixed costs are characterized by land costs and price, depreciation and maintenance of capital goods (Table 26).

In this scenario, the algae biomass showed positive values, because the co-products were not used for commercialization. The microalgae-based biomethane project was shown to be unprofitable under the considered conditions. This fact can be confirmed by the negative values for total results, ROI and pay-back time of all scenarios analyzed (Table 27).

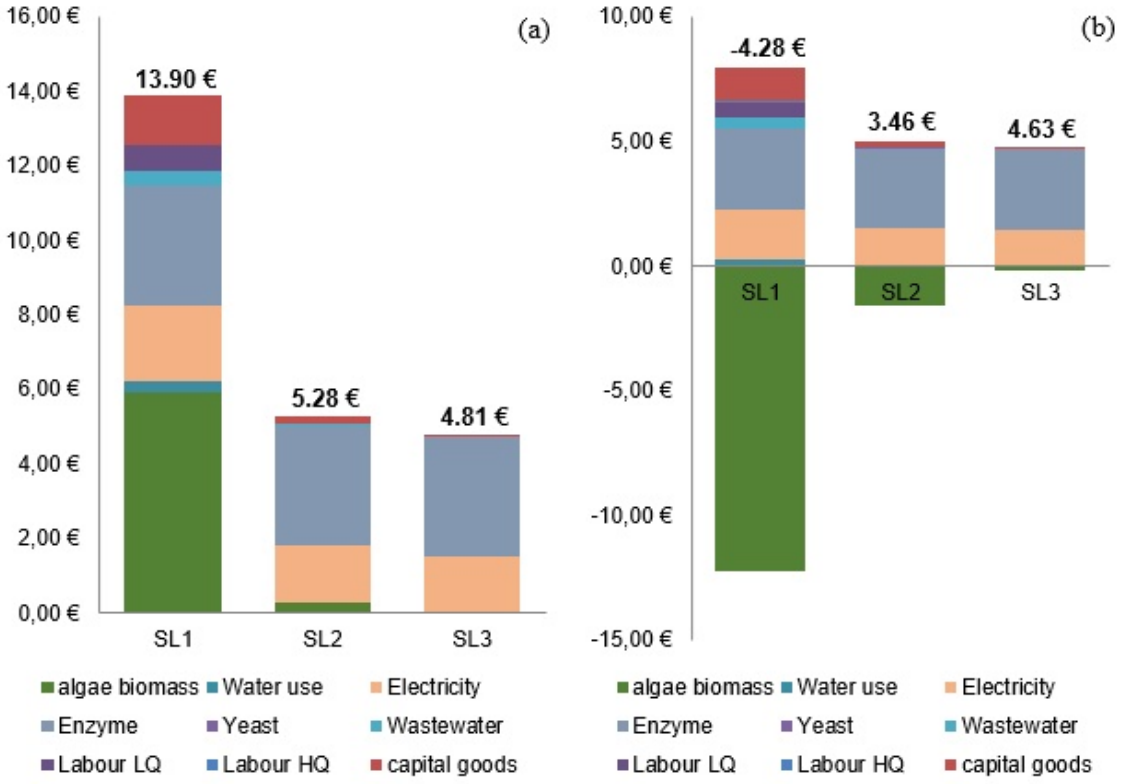


Figure 28: Prices obtained for *P. tricornutum* bioethanol production on SL1, SL2 and SL3 scenarios. (a) Bioethanol production without co-products valorization (B analysis); (b) Bioethanol production with co-products valorization (CP analysis).

