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Faculty of Applied Sciences

Renato Sano COELHO

Biodiesel production from heterotrophic microalgae

Produção de biodiesel a partir de microalgas heterotróficas

Tese

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Orientador: Prof. Dr. Telma Teixeira Franco
Co-orientador: Prof. Dr. Luuk van der Wielen

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Resumo

Esta tese descreve os resultados da pesquisa de doutorado executada na Universidade de Campinas e na Universidade Técnica de Delft, como parte do program de Doutorado de Dupla Titulação entre as duas universidades. O projeto de pesquisa foi desenvolvido em parceria com a Petrobras S. A., que proveu a maior parte do suporte financeiro assim como suporte técnico, com o objetivo de avaliar o potencial de microalgas heterotróficas para a produção de biocombustíveis.

Microalgas têm gerado muito interesse devido a seu inquestionável potencial para produção de biomassa e lipídeos através de fotossíntese. Nas últimas duas décadas, a busca por novas fontes de bio-energia causou um salto na pesquisa científica sobre cultivo de microalgas, o que impulsionou rapidamente o estado da arte. Apesar disso, a produção em larga escala ainda enfrenta obstáculos significativos, que encarecem os custos de produção and impedem que as microalgas se tornem uma fonte viável de bioenergia. A maior limitação das microalgas autotróficas é a necessidade da luz para o crescimento e o inevitável efeito de auto-sombreamento que ocorre com o aumento populacional. Quando a cultura se torna mais densamente povoada, a luz não consegue atingir camadas mais profundas, consequentemente desacelerando o crescimento. Isto limita a biomassa a baixas concentraçoes e, consequentemente, aumenta os volumes de cultivo e a demanda de grande quantidade de energy para separação da água.

Apesar de extensa bibliografia sobre microalgas ter sido produzida nas últimas duas décadas, apenas uma pequena fração dos estudos se focaram no potencial heterotrófico desses versáteis microorganismos. Microalgas heterotróficas utilizam carbono orgânico como fonte energética e estrutural, ao invés de absorver carbono da atmosfera. Nesta condição, as microalgas podem crescer sem limitações pela luz e alcançar altas concentrações de biomassa e lipídeos. Porém, o cultivo heterotrófico e autotrófico não são comparáveis, já que o primeiro necessita de uma fonte de carbono orgânica e o segundo absorve carbono atmosférico. A tecnologia e os custos associados a cada um dos processos diferem fortemente.

O desenvolvimento do cultivo heterotrófico inicia com a seleção de cepas adequadas para a produção de biocombustíveis e outros produtos de interesse. Este ainda é um campo de pesquisa pouco explorado, já que o cultivo heterotrófico representa apenas uma pequena fração de toda a literatura sobre algas. No capítulo 2, cepas de microalgas foram avaliadas em relação a sua capacidade de crescimento heterotrófico e produção de lipídeos. Após a análise do crescimento e composição celular, potenciais aplicações comerciais foram sugeridas para cada espécie estudada, já que diferentes composições de biomassa e lipídeos podem ser adequadas a diferentes produtos, como combustíveis, alimentos e produtos químicos. *Chlorella vulgaris* CPCC 90 foi identificada como uma opção adequada para a produção de biodiesel devido ao seu alto conteúdo lipídico e alta produtividade. Uma cepa produtora de ácidos graxas omega-3 poliinsaturados foi identificada e um breve estudo de otimização foi conduzido para aumentar a produção do ácido graxo de alto valor agregado.

Após a seleção da cepa mais adequada para a produção de bio-combustíveis, o próximo passo foi o desenvolvimento de um cultivo altamente produtivo. A maior vantagem do cultivo heterotrófico é a possibilidade de alcançar altas concentrações de biomassa e conteúdo lipídico e, consequentemente, maiores produtividades volumétricas. Porém, o acúmulo de lipídeos ocorre quando células de microalgas são expostas a certas condições limitantes, que afetam negativamente o crescimento da biomassa. Desta forma, as condições de cultivo devem ser equilibradas de modo a promover o crescimento da biomassa e aumentar o conteúdo lipídico. Inicialmente, cultivos em batelada alimentada foram avaliados quanto ao acréscimo na concentração de biomassa e teor de lipídeos. A separação do crescimento e acúmulo de lipídeos em dois diferentes estágios permitiu a obtenção de uma cultura altamente concentrada e com elevado teor lipídico. Os lipídeos resultantes foram extraídos da biomassa e convertidos a biodiesel. Os rendimentos totais dos processos de cultivo, extração e reação foram calculados e discutidos (Capítulo 3).

Apesar do cultivo em batelada alimentada ter-se mostrado altamente produtivo, o cultvo contíinuo tem o potencial de reduzir o tempo ocioso da planta e aumentar a produtividade global e, consequentemente, reduzir custos de produção. Porém, manter cultivos contínuos com altas concentrações celulares não é trivial. O equilíbrio entre a vazão específica e a concentração de biomassa é crucial para a manutenção de alta produtividade. Cultivos em batelada alimentada e contínuos foram comparados quanto às produtividades totais, e o efeito da vazão específica sobre a concentração e produtividade de biomassa foi estudado (Capítulo 4). Cultivos contínuous também permitem um melhor controle da qualidade do produto final. A vazão específica e outros parâmetros, tais como a razão de alimentação de Carbono e Nitrogênio, afetam significativamente a composição de biomassa e o perfil de ácidos graxos dos lipídeos intracelulares. Através da variação destes parâmetros sob regime estacionário, tanto o conteúdo lipídico como a composição de ácidos graxos foi afetadas. Através da modelagem destes efeitos, é possível otimizar o processo, de acordo com o produto lipídico desejado (Capítulo 5).

A integração de processos com outros setores da indústria pode, potencialmente, aumentar a viabilidade da produção de biocombustíveis de microalgas. Como o cultivo heterotrófico exige grande disponibilidade de fontes de carbono baratas, a integração com a indústria de cana-de-açícar é uma opção atraente. Existem também potenciais ganhos para a industria da cana-de-açúcar, já que um terço de suas emissões de carbono resulta da queima de grandes quantidades de diesel de origem fóssil em operações agrícolas e de transporte. A produção de biodiesel de microalgas heterotróficas a partir de substratos da cana-de-açúcar representa uma oportunidade de de substituir a utilização de combustível de origem fóssil e aumentar a renovabilidade das refinarias de cana-de-açúcar. No Capítulo 6, é proposto um modelo de integração em que o melaço da cana-de-açúcar, vapor e eletricidade gerados na biorefinaria de cana-de-açúcar são utilizados para a produção de biodiesel de microalgas. Os resultados das simulações mostraram que a viabilidade do modelo proposto depende ainda da maturação da tecnologia, assim como de fatores externos, tais como o preço do petróleo e políticas e incentivos favoráveis a tecnologias sustentáveis.

Esta tese representa uma contribuição ao estado da arte do desenvolvimento de biocombustíveis e outros produtos a partir de microalgas heterotróficas, especificamente focado no uso de culturas com alta densidade celular. Oferece ainda uma visão geral de alguns dos desafios que devem ser superados e das mais importantes variáveis na obtenção de um processo altamente produtivo e economicamente viável.

Abstract

This thesis summarizes the results of a doctoral research executed in the State University of Campinas and in the Technical University of Delft as part of the PhD Dual Degree Program between the two universities. The research project was designed in partnership with Petrobras S. A. (Brazilian Petroleum Corporation), which provided most of the financial support as well as technical cooperation, with the goal of evaluating the potential of heterotrophic microalgae for biofuels production.

Microalgae have generated a lot of interest due to their undoubted potential for the production of biomass and lipids through photosynthesis. In the last two decades, the search for new bio-energy feedstocks created a boom in scientific research on microalgae cultivation, which has improved the state of art of the technology at a rapid pace. However, large scale production still faces significant bottlenecks, which increase manufacturing costs and prevent microalgae from becoming a feasible bioenergy source. The main limitation related to autotrophic microalgae is the need of light for growth and the inevitable self-shading effect with the increase in cell population. As the culture becomes more densely populated, the light cannot reach deeper layers, thus slowing down the growth. This limits biomass to low concentrations and, consequently, increases cultivation volumes and demands high amounts of energy for water separation.

Although extensive research about microalgae has been produced in the last two decades, only a small fraction of the studies aimed at the heterotrophic potential of these versatile microorganisms. Heterotrophic microalgae utilize organic carbon as energy source and building blocks rather than absorbing carbon from the atmosphere. In such circumstances, they can grow without light limitations and achieve high biomass and lipid concentrations. Nevertheless, heterotrophic and autotrophic cultivations are hardly comparable, since the former requires an organic carbon feedstock and the latter absorbs carbon from the atmosphere. The costs associated with each process are remarkably different, as well as the technology involved.

The development of the heterotrophic cultivation process starts with the selection of suitable strains for the production of biofuels and other products. This is still a poorly explored field of research, as heterotrophic cultivation represents only a small fraction of all literature about algae. In Chapter 2, strains of microalgae were evaluated on their capacity for heterotrophic growth and lipid production. After the analysis of growth characteristics and cell composition, potential commercial applications for each strain were suggested, as different biomass and lipid compositions may be suitable for different final products, from biofuels to food and chemicals. *Chlorella vulgaris* CPCC 90 was identified as a suitable option for biodiesel production due to its high lipid content and productivity. One polyunsaturated omega-3 fatty acid producing strain was identified and a short optimization study was performed in order to enhance the production of the high value added fatty acid.

After selection of the most suitable strain for biofuels production, the next step was the development of a highly productive cultivation process. The greatest advantage of heterotrophic cultivation is the possibility of reaching high biomass concentrations and lipid contents and, consequently, high volumetric productivities. However, lipid accumulation occurs when microalgae cells are exposed to certain limiting conditions, which negatively affect biomass growth. Therefore, cultivation conditions must be balanced in order to promote biomass growth and increase lipid content. After identification of the most suitable strain for biofuels production, fed-batch strategies were evaluated as means of increasing biomass concentration and lipid content. Decoupling biomass growth and lipid accumulation in two different stages allowed the production of a highly concentrated culture with increased lipid content. The resulting lipids were extracted from the produced biomass and converted into biodiesel. The overall yields of cultivation, extraction and reaction processes were calculated and discussed (Chapter 3).

Although fed-batch cultivation proved itself highly productive, continuous production can potentially reduce downtime operations and increase global productivity, consequently reducing production costs. Operating

continuous cultivation at high cell concentrations such as in the fed-batch process, however, is not trivial. The balance between dilution rate and biomass concentration is crucial in order to maintain high productivities. Fed-batch and continuous cultures were compared in terms of overall productivities and the effect of dilution rates was evaluated over biomass concentration and productivity (Chapter 4). Continuous cultivation also allows a better control of the final product quality. Growth rates and other parameters, such as Carbon to Nitrogen feeding ratio, significantly affect biomass composition and the fatty acid profile of intracellular lipids. By varying these parameters in steady state cultivation, lipid content and fatty acid composition were affected. By modelling these effects, it is possible to optimize the process according to the desired lipid-based product (Chapter 5).

Process integration with other industry sectors may potentially increase the feasibility of microalgae biofuels production. Since heterotrophic cultivation demands a large availability of cheap carbon feedstocks, integration with the sugarcane industry is an attractive option. There are potential gains for the sugarcane industry as well, since one third of their carbon emissions result from burning large quantities of fossil-based diesel in crops and transportation operations. The production of heterotrophic microalgae biodiesel from sugarcane feedstocks offers the possibility of replacing the fossil fuel utilization and increasing the overall renewability of the sugarcane biorefinery. In Chapter 6, an integration model is proposed in which molasses, steam and electricity of sugarcane biorefinery are used for the production of microalgae biodiesel. Simulation results showed that the feasibility of the proposed model depends on the further development of the technology, as well as on external factors, such as petroleum prices and sustainability-driven policies and incentives.

This thesis represents a contribution to the state of the art on the development of biofuels and other products from heterotrophic microalgae, specifically focused on the use of high cell density cultures. It offers an overview of some of the challenges that need to be overcome and provide insights on the most important variables for achieving a highly productive and economically feasible process.

Sammenvatting

Dit proefschrift geeft een overzicht van de resultaten van een promotieonderzoek uitgevoerd in de State University of Campinas en de Technische Universiteit Delft in het kader van het Dual Degree PhD opleidingsprogramma van de twee universiteiten. Het onderzoek werd uitgevoerd in samenwerking met Petrobras S. A. (Braziliaans Petroleum Corporation), die het grootste deel van de financiële steun bijdroeg, alsmede technische ondersteuning, met als doel het beoordelen van het potentieel van heterotrofe microalgen voor de productie van biobrandstoffen.

Microalgen staan in het middelpunt van de belangstelling vanwege hun potentieel voor de productie van biomassa en lipiden door middel van fotosynthese. In de laatste twee decennia, creëerde het zoeken naar nieuwe bio-energie grondstoffen een hausse in het wetenschappelijk onderzoek naar microalgenteelt, waarbij de stand van de techniek van de technologie in een snel tempo verbeterde. Echter, productie op grote schaal kampt nog steeds met aanzienlijke knelpunten, die de productiekosten verhogen en voorkomen dat microalgen als een haalbare bio-energiebron worden gezien. De belangrijkste beperking met betrekking tot autotrofe microalgen is de behoefte aan licht voor de groei en hetonvermijdelijke zelf -schaduweffect met de toename celpopulatie. Naarmate de biomassaconcentratie in een microalgencultuur stijgt, kan het licht de diepere lagen niet bereiken, waardoor de groei vertraagt. Dit beperkt biomassa om lage concentraties en dientengevolge een grotere teelt volumes en vraagt grote hoeveelheden energie voor water afscheiding.

Veel onderzoek naar microalgen is gepubliceerd in de afgelopen twintig jaar, maar slechts een kleine fractie van de studies om de heterotrofe veelzijdigheidvan deze micro-organismen. Heterotrofe organismen gebruiken organische koolstofbronnen als energiebron en bouwstenen in plaats van koolstof uit de atmosfeer. In dergelijke omstandigheden kunnen microalgen groeien zonder lichtbeperkingen en bereiken een hoge biomassa en lipide-concentraties. Niettemin heterotrofe en autotrofe teelten zijn nauwelijks vergelijkbaar, aangezien de eerste dient een organisch koolstof voedingsmateriaal en deze absorbeert koolstof uit de atmosfeer. De kosten van elk proces en de betrokken technologie zijn aanzienlijk anders.