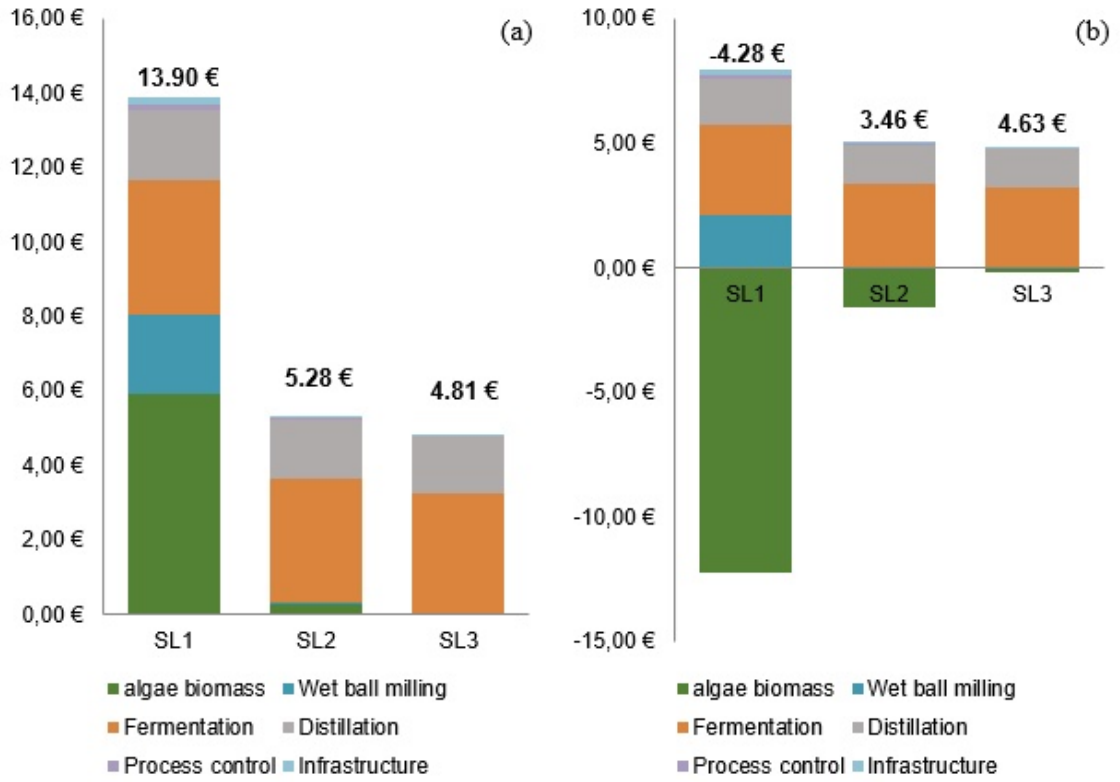


Figure 29: Prices obtained for *P. tricornutum* bioethanol production in the SL1, SL2 and SL3 scenarios and contribution of each process step to the final cost of bioethanol. (a) Bioethanol production without co-product valorization (B analysis); (b) Bioethanol production with co-products valorization (CP analysis).

Table 26: Investment of variable cost and capital goods for each scaling scenario for biomethane production.

Costs	**SL1 (€)**	**SL2 (€)**	**SL3 (€)**
	B(*)	**B(*)**	**B(*)**
Variable costs			
Algae biomass	6,742,944	3,133,486	1,705,568
labor LQ	324,555	324,555	324,555
Total	7,067,499	3,458,041	2,030,123
Capital goods			
Depreciation	560,934	560,934	560,934
Interest	856,825	856,825	856,825
Maintenance	259,644	259,644	259,644
Land	208	208	208
Total	1,677,611	1,677,611	1,677,611
Total costs	**8,745,110**	**5,135,652**	**3,707,734**

(*)B – represents the production of biomass without co-products valorization.

Table 27: Financial parameters of the project for each scaling scenario for biomethane production.

Parameters	SL1	SL2	SL3
	B(*)	B(*)	B(*)
Total return (€)	156,000	156,000	156,000
Total costs (€)	8,745,110	5,135,652	3,707,734
Total results (€)	- 8,589,110	- 4,979,652	- 3,551,734
Selling price (€)	0.30	0.30	0.30
Cost price (€)	16.82	9.88	7.13
Total goods (€)	11,249,335	11,249,335	11,249,335
ROI (%)	-89%	-57%	-44%
Pay-back (year)	-1.6	-3.2	-5.3

(*)B – represents the production of biomass without co-products valorization.

Although the economy of scale was significant for determining a reduction in the cost, it was not sufficient to make this approach feasible (Figure 30). The final cost of biomethane achieved in this study was about 16.82 € kg^{-1}, 9.88 € kg^{-1} and 7.13 € kg^{-1} for B analysis of the SL1, SL2 and SL3 scenarios, respectively (Figure 30).

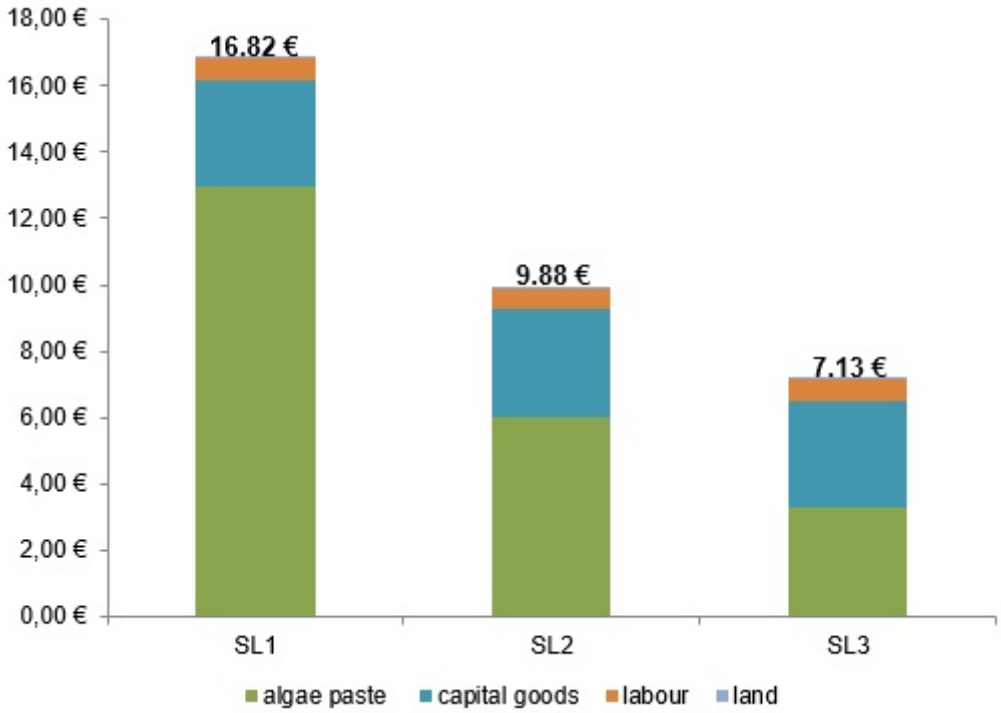


Figure 30: Prices obtained for *P. tricornutum* bioemethane production in the SL1, SL2 and SL3 scenarios.

Finally, the alga paste addressed to biomethane represented the most costly element for production of this biofuel, and it was possible to conclude that using algae biomass for anaerobic digestion in biogas production is not a good alternative for this valuable resource.

6.5 Conclusions

A biorefinery approach for microalgae-based biofuels production after valorization of algae co-products was evaluated in this study. The process data were obtained from a PBR pilot-plant localized under natural conditions in Chile. The model used for scaling-up the production of biomass showed that the valorization of algae co-products is important for improving the net result of the system, making it economically viable. However, it was observed that biodiesel, bioethanol and biomethane production immediately after microalgae biomass production is not economically viable under the conditions considered in this study. Microalgae biomass valorization allowed to the market price of biodiesel and bioethanol to be reduced, but it was not sufficient to make the selling price of these biofuels competitive with diesel fuel and gasoline. The selling price of biomethane production was very high when using algae paste as a raw material for anaerobic digestion, demonstrating the unfeasibility of this resource use. The biorefinery strategy depends on the biochemical composition of the considered strain leading to a different market portfolio. In order to use this approach, it is important to consider the location of the project and the biomass productiveness achieved by the microalgae under those specific natural conditions. Furthermore, it is essential to consider the local reality and prioritize the use of local resources, for a better exploitation of the biomass potentiality and to allocate the components in a better market combination.

Chapter 7

Conclusions and Future Prospects

Several alternatives that minimize or mitigate greenhouse gas emissions have been developed and applied in the productive sector. The feasibility of implementing these technologies has been demonstrated to be directly related to the achievement of efficient use of resources from the technical, economic and environmental points of view, and in a final positive energy balance.