De ontwikkeling van de heterotrofe kweek begint met de selectie van geschikte stammen voor de productie van geavanceerde biobrandstoffen. Dit is nog een maagdelijk gebied van onderzoek, waarbij heterotrofe kweek slechts een klein deel van alle literatuur omvat. In hoofdstuk 2 werden stammen van microalgen beoordeeld op hun vermogen tot heterotrofe groei en lipide-productie. Na de analyse van de groeikenmerken en celsamenstelling, werden commerciële potentie van elke stam voorgesteld, omdat verschillende samenstellingen van biomassa en lipiden geschikt voor verschillende eindproducten geschikt n zijn, van biobrandstoffen tot voedsel en chemicaliën. Chlorella vulgaris CPCC 90 werd geïdentificeerd als een geschikte optie voor biodieselproductie vanwege het hoge vetgehalte en productiviteit. Een meervoudig onverzadigde omega-3 vetzuur producerende stam werd geïdentificeerd en een korte optimaliseringsonderzoek werd uitgevoerd om de productie van het vetzuur verhogen.

Na selectie van de meest geschikte soort voor de productie van biobrandstoffen, was de volgende stap de ontwikkeling van een zeer productieve teelt. Het grootste voordeel van heterotrofe kweek is de mogelijkheid om een hoge biomassaconcentratie en de lipideophoping te realiseren en dus hoge volumetrische productiviteiten te komen. Echter, lipide ophoping optreedt wanneer microalgen cellen worden blootgesteld aan bepaalde randvoorwaarden die essentieel de biomassagroei verminderen. Daarom moet kweekomstandigheden worden geoptimaliseerd om zowel de groei van biomassa bevorderen en lipidegehalte. Na bepaling van de meest geschikte soort microalgen voor de productie van biobrandstoffen werden fed-batch strategieën geëvalueerd met betrekking tot de concentratie biomassa en het lipidegehalte. Ontkoppeling van biomassagroei en lipide-accumulatie in twee verschillende stadia kon de productie van een hoge celdichtheids cultuur combineren met een verhoogd vetgehalte. De resulterende lipiden werden geëxtraheerd uit de geproduceerde biomassa en omgezet in biodiesel. De totale opbrengsten van de teelt, extractie en reactieprocessen werden berpaald en geevalueerd (hoofdstuk 3).

Hoewel de fed-batch kweek zichzelf bewees voor zeer productieve, kan continue productie de downtime van het process verminderen en van de totale productiviteit verhogen, en daarmee de productiekosten verlagen. Continuecultures bij hoge celconcentratieszijn echter niet triviaal. De balans tussen verdunningsnelheid en biomassaconcentratie is van cruciaal belang om hoge productiviteiten handhaven. Fed-batch en continue culturen werden vergeleken in termen van algemene productiviteiten en het effect van verdunningssnelheidwerd geëvalueerd met betrekking tot biomassaconcentratie en -productiviteit (hoofdstuk 4). Continucultures leiden ook tot een betere controle van de samenstelling en dus kwaliteit van het eindproduct. Groeipercentages en andere parameters, zoals toevoerverhouding van koolstof en stikstof hebben een significante invloed op biomassasamenstelling en het vetzuurprofiel van intracellulaire lipiden. Variatie van deze parameters in steady state teelt op vetgehalte en de vetzuursamenstelling werd in kaart gebracht. Met behulp van modellen van deze effecten is het mogelijk om het proces te optimaliseren met betrekking tot het gewenste lipide gebaseerde product (hoofdstuk 5).

Procesintegratie met andere sectoren kan de haalbaarheid van microalgen biobrandstofproductie door microalgen verhogen. Omdat heterotrofe teelt vraagt om een grote beschikbaarheid van goedkope koolstof grondstoffen, is integratie met de suikerriet-industrie een aantrekkelijke optie. Er zijn potentiële voordelen voor de suikerriet industrie omdat een derde van hun kooldioxide-emissies afkomstig is van het verbranden van grote hoeveelheden fossiele diesel in groei en transport van biomassa. De productie van biodiesel via heterotrofe microalgen uit suikerriet biedt de mogelijkheid tot vervanging van het gebruik van fossiele brandstoffen en een verbetering van de duurzaamheid van de suikerriet bioraffinage. In hoofdstuk 6, wordt zo'n integratiemodel voorgesteld waarbij melasse, stoom en elektriciteit van suikerrietbioraffinaderij gebruikt worden voor de productie van microalgen biodiesel. Simulatieresultaten tonen aan dat de haalbaarheid van het voorgestelde bioraffinagesituatie afhankelijk is van de verdere ontwikkeling van de technologie, maar ook van externe factoren, zoals aardolieprijzen en duurzaamheid gerichte beleid en incentives.

Dit proefschrift vormt een bijdrage aan de technologische ontwikkeling van geavanceerde biobrandstoffen door heterotrofe algen, specifiek gericht op het gebruik van hoge celdichtheidskweken. Het biedt een overzicht van enkele van de uitdagingen die moeten worden overwonnen en geven inzicht in de belangrijkste variabelen voor het bereiken van een productief en economisch haalbaar proces.

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Chapter 1

Introduction and Thesis Outline

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During the 2015 UN Framework Convention on Climate Change (COP21), the international community reached an agreement to take measures to avoid a global temperature increase of 2 ºC above pre-industrial levels [1]. In order to accomplish this goal, it is estimated that between 2011 and 2050 two thirds of the existing fossil fuel reserves must remain unused. If policymakers commit to this goal, future investments in fossil fuel exploration and discovery of new reserves would render unnecessary, as increasing reserves could not lead to increased production [2]. Instead, these resources could be used in the development of renewable energy technologies. In 2015 the total investment in renewable energy reached $286 billion [3]. In comparison, by 2014 the global investment in energy supply from fossil fuels reached over $1 trillion per year, accounting for 70 % of all energy supply investment [4]. This goes to show that the investment level on renewable technologies is likely to increase multiple times in the coming years, if society, governments and corporations commit to mitigate the climate change threat.

The search for alternatives to fossil energy is pushing researchers to identify the most productive technologies and feedstocks for energy, biofuels and chemicals production. Energy can be generated from many renewable resources such as sunlight , wind, municipal and agro-industrial waste streams and landfills (through waste burning or from biogas produced by anaerobic digestion or gasification [5]). However, liquid biofuels used in transportation are still solely produced from crops that yield sugar, starch, lignocellulose or oil (lipids).

According to the United States Energy Information Administration (EIA), in 2012 $1.1 \cdot 10^{11}$ L of biofuels were produced in the world, out of which 77.3 % were ethanol and 22.7 % biodiesel. USA is currently the leading producer of ethanol (61.8 % of global production), followed by Brazil (27.4 %), and also the largest producer of biodiesel (14.8 %), followed by Germany (12.7 %) [6]. Virtually all biofuels are still produced through first generation technologies from traditional agricultural feedstocks. In the USA and Brazil, ethanol production is based largely on corn and sugarcane, respectively, whereas biodiesel is mostly produced from soybeans in America (USA, Brazil and Argentina) and rapeseed in the EU (UN 2015).

The production of biofuels from edible feedstocks became highly controversial in the two last decades, as it was assumed to create competition between food and fuels demand and, ultimately, increase food prices worldwide [7]. From 2001 to 2008, it was suggested that the prices of corn, sugarcane, soybean and rapeseed increased steeply due to the increase in oil prices, which boosted biofuels demand. The most famous example of this suggested effect happened in 2007, when a sudden increase in corn flour prices, the main staple in Mexican diet, led to a food security crisis producing widespread protests around the country [8]. However, other factors non-related to energy production also had strong impact over food prices, such as severe droughts in Australia (2005-2007), slower growth of cereal yields due to low investment during the past 20 years, increased demand on commodity futures markets due to speculation and portfolio diversification; and trade policies, such as export bans and aggressive buying by some governments [9]. Despite all the controversy, recent studies have shown that it is possible to produce bioenergy crops without leading to more food insecurity, provided some conditions are met at policy and project level. These include a) little or no displacement of agricultural land for food production and b) that bioenergy production provide additional employment opportunities and generate income for households [10]. In addition, food security benefits from the use of crop residues or lignocellulosic energy crops for the production of the so-called second generation biofuels.

Second generation biofuels can be produced by a number of technologies and can be classified in thermo-chemical and biochemical processes. Thermochemical technologies are based on conversion of the whole, or part of the biomass feedstocks through high temperatures and pressures with specific catalysts. Gasification, pyrolysis and hydrothermal liquefaction technologies convert the biomass into syngas or bio-oils, which can be converted into biofuels through refining and/or synthesis reactions (e.g. Fischer-Tropsch). Biochemical processes usually refer to

the fermentative production of bioethanol and butanol, but can also include the production of hydrocarbons produced by genetically modified yeast or digestion processes [11, 12].

Second generation ethanol technologies are rapidly evolving and production costs are decreasing, with commercial second generation ethanol plants already in operation [13]. Most of the research and investments on second generation technologies is related to the production of ethanol from lignocellulosic biomass. Lignocellulosic biomass can be seen as roughly a sugar polymer that, once hydrolysed into lignin and cellulose, can be converted into ethanol through well-known fermentation technology. However, ethanol has disadvantages as transportation fuel when compared with hydrocarbon based fuels, such as gasoline. The main issues are the lower volumetric energy density (around two thirds of the energy density of gasoline), which results in reduced vehicle range, and the corrosive nature of alcohol-based fuels, due to their high oxygen content [14]. Ethanol is already added in gasoline up to 15 % in the US and 25 % in Brazil, however only special engines, commonly known as "flex-fuel", can run on higher proportion blends containing up to 85-100 % ethanol. Such engines, which are largely applied in Brazil, use updated materials and linings in order to prevent corrosion [15].

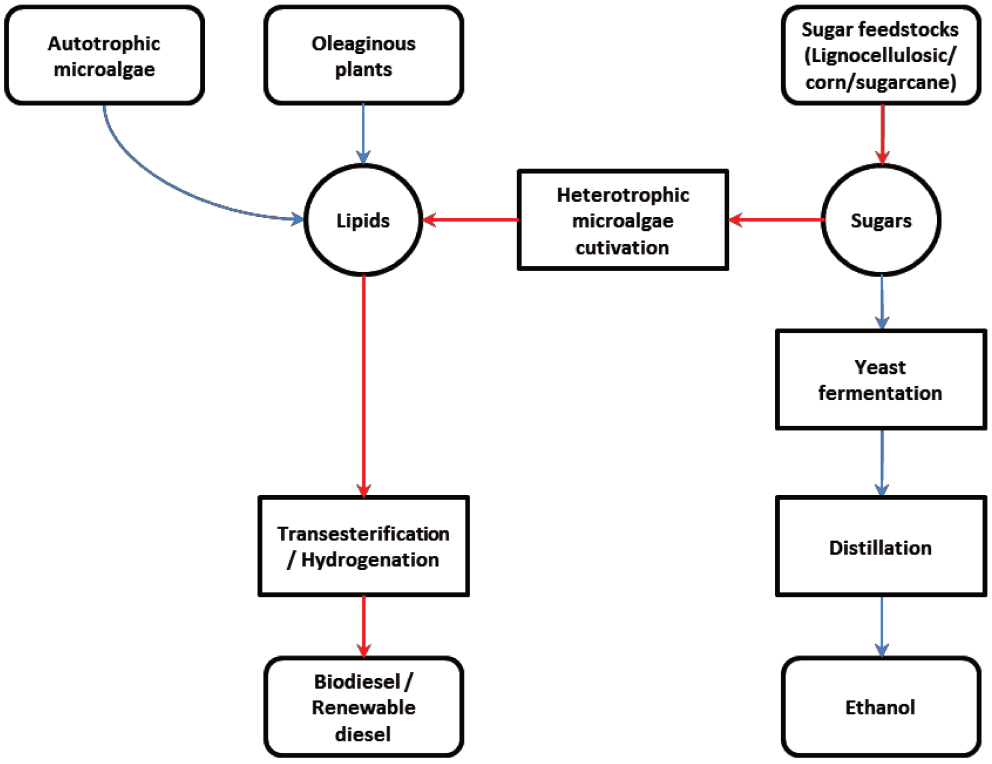


Figure 1. Simplified flow chart of heterotrophic microalgae biodiesel production in comparison with autotrophic microalgae and traditional biofuels production (ethanol and biodiesel)

Biodiesel is the second most produced biofuel in the world. The energy density of biodiesel is approximately 90 % of that of petrol diesel and can be directly used in diesel engines. Presently most biodiesel is produced from oil extracted from food-related oleaginous crops, such as soybean and rapeseed. While these traditional oleaginous crops used for biodiesel production have productivities around 400-1200 L/ha/yr, palm oil can reach up to 6000 L/ha/yr. However, palm is only cultivated in a narrow range around the Equator, which is also the area where most tropical rainforest is located. As consequence, there is a big concern that countries growing palm crops are doing it at the expense of rain forest being destroyed. It is estimated that between 1990 and 2005, 1.7-3.0 million ha of forest were cleared in Indonesia for expansion of oil palm plantations [16]. Thus, there is still a need for new biodiesel feedstocks that avoid pressure on food prices and environmental issues.

Microalgae have long been considered one of the most promising non-food biomass sources, due to early in-vitro studies that showed remarkable growth rates and lipid contents when compared with traditional plant-based crops. However, decades of research have shown that cultivation of algae in large scale is significantly more complex than traditional crops, and this ultimately leads to high costs of production. It is estimated that the costs of microalgae production must be reduced at least one order of magnitude in order to become an economically feasible option for biofuels production, from around €5.00 kg^{-1} dry cell weight (dcw) to around €0.70 kg^{-1} dcw [17, 18]. Costs have dropped considerably in the last years, but it is still uncertain when the technology will proof feasible, as the economic estimates found in literature have a large variability, with costs ranging by a factor of 20 [19].

In the core of the main technological bottlenecks are two key characteristics of microalgae: i) they must be grown in liquid medium and ii) they perform photosynthesis and therefore depend on direct light irradiation. These two characteristics, combined, lead to a simple issue: as the population of microalgae becomes denser, light is blocked by self-shading of cells, up to a point in which growth is no longer possible [20]. This results in low concentrations of biomass in the medium (< 0,1 %) and far lower productivities than the obtained in lab-scale growth, where light limitations are usually much less drastic [19]. Low concentrations of biomass increase upstream costs by increasing the volume and number of reactors needed, and downstream costs by increasing energy demand and equipment scale for biomass separation from the liquid medium.