Microalgae have been exploited as an important raw material for industrial applications, targeting the production of pharmaceutical, nutraceutical and chemical products and biofuels. These organisms provide an alternative to traditional crops due to the ease with which they can be cultivated in several environmental conditions and non-conventional sites, such as wastewater, saline water, seawater, freshwater, non-occupied lands, among other unusual crop alternatives. In addition to their versatility, microalgae contribute to energy security because they are a local resource and have the additional advantage of not competing with food and arable land occupation.

The microalgae biochemical potential is a key factor in determining the biotechnological application of each microalga strain. Determination of this potential is related to the local diversity and environmental conditions in which microalgae strains are cultivated. The plasticity of these organisms offers an opportunity to address the biochemical machinery of the cells, which allows a range of bioproducts to be obtained to meet market requirements.

Microalgae-based biofuels have been demonstrated as a good alternative to fossil fuels and conventional-crop biofuels. However, biofuels from microalgae are still facing immature technologies for their large-scale production and commercialization around the world. Therefore, investment in research and development is necessary to produce new insights and strategies to enable the industry to expand and adapt, according to a feasible scenario.

In this study the biochemical composition of *P. tricornutum* biomass was analyzed, cultivated under natural conditions in Chile, in order to address the main compounds for high-value products and bioenergy production. The analysis of lipid composition of

this diatom demonstrated that the FAME profile meets the requirements of international biodiesel standards, and that *P. tricornutum* can be a good alternative for biodiesel production.

On a second plane, the *P. tricornutum* cell wall was analyzed to obtain the biosilica composition and thus to verify the possibility of using the diatom's residual biomass for nanotechnology applications. This analysis revealed that the diatom's biosilica purification increased the Si content, demonstrating that the diatom biomass can be valorized and used for a range of nanotechnology applications which require a highly purified content of Si.

After biochemical characterization of the *P. tricornutum* biomass, it was possible to design three different biorefinery scenarios, targeted to the total valorization of the extractable components of this diatom. A techno-economic analysis was performed of two of the biorefining scenarios proposed, in order to analyze the profitability of applying the circular economy concept to microalgae-based products. The analysis revealed that biofuels from microalgae under the analyzed scenarios are still not feasible, due to the high costs of downstream processing.

Furthermore, developments in cultivation systems are important, since improvements in biomass productivity, features and composition are not only relevant to the upstream processing but also have perceptible effects on the market price of downstream and bioproducts.

The results of this thesis are still being analyzed to perform a Life-cycle assessment (LCA), in order to evaluate the sustainability and environmental impact of algal biofuel, performed under a Chilean scenario. Using LCA tools, the different phases of the productive process can be analyzed to identify environmental and social damage. Moreover, it will be possible to recognize the best routes and technological processes with the lowest environmental impact, according to reduction and higher efficiency in feedstock uses.

Nevertheless, the microalgae-based industry is still facing immature technologies which prevent it from becoming economically feasible in the short term. However, further reductions in production costs depend on improving downstream technologies, solving bottlenecks and, in last instance, on incentive policies to make this approach economically feasible and more widespread.