A different approach on the use of microalgae for the production of biofuels is heterotrophic growth. Only a few species of microalgae can grow in pure heterotrophy, i.e. in total absence of light, and they can do so by using carbon molecules as source of energy and building blocks. The advantage of this approach is that light is not needed and, thus, not limiting. This allows the microalgae to grow up to higher concentrations than in photosynthetic cultures and, consequently, obtain higher volumetric productivities and lower downstream costs. The main disadvantage is the cost of organic carbon sources, which can outweigh the gains in concentration and productivity.

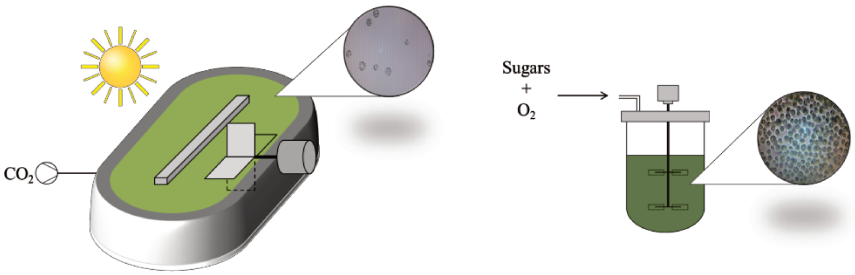


Figure 2 Autotrophic and heterotrophic cultivation schemes. Inside the circles, cell densities are illustrated

Comparing heterotrophic and autotrophic growths may not be a fair approach, as the two modes have potential different roles in the biofuel process chain. While autotrophic microalgae are an alternative to plant feedstocks as source of sugars and lipids from CO2 and light, heterotrophic microalgae have a similar role as other heterotrophic microorganisms in the production of biofuels. In other words, they convert sugar-based feedstocks into a fuel-like substance, like ethanol from yeasts, lipids from oleaginous yeasts, and hydrocarbons from modified microorganisms. Although lipids are not directly usable as fuels for transportation, they are easily converted into fatty acid esters when reacted with an alcohol and a catalyst (either a base or an acid) in a simple reaction, widely applied for biodiesel production, or by hydrogenation technologies, which are already applied in commercial scale by companies such as NESTE Oil and Honeywell UOP [21].

The recent advances in biomass pre-treatment technologies, enzymes production for biomass hydrolysis and costs reduction open up opportunities for other fermentative technologies for biofuel production, other than bioethanol. A low cost fermentable feedstock will improve the feasibility of biofuels from heterotrophic organisms, such as oleaginous yeasts and microalgae. However, the technical and economic feasibility of heterotrophic microalgae is not yet well established, and the characteristics of heterotrophic growth and biomass processing of

microalgae have not been thoroughly investigated. Therefore, the most important process parameters and bottlenecks are not well known.

This thesis is a contribution to the field of microalgae biofuels research, providing an investigation on specific topics on heterotrophic algae cultivation, biodiesel production and the economic aspects of biofuels production from microalgae. In the following chapters, the heterotrophic production of microalgae is evaluated through the selection of producing microorganisms, evaluation of high density fed-batch cultivations, comparison between continuous and batch cultivation, tailoring of lipid production and, finally, a techno-economic evaluation of the process as part of a sugarcane biorefinery in Brazil.

Thesis Outline

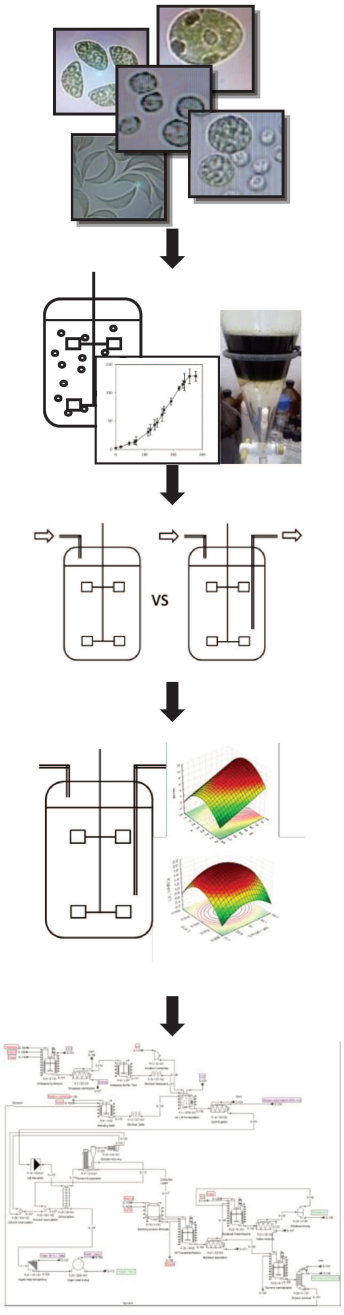
In Chapter 2, we describe a product-oriented prospection of microalgae strains for heterotrophic production. Growth characteristics and biomass compositions are evaluated and potential candidates for biofuels and other bioproducts are identified.

In Chapter 3, biodiesel production from the strain *Chlorella vulgaris* CPCC 90 is evaluated from high cell density cultivation to FAMEs conversion. Yields and product quality are determined and the main bottlenecks of the process are discussed.

In Chapter 4, fed-batch and continuous cultivations are evaluated at high cell densities for biomass and lipid production. The advantages and disadvantages of each process are briefly discussed.

In Chapter 5, the continuous heterotrophic microalgae production of lipids is evaluated. The effect of two major variables over productivities, cell composition and biodiesel quality parameters is determined. Based on the experimental results, empirical models are created for estimation of process parameters and product quality.

In Chapter 6, a process design of the heterotrophic production of microalgae biodiesel is presented. With the primary goal of replacing the diesel presently consumed in a sugarcane biorefinery, a plant with capacity for 20 million liters a year is designed. Technical and economic feasibility of the process is discussed.



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Chapter 2

Product-oriented prospection of heterotrophic microalgae

This chapter will be submitted as:
Coelho, R. S.; Franco, T. T. **Product-oriented prospection of heterotrophic microalgae**
Product-oriented prospection of heterotrophic microalgae

Abstract

The rising recent interest in microalgae production is mainly driven by its promising use as biofuel feedstock, with governments and companies presently investing great amounts of resources in order to achieve this goal. However, biofuels production from microalgae still faces important bottlenecks and years of research will still be necessary for the maturation of the technology. Meanwhile, not only biofuels, but also the production of higher value added products will benefit from the innovations resulting from this trend. The identification of new strains for heterotrophic production of biofuels and other products is a barely explored field of research, as heterotrophic cultivation represents only a small fraction of all literature about algae. In this initial experimental chapter, strains of microalgae are evaluated on their capacity for heterotrophic growth and biomass composition. A product-related analysis is performed to identify the most adequate products from each strain. Chlorella vulgaris CPCC 90 was identified as an suitable option for biodiesel production due to its high lipid content and productivity. One promising EPA (eicosapentaenoic acid, C20:5) producing strain was identified and a short optimization study was performed to enhance the production of the high value added fatty acid, enhancing the EPA productivity by 2.4 fold when compared with the non-optimized situation.

1. Introduction

In recent years, the interest on microalgae as a feedstock for biofuels has dramatically increased, due to the wide acceptance of the role of anthropogenic effects on climate change and ongoing controversy about the use of food/feed products for biofuels production [1]. But despite microalgae have been studied for decades as a potential feedstock for biofuels production, they have not yet been commercially produced for this purpose [2]. They are, in turn, produced for high value added products, such as carotenoids and long chain polyunsaturated fatty acids [3]. Some of the companies with established industrial production of microalgae products are DSM (β-carotene, polyunsaturated fatty acids, DHA and EPA), BASF (β-carotene), Fuji Chemicals (asthaxanthin) and Solazyme (personal care products) [4].

Production costs are still too high for use as biofuels and must be reduced by an order of magnitude to allow feasible production of commodity products. One of the main

reasons affecting production costs are the low concentrations of biomass (0.1 – 1.0 g L^{-1}) achieved in large scale cultures (usually open ponds). Harvesting of the biomass and extraction of products from such diluted cultures is a limiting factor for the feasibility of commercial algal fuels [5].

Heterotrophic cultivation is an alternative approach for reaching high titers of microalgal biomass and lipids [6]. Photoautotrophic cultures can be highly productive under optimal conditions, but are usually limited by the incidence of light and environmental conditions. Heterotrophic cultures, on the other hand, benefit from the potential productivity of microalgae without being affected by light and environmental limitation and, therefore, can reach volumetric productivities more than 10 times higher than in photosynthetic systems [7].

In addition to the high biomass production potential, oleaginous microalgae are amongst the most efficient lipid producing organisms and are able to reach lipid contents around 20-60 % of their dry cell weight [8]. The combination of high biomass productivities obtained in heterotrophic systems with the high potential for oil accumulation results in high lipid productivity. Recently, the best reported results from autotrophic and heterotrophic cultivation were compared and the latter achieved lipid productivities up to 25 times higher [7]. However, only a few species of eukaryotic algae are known to be able to grow in the absence of light by using organic carbon sources for energy and building material (Table 1). The identification, selection and characterization of heterotrophic microalgae is still a rather unexplored field of research and presents a wide range of possibilities.

Table 1. Most commonly reported strain of heterotrophic microalgae

Species	Target product	References
Chlorella ssp.; Auxenochlorella protothecoides	Lipids for biodiesel	[9-11]
Cryptecodinium cohnii	DHA	[12-14]
Galdieria sulphuraria	Phycocyanin	[15-17]
Scenedesmus ssp.	Lipids for biodiesel	[18-20]
Schizochytrium ssp.	DHA	[21-23]

In this study, different wild-type strains of microalgae are evaluated for their heterotrophic growth properties and biomass composition in order to identify potential intracellular products. An analysis of the fatty acids reveals the capacity for biodiesel and polyunsaturated fatty acids production by some strains. In addition a Design-of-experiments strategy approach is applied for optimization of omega-3 oils production by one of the strains.

Statistical design of experiments is an efficient procedure for planning experiments, which consists of predetermined settings of process variables. The use of such optimized planning allows information-rich data collection with a minimum number of runs. The data collected can be analyzed by statistical methods such as multivariate analysis of variance and generate models to describe responses through regression methods along with model residual analysis [24].

2. Material and Methods

2.1 Strains and maintenance

The strains *Chlorella vulgaris* CPCC90 was purchased from the Canadian Phycological Culture Centre. *Chlamydomonas sp.*, *Ankistrodesmus fusiformes* and *Scenedesmus bijugus* were donated by the Department of Botany of Federal University of São Carlos. *Tetraselmis sp., Dunaliella sp.*, *Monoraphidium sp.* and AX1 (unidentified strain) were isolated in the surroundings of the State University of Campinas.

Stock cultures were maintained axenically on synthetic modified Bold's Basal medium with the following composition (mg L^{-1}): Na_2EDTA (50), KOH (3.1), $CaCl_2.2\ H_2O$ (25), $MgSO_4.7H_2O$ (75), K_2HPO_4 (75), KH_2PO4 (175), NaCl (25), MoO_3 (0.71), $Fe_2SO_4.7\ H_2O$ (4.98), H_2SO4 (1 µL/L), H_3BO_3 (11.42), $ZnSO_4.7\ H_2O$ (8.82), $MnCl_2.4\ H_2O$ (1.44), $CuSO_4.5\ H_2O$ (1.57), $Co(NO_3)_2.6\ H_2O$ (0.49) (Stein-Taylor, 1973). Although the Bold's Basal medium does not provide the optimal ratio of nutrients for every strain, it is successfully used for a wide range of green algae [25].

2.2 Screening of heterotrophic microalgae in different carbon sources

Glucose, sucrose, glycerol, xylose and fructose were evaluated as carbon sources for heterotrophic growth of microalgae. The strains were inoculated in petri dishes containing semi-solid modified BBM medium with 15 g L^{-1} agar and supplemented with carbon sources at concentration of 10 g.L^{-1}. NaNO3 was added as nitrogen source as a function of carbon source to achieve the carbon:nitrogen(C:N) ratio of 20 g of carbon per g of nitrogen. Cultures were incubated at 25 ºC in a closed dark incubator and cultures growth was monitored visually every 12 h during 7 days.

2.3 Shake flask assays

Microalgae cultures were cultivated in 250 mL Erlenmeyer shake flasks with 100 mL modified BBM medium. Each flask was supplemented with glucose 10 $g.L^{-1}$. Cultures were incubated at 25 ºC in the dark with agitation rate of 150 rpm. Each flask was inoculated with an initial biomass concentration of 0.3 $g.L^{-1}$. The inoculated flasks were cultivated until all the substrate was consumed. For experimental design runs, the samples were prepared the same way as described above, but cultivated up to 96 h.

2.4 Analytical methods

Cell growth was measured by absorbance reading of cell suspensions at 680 nm wavelength. The biomass concentration ($g.L^{-1}$) was calculated by a previously established calibration curve. For the construction of the calibration curve, biomass dry cell weight was determined gravimetrically by filtering a 5 mL sample of the culture broth through a 0.22 µm porosity membrane and drying of the filter in an oven at 50 °C until constant weight.

Glucose content in the broth was analyzed using a commercial enzymatic glucose-oxidase assay kit by Bioliquid®. Carbohydrate content was analyzed through the phenol sulfuric acid [26]. Protein content was calculated from the total nitrogen content, quantified using a TOC-V CSH total organic carbon analyser (Shimadzu, Japan) equipped with a TNM-1 total nitrogen measuring unit (Shimadzu, Japan), multiplied by 6.35 [27]. For lipid content determination, cells were harvested by centrifugation at 8000 x g for 10 min, frozen at -80 °C and freeze-dried at -30 °C and 50 mmHg. 400 mg of lyophilized biomass were digested in 5 mL of HCl 2 M at 80 °C during 1h. After digestion, extraction process started with the centrifugation (500 g for 2 min) of the digested biomass and addition of 4 mL of methanol, 2 mL of chloroform, and 3.6 mL of distilled water to the biomass pellet. The mixture was agitated (2 min) and centrifuged (500 g for 2 min). The lower phase containing chloroform and lipids was transferred to a test tube. Re-extraction was carried out in upper phase by addition of 4 mL of a 10% v/v methanol:chloroform solution, followed by agitation and centrifugation (500 x g for 2 min). The lower phase was added to the test tube and the chloroform was removed in a rotary vacuum evaporator. The remaining material (total lipids) was left in the oven at 50 °C for 24 h, cooled down to room temperature and weighed.

Fatty acid composition was determined through GC-MS analysis. The crude lipid was initially converted into fatty acid methyl ester (FAME) according to Lewis *et al.* [28] and then analysed using a GC/MS gas chromatography (Shimadzu, Japan) system according to Tapia *et al.* [29]

2.5 Design of experiments for optimization of *A. fusiformes*

Biomass composition analyses showed that *A. fusiformes* was the only strain able to produce eicosapentaenoic acid, a long-chain polyunsaturated omega-3 fatty acid with high commercial value (section 3.3). However, under non-optimized conditions, the strain showed low growth rates and productivities. Although the Bold's Basal medium, utilized as mineral base in this study, is successfully used for a wide range of green algae, it does not provide the optimal ratio of nutrients for every strain [25]. Optimal cultivation conditions such as temperature, pH and C:N ratio are often strain specific and may need optimization. In order to optimize the cultivation conditions of *A. fusiformes*, a central composite experimental design was arranged. Three independent variables were selected: temperature, pH and C:N ratio. Previous studies have shown a significant impact of cultivation pH and temperature in the accumulation of EPA and DHA fatty acids, especially when the microalgae were cultivated below the optimal temperatures for growth [30, 31]. C:N ratio is usually described as one of the most important variables in lipid accumulation for heterotrophic microorganisms[32].

The codified matrix of experiments was designed with the software Statistica 10.0 and is shown in Table 2.

Table 2. Codified matrix of experimental design for optimization of growth and EPA content in A. fusiformes.

Runs	T	pH	C:N
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.68	0	0
10	1.68	0	0
11	0	-1.68	0
12	0	1.68	0
13	0	0	-1.68
14	0	0	1.68
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0

The experimental design matrix is first defined in coded values. In order to translate the coded values to real values, Table 3 shows the correspondence between them.

Table 3. Coded and real values of experimental design for optimization of growth and EPA content in A. fusiformes.

	Levels				
Factors	-1.68	-1	0	+1	+1.68
T	19.3	22	26	30	32.7
pH	6.33	7	8	9	9.7
C:N	3.2	10	20	30	36.8

Temperature range was set to emulate environmental average temperature and fluctuations at the site of isolation of the species. pH range was based on the optimum pH range of most algae species [33] For C:N ratio, approximately 20 was reported as the limit between carbon and nitrogen limitation, therefore it was selected as the central point in the experimental design [34].

2.6 Productivities definition

The productivity of biomass is defined as:

$$Px = \frac{Xf - X_0}{t}$$

Where X_f = final biomass concentration; X_0 = initial biomass concentration; t = time of cultivation

Productivity of EPA is defined as

$$P_{EPA} = Px \times \frac{Lipid}{100} \times \frac{EPA}{100}$$

Where Lipid = lipid content in the biomass, in % dry cell weight (dcw); and EPA = EPA content in the lipid, in % of total fatty acids (FAs).

2.7 Biodiesel quality parameter estimation.

Some of the most important biodiesel quality parameters of fatty acid mixtures can be predicted by empirical models, based on the properties of the individual fatty acids. These models were used to predict the quality of the biodiesel produced using the lipids from the microalgae strains as feedstock. The models utilized in the calculations are described in Table 4.

Table 4. Estimation equation for biodiesel quality parameters from fatty acid composition

Property	Equation	Reference
Cetane Number (CN)	$CN = \sum \frac{X_{ME} \times CN_{ME}}{100}$	[35]
Oxidation stability (OSI)	$OSI = \Sigma X_{Me}\ (wt.\%) \cdot OS_{Me}$	[36]
Cold filter plugging point (CFPP)	$CFPP = 3.1417 \cdot (0.1 \cdot C16{:}0(wt.\%) + 0.5 \cdot C18{:}0\ (wt.\%) + 1 \cdot C20{:}0\ (wt.\%) + 1.5 \cdot C22{:}0\ (wt.\%) + 2 \cdot C24{:}0\ (wt.\%)) - 16.477$	[37]
Lubricity (Lub ws 1.4)	$Lub = \sum \frac{X_{ME} \times Lub_{ME}}{100}$	[35]
Kinematic viscosity (ν)	$\ln(\nu) = \sum \frac{X_{ME} \times ln(\nu_{ME})}{100}$	[38]
Heating value (Hv)	$Hv = \sum \frac{X_{ME} \times Hv_{ME}}{100}$	[37]

X_{Me} (wt.%) is the percentage of the fatty acid alkyl ester in the mixture, CN_{Me} is the measured cetane number of the fatty acids, OS_{Me} is the measured oxidation stability of the fatty acids; D_{Me} is the number of double bonds in each fatty acid; MW is the molecular weight of fatty acids. Lub_{Me} is the measured lubricity of each fatty acid; ln(v) is the logarithm of mixture viscosity; ln(v) is the logarithm of each fatty acids measured mixture viscosity Hv_{Me} is the measured heating values of each fatty acid.

For the calculation of quality parameters of fatty acids mixtures described in Table 4, properties of individual fatty acids were taken from literature and are described in Table 5.

Table 5. Fuel properties of individual fatty acids

Property	Fatty acid									
	C5	C16:0	C16:1	C16:2	C16:3	C17:0	C18:0	C18:1	C18:2	C18:3
CN	17.8	74.5	51	33.1	20.7	87.75	101	59.3	42.2	22.7
OSI (h)	40	24	2.1	0.25	0.07	24	24	2.79	0.94	0.2
Lub ws1.4 (µm)	357	357	246	211	168	339.5	322	290	236	183
ν (mm^2/s)	0.526	4.32	3.67	4.08	2.59	4.53	4.74	4.51	3.27	3.14
Hv (kJ/kg)	28.12	39.47	39.32	39.14	38.86	39.785	40.1	39.93	39.72	39.37

Experimental values taken from Knothe [39], Knothe [40] and Gopinath et al. [41]. CN: cetane number; CFPP: Cold flow plugging point; OSI: oxidative stability index; dH: heating value; Lub: Lubricity; v: kinematic viscosity. See Table 4 for more information.

3. Results

3.1 Screening of heterotrophic microalgae in different carbon sources

The screening of the species started with qualitative growth tests in petri dishes, containing agar medium and varying substrates. The results were described in positive or negative growth after 7 days of cultivation. Only four species were able to grow in heterotrophic cultivation by using the substrates tested. *C. vulgaris* showed more intense and rapid growth on glucose, but surprisingly couldn't use fructose despite it being an glucose isomer. The strains *Tetraselmis sp. Dunaliella sp.* could not grow in any of the substrates. Glycerol was reported as a possible substrate for Chlorella species [42], however the tested strain showed no sign of growth. *Chlamydomonas* sp. grew well on both glucose and fructose, indicating the possible use of inverted sucrose as substrate for this strain. This result is surprising as there has been reported that strains of genus *Chlamydomonas* (e.g. *C. reinhardtii)* could only use two carbon molecules, like acetate, for heterotrophic metabolism. The *Ankistrodesmus fusiformes* strain could also grow well in both glucose and fructose. To our knowledge, the only work that described heterotrophic growth of an *Ankistrodesmus* species was Bollman *et al.* [43]. *Monoraphidium* and *Scenedesmus bijugus* could grow, but at seemingly much slower rates than the other strains. Sucrose and xylose were not consumed by any of the strains.

3.2 Shake flask cultivations

Four strains were selected for shake flask cultivations: *C. vulgaris, Chlamydomonas sp., A. fusiformes* and AX1. *Tetraselmis sp. Dunaliella sp. Monoraphidium sp.* and *Scenedesmus bijugus* were discarded, as they showed very slow or no growth in the qualitative tests. The growth profiles of the selected strains are shown in Figure 1.

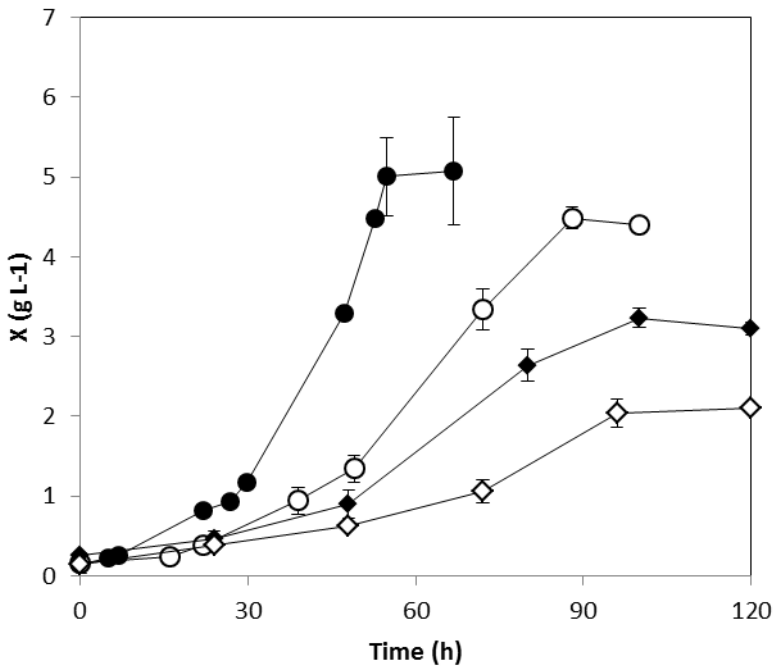

Figure 1. Heterotrophic growth of four selected strains on glucose. ● AX1; ○ C. vulgaris; ♦ Chlamydomonas sp.; ◊ A. fusiformes

Table 6 shows that the growth rates of the fours strains of microalgae greatly differed. Strain AX1 was the fastest growing microalgae reaching 5 g L^{-1} and exhaustion of substrate after 60 hours. Maximum specific growth rate of strain AX1 reached 0.08 h^{-1}, which is comparable to the highest reported value for heterotrophic microalgae

[9]. The culture of the strain AX1 became very viscous at the end of cultivation, suggesting the excretion of a gel-like substance, possibly a polysaccharide. This could be an interesting sub-product of the cultivation, as some microalgal exopolysaccharides have been shown to promote health benefits in humans, such as prevention of cardiovascular disease, anti-oxidant and anti-inflammatory effects [44]. However, at higher cell densities the increase in viscosity of the medium may lead to a decrease in oxygen and mass transfer operations and reduce final productivities [23]. The second highest μ_{max} strain was observed for *Chlorella vulgaris* (0.04 h^{-1}), followed by *Chlamydomonas sp.* (0.03 h^{-1}) and *A. fusiformes* (0.02 h^{-1}).

Table 6. Results of shake flask cultivation of selected heterotrophic microalgae.

Carbon Source	X_{max} ($g.L^{-1}$)	μ_{max} (h^{-1})	Yx/s (g/g)	P_x ($g.L^{-1}.d^{-1}$)
C. vulgaris	4.6 ± 0.2	0.04 ± 0.01	0,49 ± 0.02	1.2 ± 0.1
Chlamydomonas sp.	3.4 ± 0.4	0.03 ± 0.01	0,63 ± 0.01	0.7 ± 0.1
A. fusiformes	2.2 ± 0.3	0.02 ± 0.01	0,52 ± 0.01	0.4 ± 0.1
AX1	5.1 ± 0.7	0.08 ± 0.01	0.51 ± 0.01	2.0 ± 0.1

Maximum cell density: X_{max}, ($g.L^{-1}$); maximum specific growth rate: μ_{max} (h^{-1}); yield of cells on substrate: $Y_{X/S}$; cell productivity: $P_x = \Delta X/\Delta t$ ($g.L^{-1}.d^{-1}$).

The final biomass concentration for *C. vulgaris* and AX1 were not significantly different (4-5 g L^{-1}), while *Chlamydomonas sp.* and *A. fusiformes* produced a lower amount of biomass (2-3 g L^{-1}). It must be stressed that neither the culture medium nor the cultivation conditions were optimized for any species.

3.3 Biomass composition

The biomass composition of the four selected strains was evaluated in terms of the macronutrients protein, carbohydrates and lipids (figure 2). Ash content was not determined and thus not considered in the biomass composition analysis. Ash content in microalgae produced in closed reactors is usually around 4-6 % of dry cell weight [45]. Although the growth conditions were not set to induce any specific product production, it is important to compare the strains on their lipid and carbohydrate accumulation capacity in growth favourable conditions. Lipid and carbohydrate synthesis are known to be enhanced during stress conditions that may decrease growth rates and total productivity [46]. Therefore, it is important to identify species that can produce lipids while maintaining active growth. Some species of microorganisms have the capacity of accumulating high contents of neutral lipids, usually triacylglycerols, and therefore are called oleaginous [47]. Sometimes an arbitrary limit of 20% (m/m) is used to classify the microorganisms as such [48]. Not all algae have the capacity of accumulating high contents of TAGs and their lipids are composed mostly by structural lipids such as glyco- and phospholipids and sterols, all of which have structural functions in the cell membrane [49].

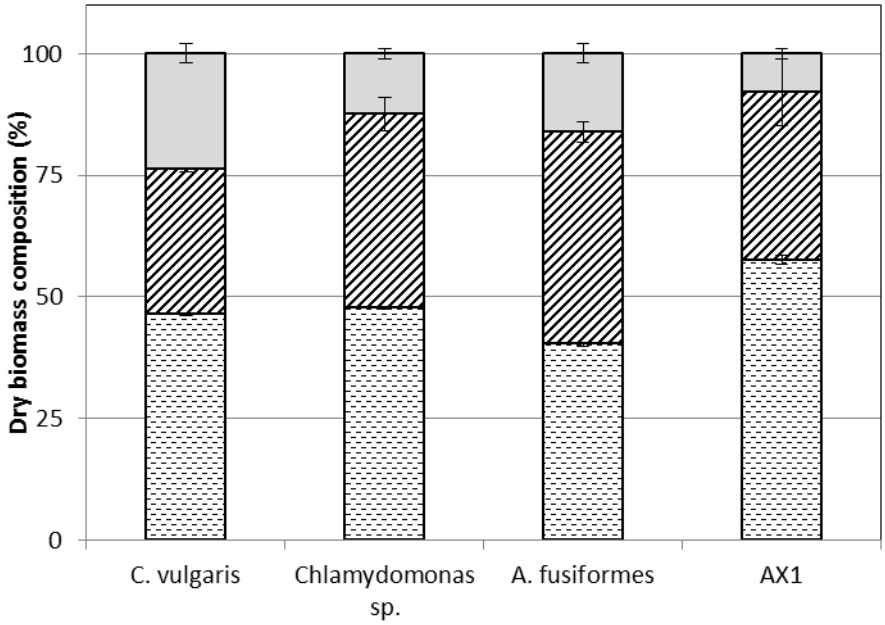

Figure 2. Biomass composition of selected strains considering only macronutrients (ashes not accounted). ▭ *Lipids;* ▨ *Carbohydrates;* ▭ *Proteins*

The results show that *Chlorella vulgaris* was the only strain that could accumulate more than 20% (23 %) of its weight in the form of lipids, followed by *A. fusiformes* (14 %), Chlamydomonas (11 %) and AX1 with only 9 %. Although AX1 reached the fastest growth rate and biomass productivity, it had the lowest lipid content of the four strains. C*hlorella vulgaris* also reached the highest lipid productivity, followed by AX1, *Chlamydomonas sp.* and *A. fusiformes*. The carbohydrate content was not significantly different among the different strains (35-39 %), with the exception of *C. vulgaris* (28 %). Some strains of microalgae tend to accumulate carbohydrates rather than lipids as carbon reserve, and significant differences in the lipid/carbohydrate accumulation pattern are observed even between strains of the same genus [50]. The protein content in AX1 was significantly higher than in the other strains. The high growth rates and protein content found in this strain make it a promising candidate for protein production for human and animal nutrition.