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Appendix A

Supplementary Materials

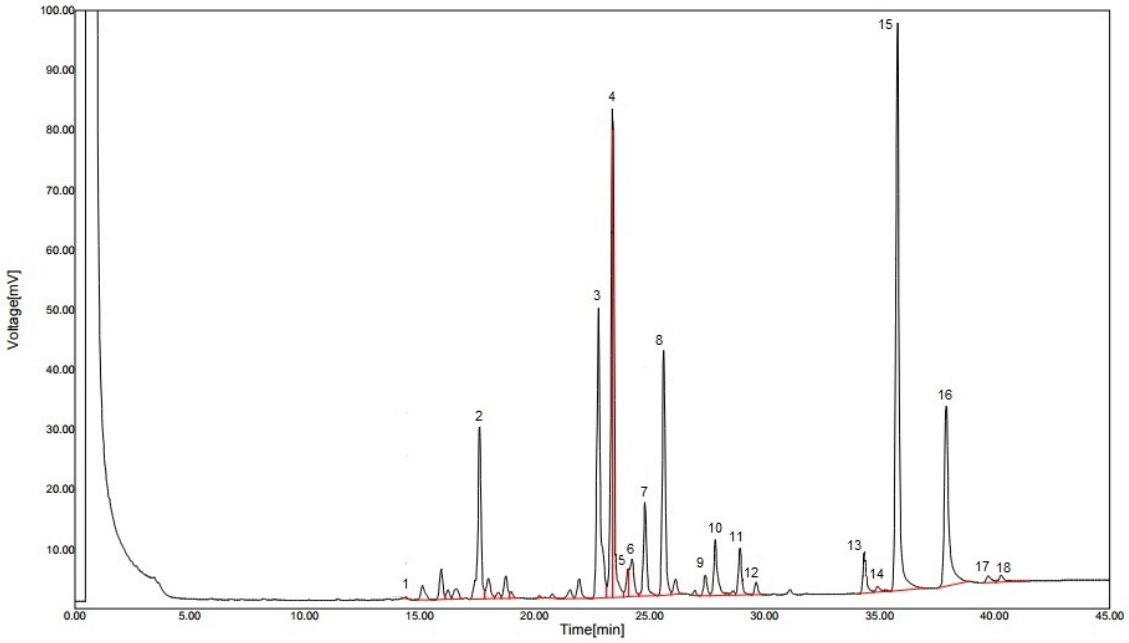

Figure A.1: Gas Chromatography peaks from *P. tricornutum* Fatty Acid Methyl Esters. Numbers indicate Fatty Acid Methyl Esters listed in Table A.1.

Table A.1: Total Fatty Acid Methyl Esters obtained from *P. tricornutum* Gas Chromatography transesterificated oil.

Number	**Fatty Acid Methyl Esters**	**Chain**	**Retention Time (min)**	**Area Vs^{-1}**
1	Methyl Undecanoate	C11:0	14.4000	2.1207
2	Methyl Dodecanoate	C12:0	17.6000	285.7194
3	Methyl Pentadecanoate	C15:0	22.7667	505.5615
4	Methyl Pentadecenoate	C15:1 cis-10	23.4000	429.1050
5	Not identified	- -	24.0500	33.8629
6	Not identified	- -	24.2333	85.9821
7	Methyl Palmitoleate	C16:1 cis-9	24.7833	156.1439
8	Methyl Heptadecanoate	C17:0	25.6000	385.3978
9	Methyl Heptadecenoate	C17:1 cis-10	27.4000	35.8380
10	Methyl Stearate	C18:0	27.8500	101.6849
11	Methyl Oleate	C18:1 cis-9	28.9333	78.9888
12	Methyl Linoleaidate	C18:2 trans-9,12	29.6333	21.3046
13	Methyl Heneicosanoate	C21:0	34.3333	72.5130
14	Methyl Eicosadienoate	C20:2 cis-11,14	34.9000	16.2862
15	Methyl Eicosatrienoate	C20:3 cis-8,11,14	35.7833	948.9099
16	Internal Standard	- -	37.9000	414.7108
17	Methyl Arachidonate	C24:4 cis-5,8,11,14	39.7167	17.1807
18	Methyl Docosadienoate	C22:2 cis-13,16	40.2667	19.4047

Table A.2: Input parameters for biomass drying process to biodiesel production.

Input parameters	**Variables**	**Unit**
Dried biomass	231	ton year^{-1}
Dry matter content	15	wt%
Algae biomass (wet algae paste)	1541	ton year^{-1}
Dry matter content after drying	80	wt%
Water before	1309	ton year^{-1}
Water after	58	ton year^{-1}
Water to be evaporated	1252	ton water year^{-1}
Capacity of dryer	15.7	kg hwater^{-1}
Required energy for evaporation at 100°C	2829664	MJ year^{-1}
Electricity	786018	kWh year^{-1}
Labor LQ	400	h year^{-1}
Labor HQ	12	h year^{-1}
Output (80% DW algae)	289	ton year^{-1}

Table A.3: Input parameters cell disruption by dry milling process to biodiesel production.