3.4 Fatty acid composition

The industrial application of lipids is highly dependent on its fatty acid composition, as these can influence the chemical and physical properties of the final products [40]. In biodiesel production, the trans-esterification reaction does not modify the fatty acid basic structure and thus the biodiesel is directly related to the fatty acid composition of the feedstock [51]. Therefore, the analysis of the fatty acid composition (Figure 3) in the lipids can indicate what applications are more suitable for each feedstock.

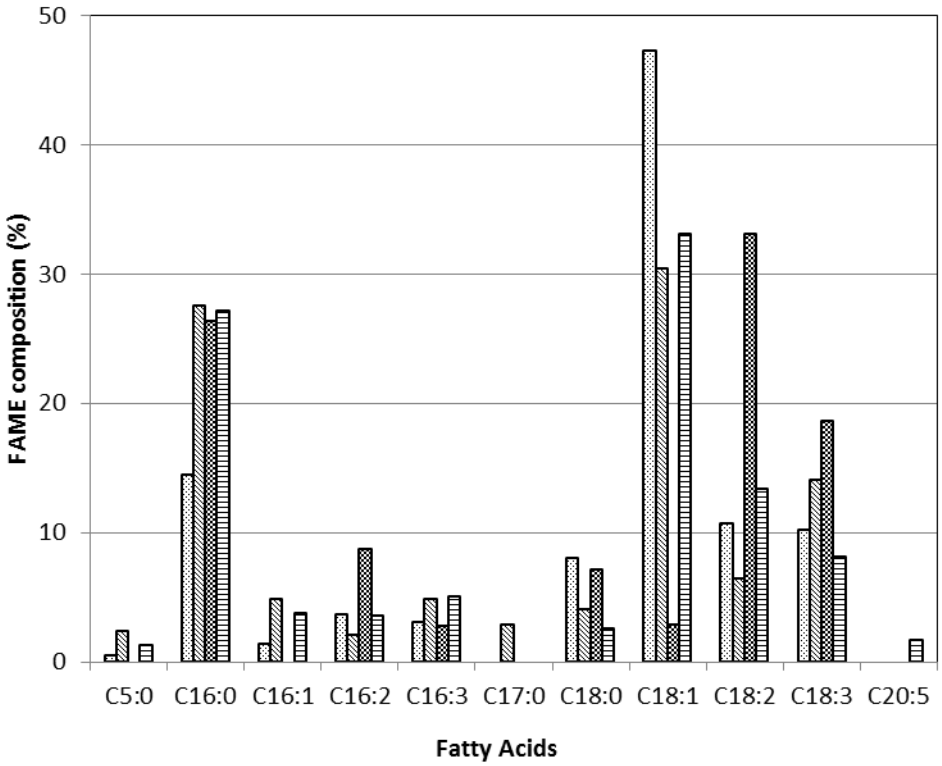

Figure 3. Fatty acid composition of four selected strains. Chlorella vulgaris; Chlamydomonas sp.; AX1; A. fusiformes.

The fatty acid composition of the selected microalgae showed that *Chlorella vulgaris* and *Chlamydomonas sp.* strains had similar composition with the major fatty acid being oleic acid (C18:1). *Chlamydomonas sp*, however, had a higher content (27.6 %) of saturated fatty acids, mainly palmitic acid (C16:0), against 14.5 % in *Chlorella vulgaris.* The fatty acids found in the lipids of *A. fusiformes* had a similar composition to C*hlamydomonas sp.*, however it is worth noting that the *A. fusiformes* strain was the only one to produce eicosapentaenoic acid (C20:5) or EPA. Many clinical studies have shown health benefits from long chain polyunsaturated fatty acids, especially EPA and DHA (C22:6). These two fatty acids are consumed in large quantities worldwide and fish oil is presently their major source [52]. The isolation of microorganisms able to produce these high value fatty acids could greatly improve productivity, as their production could then take place in controlled optimized bioreactors. In addition, recent projections show that the annual demand for fish oil will exceed the current global supply in a few years, which means that new sources of long chain polyunsaturated fatty acids must be developed [53]. The concentrations found in fish oil vary from 10-20 % of the total fatty acids [54], which is approximately 5 to 10 times the amount detected in *A. fusiformes,* however optimization of the cultivation conditions may lead to improvements in its fatty acid content. The final productivity of EPA of *A. fusiformes* was calculated as 0.75 mg L^{-1} d^{-1}.

Table 7. Fatty acid classes of lipids from selected strains

Strains	Fatty acid classes (% total fatty acids)				
	SFA	MUFA	PUFA	ω-3	EPA
C. vulgaris	23,1	48,7	27,9	13,4	0
Chlamydomonas	34,2	35,4	27,6	19,0	0
A. fusiformes	31,2	37,0	32,0	15,0	1,7
AX1	33,6	2,9	63,5	21,5	0

SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ω-3: omega-3 fatty acids; EPA: eicosapentaenoic acid (C20:5)

The fatty acid composition of the strain AX1 differed from the other strains for having a low content of C18:1 (3 %) and instead a high content of C18:2 (32 %). The polyunsaturated fatty acids accounted for 64 % of the total fatty acids and saturated fatty acids for 34 % (Table 7), approximately. This is a very unique fatty acid composition, as oleic acid is usually one of the major components of the fatty acid pool in most microalgae [55]. From an extensive review of literature it was reported that only one out of twelve families of microalgae contained less than 10 % of monounsaturated fatty acids on average, namely Porphyridiophyceae. The families Prasinophyceae, Chlorophyceae and Dinophyceae contained less than 20 % of monounsaturated fatty acids and more than 50 % of polyunsaturated fatty acids [51]. Due to its specificity, these data could help in the process of identification of the isolated strain AX1. In comparison with commercial vegetable oils, AX1 fatty acid composition was similar to soybean oil, which contains circa 35 % of linoleic acid (C18:2) [8]. The content of ω-3 fatty acids in AX1 was also found to be the highest among the four strains, however it was mainly composed by C16:3 and C18:3. Although C18:3 (linolenic acid) is an essential ω-3 fatty acid, its market value is not as high as EPA and DHA since it can be found in vegetable oils, unlike the two longer chain fatty acids. However, the highly unsaturated fatty acid composition combined with its high protein content reinforce the possibility of using this algae for animal feed production [56].

Although microalgae have been cultivated for more than 50 years for human and animal nutrition and high value added products [57], biofuel production is arguably the subject of most of the recent algae-related literature, due to the uncertainty in future energy prices and advantages over plant feedstocks [58]. Governments and the private sector are investing heavily on the development of algae-based biofuels, including the oil and gas industry, whose investments in algae research reached around $ 1 billion in recent years [1].

As mentioned before, the quality of the biodiesel is directly related to the fatty acid composition of the feedstock. In general ways, the unsaturation degree, chain length and branching of the chain are the main structural features of fatty acids that influence the fuel properties of the corresponding fatty acid esters (biodiesel) [59]. These quality properties are influenced in a different and, sometimes, antagonist way. For instance, the cetane number, a measure of the ignition quality of the biodiesel, is reported to be enhanced with low unsaturation degrees and longer chain lengths, while the cold filter plugging point (CFPP), an important measure for operation at low temperatures, benefits from high unsaturation degrees and shorter chain lengths [41]. Thus, the ideal fatty acid composition for a high quality biodiesel depends on the balance of these properties. Other important properties that can be predicted from previously established models are heating value, defined as the energy liberated by the combustion of the biodiesel; Lubricity, which is the ability to reduce friction between moving parts of the engine; and kinematic viscosity, which affects atomization of the fuel for the injection in the combustion chamber [35, 41]

In Table 8, the estimated values of some of the most important biodiesel (fatty acid esters) quality parameters for the four strains are portrayed.

Table 8 estimated biodiesel properties from selected strains of microalgae

Strains	Estimated biodiesel properties					
	v (mm^2/s)	Hv (kJ/kg)	OSI (h)	CN	Lub ws1.4 (µm)	CFPP (ºC)
C. vulgaris	4,1	**39,6**	7,1	**55,7**	271,3	0,8
Chlamydomonas	3,9	39,3	**10,3**	**55,7**	286,1	1,4
A. fusiformes	4,0	39,5	9,0	55,0	**288,0**	**- 3,9**
AX1	3,7	39,5	8,5	49,5	255,4	3,1
ASTM D6751-08	1.9-6.0	-	3 (min)	47 (min)	**-**	-
EN 14214/14213	3.5-5.0	35 (min)	6 (min)	51 (min)	**-**	5 (máx)

Regarding the limits established in the standards ASTM D6751-08 and EN 14214/14213, the estimated properties of the four strains were compliant. These estimates, however, may not be an accurate representation of the real behavior of the fuel produced from the microalgae lipids, as contaminants usually found in biodiesel may change dramatically the quality properties [60]. The estimated biodiesel from *Chlorella vulgaris* had the highest Cetane numbers and heating value, as well as a low cold filter plugging point, however *Chlamydomonas sp*. and *A. fusiformes.* had more balanced profiles, with equivalent Cetane numbers and heating values, lower CFPP, higher oxidation stability (OSI) and higher lubricity. *Chlamydomonas sp.* had a slight higher OSI and cetane number, while *A. fusiformes* had lower CFPP and higher lubricity. AX 1 had the worst predicted performance for biodiesel quality, with the lowest CN, lowest lubricity and highest CFPP of all strains. This result is due to the unique fatty acid composition observed for AX1, with high contents of saturated and polyunsaturated fatty acids, both of which may decrease the quality profile of the biodiesel at high concentrations.

3.5 Summary of results

Based on the experimental results, a summary of the observations for each strain is described in Table 9.

Table 9. Summary of results and observations for selected strains

	C. vulgaris	*Chlamydomonas sp.*	*A. fusiformes*	AX1
Growth in different substrates	Glucose	Glucose / Fructose	Glucose / Fructose	Glucose
Growth rates	Medium growth rate	Medium growth rate	Low growth rate	High growth rate
Biomass composition	High Lipid content	Low lipid content	Low protein content	High protein and carbohydrate content (possible polysaccharides)
Fatty acid composition	High MUFA content	High SFA content	EPA producer	High ω-3
Potential products	Biodiesel	Protein / Biodiesel	EPA / Animal feed (aquaculture)	Human and animal feed / polysaccharides

The qualitative growth tests in different substrates showed that none of the strains tested could utilize low cost waste substrates, such as xylose (co-product of lignocellulosic hydrolysis) or glycerol (co-product of biodiesel production). *Chlamydomonas sp.* and *A. fusiformes* could utilize fructose, which creates the possibility of using hydrolyzed sucrose or molasses, both abundant in Brazil. The cultivation test showed that the newly isolated strain AX1 can reach growth rates as high as the fastest reported for heterotrophic microalgae. The second fastest strain in the test was *C. vulgaris,* which was two times slower than AX1. *A. fusiformes* was the slowest growing strain, which could be the result of non-optimized cultivation conditions or a specific nutritional deficiency for this strain.

Regarding the biomass composition of the strains, only Chlorella surpassed the mark of 20 % (m/m) lipid content while the other strains reached between 9-14 %. The fast growing AX1 showed a high protein content, which shows that this strain was very efficient in utilizing the carbon source for cell division. The fatty acid composition analysis showed that all four strains could be adequate feedstocks for biodiesel, as the predicted biodiesel quality parameters were compliant with international standards limits. However, AX1 singular fatty acid composition, formed by saturated and polyunsaturated FAs, had the worst predicted performance between the fours strains. *A. fusiformes* was the only strain that produced the long chain polyunsaturated fatty acid EPA, which has a high market value. The EPA content, however, is much lower as the one found in fish oil, the present major commercial source. Since growth conditions were not optimized, growth rates and EPA content can probably be enhanced through nutrient and abiotic conditions optimization.

Despite displaying the highest growth rate of all strains tested, given its low lipid content and fatty acid composition the strain AX1 is probably not a good option for biodiesel production. Instead, the production of protein rich biomass for human and animal feed seems to be the best option. Aquaculture could be an interesting target market as the availability of fishmeal is likely to decrease in the coming years and the need of a protein and PUFA rich biomass opens up a market opportunity [61].

The high lipid content, relatively high growth rates and fatty acid characteristics make *Chlorella vulgaris* a better option for biodiesel production. For biofuels, profit margins are usually very tight [62] and, therefore, a high

volumetric productivity as well as a high product concentration are key for reducing upstream and downstream costs [63].

3.6 Optimization of EPA production of *A. fusiformes*

In the previous experiments, an EPA producing strain was identified. The initial EPA content detected in *A. fusiformes* was much lower than the observed for its present commercial sources, and the growth rates of this strain were the lowest amongst the strains tested. Since the cultivation tests were not performed at optimal conditions, an experimental design was assembled as a tool for identifying optimal culture conditions for higher biomass and EPA productivity. The results are described in Table 10.

The productivity of EPA depends on three different responses: the biomass productivity of the microalgae, the lipid content in the microalgae biomass, and the EPA content in the total lipids. However inter-connected, these responses are affected in different ways by cultivation conditions. For this reason, they were also considered in the experimental design assessment as responses in the effect analysis. The inclusion of these extra responses may help understand the processes by which EPA productivity is affected. In addition, increasing the EPA content in the total lipids may be just as, or even more, important that increasing EPA overall productivity, as it determines the quality and value of the extracted oil [64].

Table 10. Results of experimental design for optimization of EPA production from A. fusiformes.