Input parameters	**Variables**	**Unit**
Algae biomass (DW)	231	ton $year^{-1}$
Dry matter content	80	wt%
Biomass (undried)	289	ton $year^{-1}$
Capacity mill	36.1	kg h^{-1}
Efficiency disruption	95%	–
Energy use	1.87	kWh kg^{-1} $_{dry\ biomass}$
Energy use	432251	kWh $year^{-1}$
Labor LQ	400	h $year^{-1}$
Labor HQ	12	h $year^{-1}$
Output (processed paste)	289	ton $year^{-1}$
Output (DW processed algae)	231	ton $year^{-1}$

Table A.4: Input parameters for lipid extraction process to biodiesel production.

Input parameters	**Variables**	**Unit**
Algae biomass (DW)	231	ton $year^{-1}$
Dry matter content	80	wt%
Biomass (undried)	289	ton $year^{-1}$
Capacity SC-CO_2 extractor	10.0	L
Void Volume	0.2	–
Amount processed per batch	8.0	kg $batch^{-1}$
Number of batches per year	36118	–
Efficiency extraction lipid	95	%
Lipid content cells	9	%
Energy use	0.8	kWh kg^{-1} $_{dry\ biomass}$
Energy use	231150	kWh $year^{-1}$
Labor LQ	9030	h $year^{-1}$
Labor HQ	12	h $year^{-1}$
Output (lipid)	19763	kg $year^{-1}$
Output (residual biomass dry wt)	211387	kg $year^{-1}$
Flowrate CO_2 in system	10	kg h^{-1}
Amount of CO_2 required per batch	20	kg
Amount CO_2 recycled from each batch	80	%
Amount of CO_2 required per year	144	ton

Table A.5: Input parameters for lipid refining process to biodiesel production.

Input parameters	**Variables**	**Unit**
Mass of lipid material incoming	20	ton $year^{-1}$
Amount of water for wash step	790	kg $year^{-1}$
Amount of 85% phosphoric acid	2.0	kg $year^{-1}$
Amount of 0.1M NaOH	6.1	kg $year^{-1}$
Amount of citric acid	1.0	kg $year^{-1}$
Total volume	20	ton $year^{-1}$
Capacity of mixer/settler process	2.6	kg h^{-1}
Power requirement for heat	88	kJ kg^{-1}
Power consumption heat	1809543	kJ $year^{-1}$
Electric power use heat mixer settler	502.7	kWh $year^{-1}$
Number of hours of material collected/batch	8	h
Capacity vessel	0.0216	m^{-3}
Size of mixer settler	22	L
Power requirement for mixing	5	kw m^{-3}
Residence time	1	h
Energy used for mixing	108	kWh $year^{-1}$
Total energy used	611	kWh $year^{-1}$
Labor LQ	988	h $year^{-1}$
Labor HQ	12	h $year^{-1}$
Waste water (mix of salt, water, lipid)	1.01	m^3 $year^{-1}$
Volume of outgoing refined stream	20.60	m^3 $year^{-1}$

Table A.6: Input parameters for transesterification process to biodiesel production.

Input parameters	**Variables**	**Unit**
Mass incoming stream	20	ton $year^{-1}$
Amount of KOH needed per year	196	kg $year^{-1}$
Amount of methanol needed per year	4248	kg $year^{-1}$
Amount of sulphuric acid for neutralization required	171	kg $year^{-1}$
Amount of water for washing biodiesel	4891	kg $year^{-1}$
Capacity of mixer settler	0.00389	m^3 h^{-1}
Number of hours of material collected/batch	8	h
Capacity vessel	0.0312	m^{-3}
Size mixer settler	31	L
Power requirement for mixing	5	KW m^{-3}
Residence time	1	h
Energy used for mixing	156	kWh $year^{-1}$
Excess MeOH	2124	kg $year^{-1}$
Crude Glycerol Stream - glycerol quantity	1994	kg $year^{-1}$
Biodiesel production - FAME quantity	19260	kg $year^{-1}$
Glycerol output stream	2015	kg $year^{-1}$
Biodiesel output stream	19270	kg $year^{-1}$

Continues on the next page ...

Continuation of Table A.6.

Input parameters	**Variables**	**Unit**
Energy consumption for purification	45	Wh L^{-1} $_{biodiesel}$
Volume of outgoing glycerol stream	1791	L $year^{-1}$
Volume of outgoing biodiesel stream	21913	L $year^{-1}$
Energy used for purification	977	kWh $year^{-1}$
MeOH recovered	1891	kg $year^{-1}$
Waste (water, soaps, salt, MeOH)	5094	kg $year^{-1}$
Labor LQ	1630	h $year^{-1}$
Labor HQ	12	h $year^{-1}$

Table A.7: Input parameters for cell disruption by wet milling process to bioethanol production.