Runs	T (°C)	pH	C:N (g_C / g_N)	Pmax (g L^{-1} h^{-1})	Lipids (% dcw)	EPA (% total FA)	P_{EPA} (mg L^{-1} d^{-1})
1	22	7	10	0,10 ± 0.02	7.2 ± 0.7	2.38 ± 0.02	0.17 ± 0.04
2	30	7	10	0,43 ± 0.01	10.3 ± 0.8	1.74 ± 0.04	0.77 ± 0.01
3	22	9	10	0,09 ± 0.01	6.6 ± 0.8	2.83 ± 0.06	0.16 ± 0.02
4	30	9	10	0,47 ± 0.01	7.6 ± 0.6	1.82 ± 0.05	0.65 ± 0.05
5	22	7	30	0,13 ± 0.01	7.3 ± 0.9	2.51 ± 0.05	0.24 ± 0.01
6	30	7	30	0,55 ± 0.01	7.9 ± 0.7	3.19 ± 0.05	1.4 ± 0.1
7	22	9	30	0,10 ± 0.01	8.7 ± 0.7	2.07 ± 0.02	0.17 ± 0.02
8	30	9	30	0,27 ± 0.01	11.8 ± 0.7	1.74 ± 0.02	0.56 ± 0.03
9	19.3	8	20	0,05 ±0.01	8.1 ± 0.7	2.47 ± 0.02	0.092 ± 0.005
10	32.7	8	20	0,46 ± 0.05	9.6 ± 0.9	3.06 ± 0.06	1.34 ± 0.02
11	26	6.3	20	0,31 ± .01	8.8 ± 0.7	2.27 ± 0.05	0.62 ± 0.04
12	26	9.7	20	0,19 ± 0.04	9.0 ± 0.8	2.56 ± 0.05	0.4 ± 0.1
13	26	8	3.2	0,13 ± 0.03	9.1 ± 0.6	2.62 ± 0.03	0.31 ± 0.02
14	26	8	36.8	0,20 ± 0.03	12.5 ± 0.7	1.87 ± 0.03	0.47 ± 0.01
15	26	8	20	0,20 ± 0.03	7.5 ± 0.7	3.0 ± 0.1	0.50 ± 0.01
16	26	8	20	0,19 ± 0.03	4.6 ± 0.7	2.82 ± 0.06	0.43 ± 0.01
17	26	8	20	0,19 ± 0.03	7.8 ± 0.7	2.91 ± 0.02	0.48 ± 0.01
18	26	8	20	0,10 ± 0.03	9.1 ± 0.7	2.7 ± 0.1	0.17 ± 0.04

The maximum content of EPA (3.19 % of total fatty acids) was obtained in run 6 (Table 11), which combined high temperature and C:N and neutral pH. Highest Px was observed in runs 2, 4, 6 and 10, which were cultivated at temperatures 30 or 32.7 ºC. Highest lipid content was observed at run 14, which was cultivated at the highest C:N level (36.8). Despite not having the highest lipid content, run 6 had the highest EPA productivity due to the combination of high biomass productivity and high EPA content in the lipids.

Table 11. Effect analysis of responses of experimental design for optimization of EPA production from A. fusiformes

	P_x		Lipid content		EPA in Lipid		P_{EPA}	
Factors	Effect	p-value	Effect	p-value	Effect	p-value	Effect	p-value
T	**0,293**	**2,88E-05**	**1.504**	**0.056**	-0.045	0.850	**0.001**	**0.000**
T^2	0,057	0,135	0.002	0.998	-0.261	0.335	**0.000**	**0.070**
pH	**-0,070**	**0,055**	0.333	0.627	-0.128	0.595	**0.000**	**0.018**
pH^2	0,056	0,138	0.053	0.944	**-0.509**	**0.084**	0.000	0.809
C:N	0,007	0,833	**1.403**	**0.070**	-0.076	0.749	0.000	0.113
$C:N^2$	0,006	0,861	**1.377**	**0.098**	**-0.629**	**0.042**	0.000	0.427
T x pH	-0,046	0,283	0.102	0.909	-0.345	0.288	**0.000**	**0.034**
T x C:N	-0,028	0,512	-0.109	0.902	0.500	0.139	0.000	0.208
pH x C:N	**-0,085**	**0,072**	**2.148**	**0.041**	**-0.605**	**0.083**	**0.000**	**0.053**

The analysis of effects (Table 11) confirms that biomass productivity was positively affected by higher cultivation temperatures and lower pH with, at least, 90 % confidence level. For the Lipid content, the first and second order factors of C:N had positive and significant effect, as well as the interaction factor between pH and C:N, which suggests a synergistic relation between the two variables. Lipid content is usually benefited by higher C:N ratios, as biomass growth is limited by nitrogen concentration, shifting the absorbed carbon surplus to lipid accumulation [65]. Recently, it was shown that the increase of pH combined with nitrogen starvation enhanced lipid yields in *Neochloris oleoabundans* [66]. Hence, the positive effect of the interaction factor between pH and C:N ration suggests that a similar process happened to *A. fusiformes.*

EPA content in microalgae lipids was only affected by pH and C:N variation. The second order factors for pH and C:N and the interaction factor between them were negative and statistically significant at 90 % confidence level. In our experiments, the effect of temperature on the content of EPA in the lipids was not statistically significant in the range tested, although many studies have shown that cultivation at lower temperatures may increase the unsaturation level of the fatty acids [50]. However, in most of these studies the applied temperatures were lower as those used in this study, e.g., *P. tricornutum* EPA production was enhanced at 15 ºC [30], *Thalassiosira weissflogii* at 16 ºC [67] and the diatom *Nitzschia laevis* at 18 ºC [68]. It is possible that the cultivation of *A. fusiformes* at lower temperatures could yield higher contents of EPA in the microalgae lipid fraction, however the negative effect of lower temperatures on biomass growth would probably reduce the overall productivity of EPA.

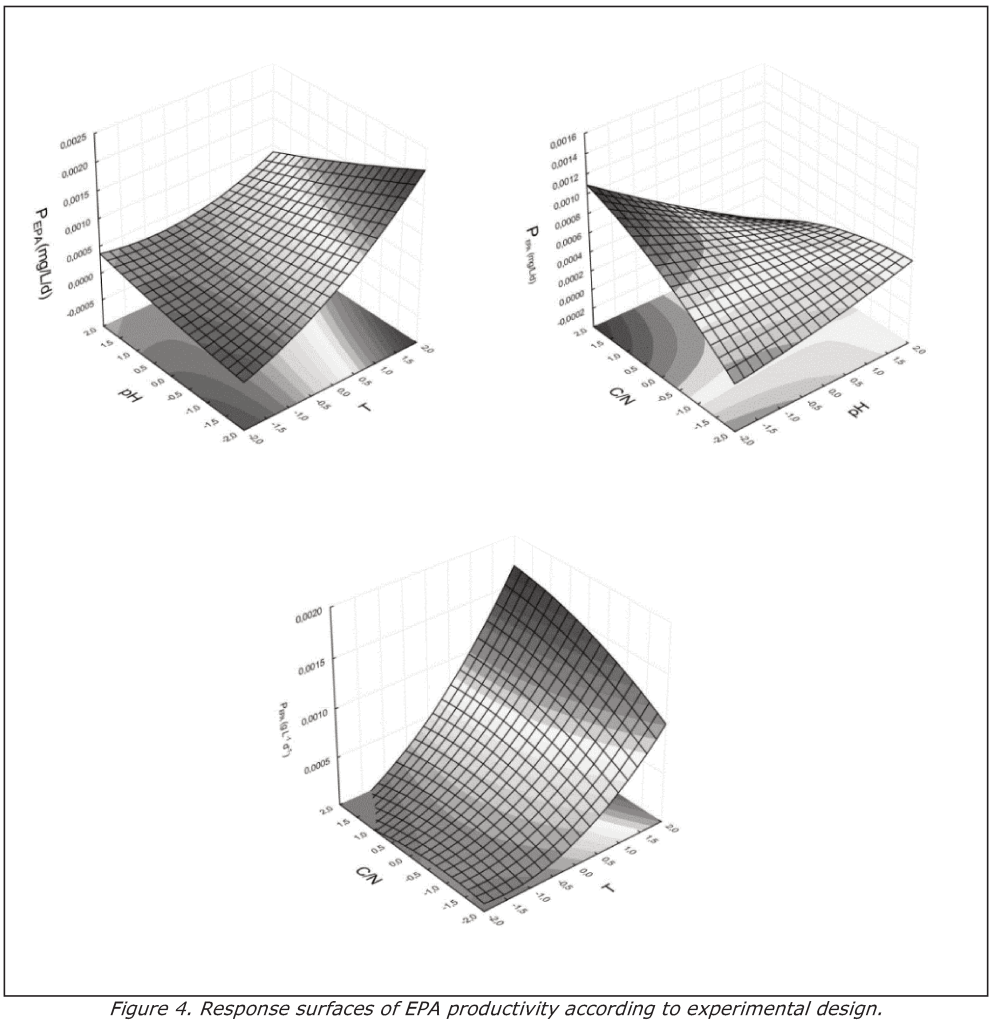

Figure 4. Response surfaces of EPA productivity according to experimental design.

For the EPA productivity (figure 4), the first and second order factor of temperature had significant positive effects, probably due to the effect on the biomass productivities. Also, two interaction effects were significant :Temperature x pH, as well as pH x C:N ratio had negative significant effects over EPA productivity. It is very difficult to estimate the nature of these interactions, as they can be result of many different mechanistic reactions, however synergistic or antagonistic interactions effects between temperature and pH are very often described in biochemical processes [69].

Regression analysis of the experimental design matrix generated the following equation (only statistically significant factors at 90 % confidence level were considered):

$$P_{EPA} = 0.00046 + 0.00035 \times T + 0.00008 \times T^2 - 0.0001 \times pH - 0.0001 \times T \times pH - 0.0001 \times pH \times C/N$$

In order to test the validity of this model, analysis of variance (ANOVA) was performed over the experimental design matrix. The results of the analysis of the regression and Lack of Fit variances are described in Table 12.

Table 12. Analysis of Variance (ANOVA) of experimental design for optimization of EPA production from A. fusiformes.

Source	SS	df	MS	F	$F_{0.05}$	p-value
Regression	2,01E-06	5	4,02E-07	24,6	3,20	1,25E-05
Residue	1,79E-07	11	1,63E-08			
Lack of Fit	1,76E-07	9	1,96E-08	12,9	19,4	0,0739
Pure Error	3,03E-09	2	1,52E-09			
Total variation	2,19E-06	16				

The results of the Analysis of Variance show that the regression was valid at 95 % confidence, while Lack of Fit was not significant at 95 % confidence level. These results confirm the validity of the model in describe the experimental design results.

3.6.1 Verification of model prediction.

The solution of the model for the maximum productivity was calculated within the tested range as T = 32.7 ºC; pH = 6.3 and C:N = 36.8, resulting in maximal estimated EPA? productivity of 2.02 mg L^{-1} d^{-1}. A validation run was performed in triplicate yielding the final EPA productivity of 1.81 ± 0.07 mg L^{-1} d^{-1}, which was 89 % of the estimated value. The final productivity was 2.4 fold the obtained in the non-optimized condition (0.75 mg L^{-1} d^{-1}) . The final biomass productivity (0.63 ± 0.05 g L^{-1} d^{-1}) and lipid content (13 ± 1 %) were the highest obtained, however the EPA content was lower than the observed in some of the runs of the experimental design. As discussed in the effect analysis, the significant factors for the EPA content were different than for EPA productivity, therefore the optimal conditions for each response are also different.

The content of EPA may determine the commercial target for the microalgae production. For the commercialization of EPA rich oil, the EPA content and productivity from *A. fusiformes* are still far from the observed in commercial production. However, while a high EPA/DHA content is adequate for a substitute of fish oil for human consumption, a lower EPA content combined with a high protein content may be a good source of nutrition for aquaculture, as fish tend to concentrate the EPA and DHA oils from the ingestion of microalgae [70].

4. Conclusion

The different aspects of growth, biomass composition and fatty acid profile evaluated in this study allowed a preliminary prospection of different strains regarding their capacity for heterotrophic growth and identification of commercial potential according to their characteristics. Chlorella vulgaris CPCC 90 was identified as the strain with the highest potential for biodiesel production, due to its adequate fatty acid composition and high lipid content. The unidentified strain AX1 has shown promising growth rate and biomass productivity, but its low lipid content limits its commercial applications. However, it may be a promising protein producer for animal and human nutrition. *A. fusiformes* was identified as an EPA producing strain and an experimental design was performed for the enhancement of EPA production. The optimization succeeded in increasing the EPA productivity 2.4 fold the obtained in non-optimized conditions. The combination of high protein content, presence of EPA and heterotrophic capacity suggest that *A. fusiformes* may be a promising candidate as aquaculture feed. This study have shown that simple preliminary tests may be used as prospection tools for new commercially interesting microalgae strains.

5. Acknowledgements

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Chapter 3

Biodiesel production from high cell density cultures of heterotrophic microalgae

This chapter will be submitted as:
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Biodiesel production from high cell density cultures of heterotrophic microalgae

Abstract

Microalgae are currently being studied widely for biofuels production, due to their relatively high growth rates and capacity for accumulating lipids. However, low biomass concentrations and volumetric productivities are still a major bottleneck for microalgae biodiesel to become feasible. A few microalgae can grow under pure heterotrophic conditions without depending on light, and thus can reach higher biomass concentrations. In this study we offer an evaluation of biodiesel production from heterotrophic microalgae, from cultivation to lipid esterification, by assessing yields and discussing specific aspects and bottlenecks of each stage. First we tested the capacity of strain *C. vulgaris* CPCC 90 to grow up to high cell densities under heterotrophic conditions. A two stage cultivation strategy was adopted to decouple biomass growth and lipid accumulation, resulting in final biomass and lipid productivities of 7.3 ± 0.3 % (m/m) and 2.0 ± 0.3 g L^{-1} d^{-1}. The yield of lipid on substrate was 0.14 $g_{Lipids}/g_{glucose}$. Although cell densities up to 77 g/L were obtained, the results suggested a growth inhibition effect, even when nutrient and oxygen limitation were avoided. The produced lipids were extracted from wet biomass after acid hydrolysis with recovery yield of 89 % of the total lipids. The lipids were then converted to fatty acid methyl esters (FAMEs) in a two stage reaction (acid esterification and alkaline trans-esterification) reaching final conversion yield of 77 % of FAMEs from the extracted lipids.