Input parameters	**Variables**	**Unit**
Algae biomass (DW)	231	ton $year^{-1}$
Dry matter content	15	wt%
Biomass (wet paste)	1541001	kg $year^{-1}$
Biomass (wet paste)	193	kg h^{-1}
Biomass (wet paste)	4623	kg day^{-1}
Passes	2	–
Capacity mill	385	kg h^{-1}
Efficiency disruption	95	%
Mill drive	11	kW
Cooling water	1.2	m^3 h^{-1}
Cooling water	9336	m^3 $year^{-1}$
Waste water	9336	m^3 $year^{-1}$
Energy use	11	kWh
Energy use	0.06	kWh kg^{-1} $_{paste}$
Energy use	0.38	kWh kg^{-1} DW
Energy use	88000	kWh $year^{-1}$
Labor LQ	667	h $year^{-1}$
Labor HQ	12	h $year^{-1}$
Output (processed paste)	1541	ton $year^{-1}$
Output (DW processed algae)	231	ton $year^{-1}$

Table A.8: Input parameters for fermentation process to bioethanol production.

Input parameters	**Variables**	**Unit**
Algae biomass (DW)	231	ton $year^{-1}$
Biomass, disrupted cells	219	ton $year^{-1}$
Dry matter content	15	wt%
Biomass (wet paste)	1541	ton $year^{-1}$
Biomass (wet paste)	193	kg h^{-1}
Biomass (wet paste)	4623	kg day^{-1}
Batches per 24 hr	4	
Biomass per batch	1155	kg
Carbohydrate content of dry matter	8	wt%
Percentage of carbohydrate fermentable to ethanol	65	wt%
Percentage of fermented to ethanol	100	wt%
Weight yield ethanol from sugar fermentation	51	wt%
Max ethanol from dry matter	5726	kg $year^{-1}$
Max ethanol concentration	0.4	wt%
Enzymes	3082	L $year^{-1}$
Yeast	2.54	kg $year^{-1}$
Electricity stirrer	2800	kWh $year^{-1}$
Electricity pump	116	kWh $year^{-1}$
Labor LQ	400	h $year^{-1}$
Labor HQ	12	h $year^{-1}$
Output (ethanol + dry biomass)	226	ton $year^{-1}$
Output (ethanol + wet biomass)	1535	ton $year^{-1}$
Output (CO_2)	5479	kg $year^{-1}$

Table A.9: Input parameters for distillation process to bioethanol production.

Input parameters	**Variables**	**Unit**
Input (ethanol + wet biomass)	1535	ton $year^{-1}$
Max ethanol yield	5726	kg $year^{-1}$
Water in biomass	1309851	L $year^{-1}$
Max ethanol concentration	0.4	wt%
Energy requirement to 94% ethanol	44	MJ kg^{-1} $_{ethanol}$
Energy requirement to 94% ethanol	254076	MJ $year^{-1}$
Electricity to 94% ethanol	70577	kWh $year^{-1}$
Wastewater	1309	m^3 $year^{-1}$
Labor LQ	400	h $year^{-1}$
Labor HQ	12	h $year^{-1}$
Output (94% ethanol)	6091	kg $year^{-1}$
Output (wastewater)	1309	ton $year^{-1}$
Output (dry rest)	220	ton $year^{-1}$

Table A.10: Input parameters for biomethane production.

Input parameters	**Variables**	**Unit**
Algae paste (15% dry matter)	0.276	CH_4 m^3 kg^{-1} $_{ODM}$
Methane	0.668	kg m^{-3}
CO_2	1.842	kg m^{-3}
Biogas	1.232	kg m^{-3}
Electric efficiency CHP	36	%
Heat efficiency CHP	60	%
CH_4 content	52	%
CO_2 content	48	%
Electric efficiency CHP	36	%
Labor LQ	8000	h $year^{-1}$
Heating value methane	36	MJ m^{-3}