1. Introduction

Scientists and policy makers agree that the global temperature rise caused by greenhouse effect must be addressed by the immediate reduction in fossil fuels consumption [1]. Biofuels are considered an alternative to reduce carbon emissions associated with liquid transportation fuels, however a number of studies have raised concerns that the indirect land use of crop-based biofuels may endanger ecosystems and result in little or no reduction in GHG emissions [2]. New biofuel technologies such as biodiesel produced from microbial fatty acids were shown to potentially produce less carbon emissions than traditional biofuels, such as sugarcane ethanol and soybean biodiesel [3].

Microalgae are promising feedstocks for the production of microbial biodiesel, however most of the research on the topic is concentrated on photosynthetic growth, which is limited by light and usually yields low concentrations of biomass resulting in high harvesting costs [4]. Heterotrophic microalgae are organisms capable of growing without performing photosynthesis by using organic carbon as source of energy and building blocks. Some of these organisms can also accumulate part of the absorbed carbon in the form of fatty acids which can later be converted to biodiesel. Since light is not limiting in this process, higher biomass concentrations and volumetric productivities can be achieved, potentially reducing cultivation volumes and harvesting costs. However, the need of an organic feedstock rather than utilizing light and carbon dioxide is an important trade-off [5]. In order to increase the economic feasibility of the process, biomass and lipid production must be optimized; however, lipid accumulation usually occurs under stress conditions that limit growth, thus causing a trade-off between biomass growth and lipid synthesis [6]. In order to circumvent this issue and maximise lipid productivity, biomass growth and lipid accumulation can be decoupled in different process stages, which can be separately optimised leading to overall higher productivities [7].

Cultures with high biomass concentration can improve productivity and reduce costs by decreasing equipment size, residual liquids and energy requirements for pumping and downstream processes. [8, 9]. The cultivation of biomass with high concentrations of bacteria and yeast is well established and limits for maximum possible concentrations have been observed (Kleman & Strohl, 1994). Cultures of bacteria and yeast can reach

biomass concentrations of up to 200 g L^{-1}, above which loss of fluidity occurs , which interferes in the transfer processes of nutrients and oxygen (Bunch, 1994; Shiloach & Fass, 2005). For microalgae, however, few studies have reached similar concentrations, since most of the literature consists of light limited photosynthetic growth. (Bumbak et al., 2011; Perez-Garcia et al., 2011). A few studies have achieved significant high concentrations of biomass in heterotrophic systems, e.g. Siegler *et al.* [10], which reached biomass concentration of 144 g L^{-1}, yet the mechanisms that limit biomass concentrations in microalgae are not well understood, as not many studies focused on obtaining high cell densities. Some examples of studies that reached high biomass concentrations through different strategies are shown in Table 1.

Table 1. Examples of high biomass concentration obtained in heterotrophic microalgae cultivation

Final dry biomass concentration (g L^{-1})	Lipid productivity (g L^{-1} d^{-1})	Microalgae strain	Process characteristics	Reference
24.01	1.19	*C. protothecoides*	Fed-batch, pulsed feeding with sugarcane bagasse hydrolysate	[11]
103.8	4.2	*C. sorokiniana* UTEX 1602	Fed-batch, two stage pulsed feeding (nitrogen limitation)	[12]
51.7	*n.a.*	*Crypthecodinium cohnii* CCMP 316	Fed-batch, Single use reactor; constant feeding with manually adjusted flow	[13]
65.1	4.3	*C. protothecoides* UTEX 25	Fed-batch, Glycerol as carbon source; pulsed feeding in two stages	[14]
144	8.1	*Auxenochlorella protothecoides* UTEX 25	Fed-batch, adaptive, non-linear model based strategy for separate nitrogen and carbon sources feeding	[10]
117	2.8	*Chlorella vulgaris* BEIJ., 1996/H 14	Fed-batch, pulsed feeding	[15]
47	2.06	*C. protothecoides* UTEX 256	Fed-batch, pulsed feedings every 24 h	[16]
70.6	n.a	*C. pyrenoidosa* 15-2070	Fed-batch, exponential feeding in multiple stages	[17]

n.a.: non-available data

Chlorella vulgaris is one of the most commercially used species, due to its high growth rates and productivities and capacity of growing in selective environments,[11-13]. Few studies have reported the heterotrophic capacity of *C. vulgaris* to grow up to high biomass concentrations. In fact, only one study reported reaching high cell density (117 g L^{-1}) through heterotrophic cultivation (Table 1).

In chapter 1, the strain *C. vulgaris* CPCC 90 was identified as a promising lipid producer through heterotrophic cultivation. In this study, we provide an evaluation of the complete process of microalgae biodiesel production, from cultivation to FAMEs conversion from *C. vulgaris* CPCC 90. First, we evaluate the capacity of the strain for growing up to high cell densities for lipids and biodiesel production. Growth limitations are discussed and a two stage strategy is used to decouple biomass growth and lipid accumulation. In the downstream process, lipid extraction from wet biomass is applied, followed by a two stage conversion process of free fatty acids and triacylglycerols into FAMES.

The overall process for obtaining biodiesel from microalgae is portrayed in Figure 1. Microalgae are first cultivated in a closed bioreactor up to high cell concentration and lipid content. The culture broth is then centrifuged

for harvesting of biomass, resulting in a stream of microalgae paste with 20-30 % solids and a stream of water containing the spent culture medium. The biomass paste is then hydrolyzed at 121ºC with H_2SO_4 for 30 min for cell rupture and release of cell-bound lipids[14]. For the extraction of lipids, apolar solvent is added to the hydrolyzed biomass and the mixture is agitated in order to enhance mass transfer. Organic and aqueous phase separation occurs spontaneously by gravity or assisted by centrifugation, heating and/or addition of de-emulsifiers. The organic phase containing lipids and solvent is evaporated under vacuum for solvent removal (and recycled for new extractions) and the remaining lipids are sent to a reactor for conversion into biodiesel. If the extracted lipids have high acid value, a pre-treatment stage is necessary in order to reduce acidity of the oil before alkaline transesterification. Hence, H_2SO_4 as catalyst and methanoll are added to esterify the FFAs and the mixture is reacted at 70 ºC for 2 h. This pre-esterification step results in fatty acid esters from the conversion of the FFAs, water, excess alcohol, spent catalyst and non-reacted acyl-glycerols (tri, di and mono). For the transesterification reaction, sodium hydroxide and methanol are added and the mixture is reacted again at 70 ºC for 1 h. The products of the alkaline transesterification reaction are fatty acid esters, glycerol, excess methanol and excess NaOH. Phase separation occurs spontaneously or assisted by centrifugation between an upper phase containing the fatty acid esters and a lower phase containing excess methanol, glycerol, water, and sodium hydroxide. The esters are separated by gravity and washed with water for removal of impurities.

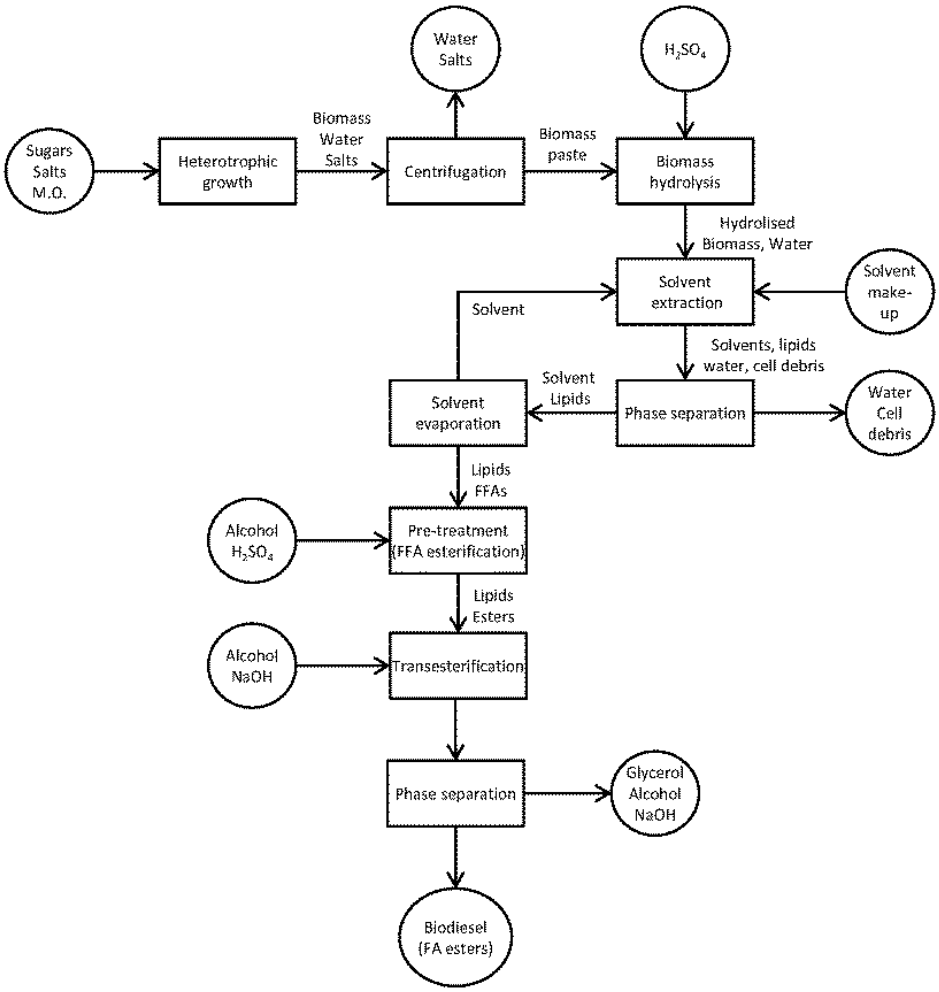

Figure 1. Flow diagram of biodiesel production from heterotrophic microalgae

2. Materials and Methods

2.1 Strain description and maintenance

The *Chlorella vulgaris* CPCC90 strain was obtained from Canadian Phycological Culture Centre. Stock cultures were maintained axenically on synthetic modified BBM medium with the following composition (mg L^{-1}): Na_2EDTA (50), KOH (3.1), $CaCl_2.2H_2O$ (25), $MgSO_4.7H_2O$ (75), K_2HPO_4 (75), KH_2PO_4 (175), NaCl (25), MoO_3 (0.71), $Fe_2SO_4.7H_2O$ (4.98), H_2SO_4 (1 µL/L), H_3BO_3 (11.42), $ZnSO_4.7H_2O$ (8.82), $MnCl_2.4H_2O$ (1.44), $CuSO_4.5H_2O$ (1.57), $Co(NO_3)_2.6H_2O$ (0.49) [15]. Glucose was added as carbon source at concentration of 10 $g.L^{-1}$ and $NaNO_3$ was added as nitrogen source at 1.21 g L^{-1} (C/N ratio of 20). Initial pH was adjusted at 6.8. All culture media was sterilized for 15 min at 121 °C.

2.2 Fed-batch cultivations

To evaluate its capacity for reaching high cell densities, *C. vulgaris* CPCC 90 was cultivated in fed-batch cultures. Different cultivation regimes were tested, in which nutrient and oxygen limitations were avoided through feeding and aeration strategies. Pulsed and feedback-activated feeding of medium were applied in order to provide constant nutrient availability, while a cascade stirring system ensured the availability of excess oxygen in all experiments.

Microalgae inoculum was cultivated in 250 mL shake flasks containing 100 mL of the same culture medium described in 2.1. The shake flasks were inoculated with 5 mL of the maintenance culture and incubated for 3 days at 26 ºC and 150 rpm agitation. The final biomass concentration in the inoculum was measured as described in 2.5 and the pre-culture was added to the bioreactors. The inoculation volume was calculated as the sufficient amount for an initial biomass concentration of 2 g L^{-1} in the bioreactors.

Fed-batch cultivation batches were carried out in a 3 L bioreactor (BioFlo 115 - New Brunswick Scientific-Edison N.J. U.S.A.) with blanketed glass vessel of 2 L maximum working volume, equipped with pH (range 2-14) and DO (PID controlled polarographic sensor, with range 0-200%) probes. Feeding medium was prepared with the same composition described in section 2.1, but 30 times more concentrated (all components). For nitrogen starved cultivation, $NaNO_3$ was not added to the feeding medium. The experiments were carried out at 26 ºC, aeration rate of 1 vvm (volume of gas per volume of culture per minute), in the dark and at initial agitation rate of 150 rpm. Whenever DO (dissolved oxygen concentration) decreased below 20 % of saturation, agitation rate increased in a cascade system up to 600 rpm (Biocommand® software was used for cultivation data processing and record as well as for cascade loop control). Although some microalgae were found to be sensitive to shear stress, strains of *Chlorella* can usually tolerate stirring rates of up to 800 rpm without losing cell viability [16]. Each experiment was performed in duplicate. All batches started with initial volume of 1.2 L. The end of the cultivations experiments was determined when the OD (optical density) of the culture did not increase after 12 h.

2.2.1 DO-stat cultivation

In the first fed batch experiments, a DO-Stat feeding system was used. DO-stat cultures are fed-batch cultivations in which feeding of medium is controlled by a DO feedback-activated system. Whenever DO level increases above a pre-determined set-point (indicating glucose depletion), feeding of fresh medium is activated. The aerobic metabolism of the fresh substrate causes a sudden decrease of DO level, after which feeding stops [17]. This way, DO-stat allows the maintenance of the DO level around the set-point, even at high cell densities, by controlling the nutrient feed rate [18]. In addition, only small quantities of substrate are added each time, reducing the risk of overfeeding and consequent substrate inhibition [19]. DO-stat fed-batch is a useful method for achieving high cell density cultures without the need of extensive tailoring of the process, and is frequently used in industrial processes, from recombinant protein vaccines to biofuels production [18, 20]. One of the possible disadvantages of using DO-stat methods is low reproducibility [21], since feeding profile is slightly different for every batch. It was also described elsewhere, that even though DO-stat cultivation works well in defined media, it often fails in media supplemented with complex nutrients such as yeast extract or peptone, because amino acid catabolism can support cellular maintenance and respiration, keeping DO levels low even during carbon source limitation/depletion [22]. An important issue during DO-stat cultivation is that the increase in cell density may result in decreasing growth rates due to the fluctuation in substrate level. The high substrate rate increase the frequency of feeding pulses and the amount of time under glucose depletion [23].

DO-stat fed-batch cultivations for biomass accumulation initiated with culture medium composition described in 2.1. After glucose depletion, feedback assisted feeding started and the loop feeding was kept until the end of biomass growth. The system was set to trigger feeding every time DO increased over 30 % of saturation.

2.2.2 Pulsed feeding fed-batch

Even though DO-stat cultivation is designed to provide nutrients on-demand and indefinitely, it is by definition nutrient limited, since feeding is only activated in case of substrate depletion. In other words, growth of the microorganism is not completely unrestrained and depends on the feedback response time of the feeding system. That condition could lead to low growth rate and lower productivity when compared with feeding strategies in which nutrient limitation is avoided. To test this hypothesis, a manually activated pulsed fed-batch was also evaluated. In this second feeding strategy, large pulses of nutrients were added to the culture whenever off-line substrate analyses indicated carbon source concentration decrease below a pre-determined level, but never reaching exhaustion. The objective behind this experiment was to create a condition of unrestrained growth, i.e., without substrate limitation. Feeding pulses were added to the reactor whenever glucose concentration was detected at or below 10 g L^{-1}. The feeding volume for each pulse was calculated as the necessary volume to increase glucose concentration up to 40 g L^{-1}, as previous tests have shown that no substrate inhibition effects were observed for glucose concentrations up to 40 g L^{-1} (Annex I). Feeding volume was calculated according to the following formula:

$$V_{F\,(L)} = \frac{(40 - S) \cdot V}{260}$$

Where

V_F = feeding volume, in liters;

V = volume of medium in the bioreactor, in liters;

S = residual glucose concentration in the bioreactor, in grams per liter.

2.2.3 Two stage fed-batch cultivation (Lipid accumulation)

Lipid synthesis usually occurs when cells are submitted to stress related abiotic conditions like temperature, light intensity, salinity and nutrient starvation or limitation [24]. Nitrogen limitation is the most common and effective method for increasing lipid content, however it also slows down growth rates and therefore can lead to lower overall

lipid productivity [25]. Under nitrogen limitation, the synthesis of proteins and amino acids is compromised and the metabolic flux of carbon is diverted to lipid or carbohydrate accumulation [25].

As growth and lipid accumulation occur optimally in different conditions, a possible strategy for increasing overall process efficiency is through decoupling of growth and lipid formation by implementing two consecutive separate stages [26].

A two stage fed-batch process was developed in order to decouple biomass growth and lipid accumulation. First, biomass was grown up to 60-65 g L^{-1} in a DO-stat process in the same conditions as described in 2.2.1. Right after, feeding composition was changed to a nitrogen-free medium in order to induce nitrogen limitation and lipid accumulation. The experiment was terminated when optical density showed no change or decreased in 12 hours.

2.3 Lipid extraction for biodiesel production (crude oil)

After the end of cultivation, all the culture medium was centrifuged for separation of biomass from supernatant at 8000 g for 15 min (centrifuge Hettich Model Rotina 420). The resulting biomass sludge contained 70 % water, approximately. Drying the biomass is energy and capital intensive, therefore extraction of lipids from wet biomass is an attractive alternative [27]. However, in the presence of water, intact cell walls prevent the solvent from making direct contact with the intracellular lipids resulting in low yields of extraction [28]. As *Chlorella* cell walls were shown to be very resilient [29] and previous disruption of cells was shown to significantly enhance the recovery of intracellular lipids [30], the centrifuged biomass was subjected to an hydrothermal acid hydrolysis before lipid extraction. Acid hydrolysis was reported to be the most efficient method for disruption of microalgae cells [31] and is an easy method to apply in lab scale tests.

For the hydrolysis, a modified version of the method developed by Talukder *et al.* [14] was used: First, 0.1 g of H_2SO_4 was added to each gram of dry cell weight (dcw) biomass, then the mixture was agitated for 1 min and autoclaved at 120 °C for 1 h (autoclave model PHOENIX AV100 Plus). The hydrolysed biomass mixture was then cooled down to room temperature before extraction.

Total biomass lipids were extracted using a mixture of ethanol and hexane (1:4) at a ratio of 2.5 mL per gram of biomass dry cell weight. The solvents were added to the hydrolysed biomass (70-80 % water content) and the mixture was stirred for 1 min and allowed to rest until spontaneous phase separation occurred (approximately 1 h; top layer: organic phase containing solvent and lipids; bottom layer: aqueous phase containing biomass and water). When strong emulsions were formed, the mixture was successfully separated by centrifugation.

As shown elsewhere, centrifugation is an effective method for phase separation in microalgae lipid extraction, however the capital costs of centrifuges may be prohibitive for the process to be feasible [32]. Therefore, alternative methods to centrifugation were also tested for breaking the emulsion: heating up to 30, 40 and 50 ºC inside a fume hood , salt addition (Na2SO4 and NaCl) at 1, 5 and 10 g/L and surfactant Tween 80 (1, 5 and 10mL/L).

After phase separation, the top phase containing hexane and lipids was removed and evaporated under vacuum for solvent removal. Hexane was recovered through condensation and mixed with fresh solvent before being added to the aqueous bottom layer (water, ethanol and biomass hydrolysate) for a new extraction cycle. This procedure was repeated 4 times. The lipids remaining after solvent evaporation were then left in the oven at 50 °C and weighed every twelve hours until constant weight.

2.4 Biodiesel production

2.4.1 Pre-treatment of oil for acidity reduction (first stage)

In order to reduce the levels of FFAs of the extracted oil, a pre-treatment method was used for esterification of FFAs as described by Van Gerpen *et al.* [33]. Extraction procedures can significantly change the amount of FFAs in the oil, due to the hydrolysis of triacylglycerols at harsh conditions. Oils containing high content of FFAs are not desirable for biodiesel production though alkaline catalysed transesterification, since FFAs can react with strong bases forming anions and water. If acid catalysts are used instead, soap is not formed but the reaction rate is considerably slower and higher solvent–to-oil ratio and higher reaction temperatures are needed [34]. Thus, a more adequate alternative is the use of a two stage process: first an acid catalysed reaction is performed for the esterification of FFAs only, followed by a second stage in which an alkaline catalyst is used for the transesterification of the TAGs [35].

For the first stage, H_2SO_4 was added in the proportion of 1 % of the total mass of FFAs and methanol was added in the molar ratio of 10:1 relative to the number of moles of FFAs (molar mass of FFAs was considered the same as the molar mass of oleic acid, MM = 282 g/mol). The mixture was
added to sealed reagent bottles and the esterification reaction took place at 60° C and 200 rpm shaking for 2 h in a thermostatic shaker. The mixture was then removed from the shaker and cooled down to room temperature for analysis of acid value.

2.4.2 Transesterification of oil free from FFAs (second stage)

For the transesterification of the oil free from FFA, sodium methoxide solution was prepared by mixing the catalyst NaOH with methanol. The mass of catalyst was calculated as 1% of the oil mass plus the amount necessary to neutralize the H_2SO_4 added in the previous step. The mass of methanol was calculated for a molar ratio of 10:1 in relation to the fatty acids in the sample (considering the molar mass of fatty acids equal to oleic acid: 282 g/mol). This mixture was added to a plastic airtight jar and shaken at 200 rpm at room temperature for 12 h or until complete dissolution of NaOH in methanol. The prepared sodium methoxide solution was added to previously acid esterified oil in an airtight reagent bottle and transesterification reaction was performed at 60° C and 200 rpm agitation for 1 hours. At the end of the reaction time, the mixture was set aside to cool down to room temperature and added to a separation funnel. Distilled water at 50° C (about 5% of the total volume of the reaction) was added to promote the separation of phases. Some reaction by-products and contaminants like soap and excess methanol can often accumulate in the interface of ester and glycerol phases and slow their separation. As these contaminants are water soluble, washing with water can help improve separation [33, 36]. After water addition, the mixture was left to rest for about 20 minutes and, after complete separation, the lower aqueous phase (containing glycerol, water, methanol and NaOH) was discarded. To the upper phase, which remained in the funnel (containing biodiesel and residues of methanol and NaOH), distilled water at 50° C was added in a ratio of 20% (v/v) of the total volume. The mixture was stirred slightly and then left to rest until the aqueous and organic phases separated once again. The lower phase was again discarded and this washing procedure was repeated three times with distilled water and once with ascorbic acid 0.08% solution to neutralize remaining traces of catalyst.

2.5 Analyses

Fresh samples of 10-15 mL were taken from the reactor every 12 h and/or before and after feeding pulses.

Glucose content in the broth was analyzed using a commercial enzymatic glucose-oxidase assay kit by Bioliquid®. After incubation with enzymatic reagents at 37 °C for 15 min, the filtered samples were analyzed in an UV/vis spectrophotometer model Genesys™-10UV at 505 nm and compared with a previously established calibration curve.

Biomass dry cell weight was measured in two ways: 1) By optical density: multiple dilutions of fresh samples were analyzed in an UV/vis spectrophotometer Genesys™-10UV at 660 nm and compared with a previously

established calibration curve (range: 0 – 1 g L^{-1}); 2) Gravimetrically: Samples of 5 mL of culture broth were filtered through a pre-weighed 0.22 µm porosity membrane (diameter 45 mm) and dried in oven at 50 °C until constant weight.

For lipid content determination a modified version of Bligh & Dyer method was used [37]: cells were harvested by centrifugation at 8000 g for 10 min, frozen at -80 °C and freeze-dried at -30 °C and 50 mmHg. 400 mg of lyophilized biomass were digested in 5 mL of HCl 2 M at 80 °C during 1h. After digestion, the extraction process started with the centrifugation of the digested biomass (500 x g for 2 min) and addition of 4 mL of methanol, 2 mL of chloroform, and 3.6 mL of distilled water to the biomass pellet. The mixture was agitated for 2 min and centrifuged (500 x g for 2 min). The lower phase containing chloroform and lipids was transferred to a test tube. Re-extraction was carried out in upper phase by addition of 4 mL of a 10% (v/v) methanol 99 %:chloroform 99 % solution, followed by agitation and centrifugation (500 x g for 2 min). The lower phase was added to the test tube and chloroform was removed by evaporation in a rotary vacuum evaporator. The remaining material (total lipids) was left in the oven at 50 °C for, at least,24 h, and weighed every 12 hours until constant weight.

Acid value of extracted oils was determined by titration as described in standard method ASTM D974-14. The acid value was expressed in milligrams of KOH needed to neutralize one gram of sample (mg KOH/g). The free fatty acids content was expressed as free oleic acid equivalent and calculated according to the following equation [38]:

*FFA (%) = ((Vsample-Vblank) * M * 0.282 * 100)/weight of sample*

Where V: Volume spent sodium hydroxide required; M = Molarity of the sodium hydroxide solution standardized; 0.282 = Equivalent of oleic acid (major fatty acid).

Fatty acid composition was determined through GC-MS analysis. The crude lipid was initially converted into fatty acid methyl ester (FAME) according to Lewis *et al.* [39] and then analysed using a GC/MS gas chromatography (Shimadzu, Japan) system according to Tapia *et al.* [40].

TAG, FFA and ester contents in oil were analysed through high pressure size exclusion chromatography (HPSEC). The analysis was performed in HPLC DGU-20A5 (Shimadzu) with refractive index detector, Phenogel columns (Phenomenex), 300 mm long, 7.80 mm in diameter, 500 pores, 100 and 50 Å; Mobile phase THF and 1 flow mL min^{-1}. Calibration curves for quantification were built from different concentrations of oleic acid (FA), methyl oleate (ester) and trioleate (TAG).

2.6 Growth models

Growth models selection was based on the capacity to describe the asymptotic behaviour of microbial growth curves. These models only describe the biomass concentration and are not based on substrate consumption such as Monod based models, which describe growth profile based on substrate limitation and/or inhibition. In our experiments, it was assumed that substrate was constantly available by nutrient feeding and that substrate concentration was kept below inhibitory concentrations. Therefore, substrate limitation and inhibition effects were not considered. These models were applied in order to help understand and predict the behaviour of growth at high cell concentrations. In all of the models described below, X_0 was the initial biomass concentration and X was fitted in relation to time.

The best known equation to describe this effect is the logistic growth model, first described by Verhulst [41].

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_m}\right)$$

Where X = biomass concentration; μ_{max} = maximum specific growth rate and X_m = maximum value microbial population

The integrated form of the equation, modified by Zwietering *et al.* [42] can be described as:

$$X = \frac{\ln\frac{X_m}{X_0}}{1 + e^{\left[\frac{4\mu_{max}}{\ln\frac{X_{max}}{X_0}}(\lambda - t) + 2\right]}}$$

Where λ = lag phase duration and X_0 = initial biomass concentration.

In this modified version, Zwietering *et al.* [42] added a term representing the lag phase duration, denoted by λ and defined as the time for the growth rate to reach μ_{max} at the start of cultivation. This was accomplished by re-parameterization of the logistic equation. The fitted parameters were X_m, μ_{max} and λ.

One of the most known and still used growth model is the Gompertz equation, which was also modified by Zwietering *et al.* [42] to include a parameter to account for lag phase duration (λ). The equation can be described as:

$$X = \ln\frac{X_m}{X_0} \cdot e^{-e^{\left(\frac{\mu_{max} \cdot e^1}{\ln\left(\frac{X_m}{X_0}\right)}(\lambda - t) + 1\right)}}$$

The fitted parameters for the modified Gompertz equation were the same as for the logistic equation.

While the logistic and Gompertz models consider a linear decrease of growth rate with the increase of cell concentration, this behavior is not observed in some cultures at high cell concentrations [9]. Mulchandani *et al.* [43] developed a power law version of the logistic equation in order to describe non-linear effects of biomass concentration over growth rate. The constant α can be interpreted as an index of the inhibitory effect which causes the deviation of growth from the logistic equation.

Hence, the modified version of the logistic equation is given by

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_m}\right)^{\alpha}$$

The integrated form of the equation is given by.

$$X = \frac{X_m}{\left\{1 + \left[\left(\frac{X_m}{X_0}\right)^{\alpha} - 1\right] e^{-\mu_{max}\alpha \cdot t}\right\}^{1/\alpha}}$$

For the Mulchandani modified logistic equation, the fitted parameters were X_m, μ_{max} and α.

Growth models were compared based on evaluation parameters described in Table 1.

Table 2. Growth model evaluation parameters [44]

Parameter	
Coefficient of determination	$R^2 = \frac{(\sum(x_i - \bar{x})(y_i - \bar{y}))^2}{\sum(x_i - \bar{x})^2 \sum(y_i - \bar{y})^2}$
Mean Squared Error	$RMSE = \sqrt{\frac{\sum(obs - pred)^2}{n}}$
Standard error of prediction	$\%SEP = \frac{100}{avg}\sqrt{\frac{\sum(observed - predicted)^2}{n}}$
Bias factor	$B_f = 10^{\frac{\sum log(pred/obs)}{n}}$
Precision factor	$A_f = 10^{\frac{\sum \lvert log(pred/obs) \rvert}{n}}$

3. Results

3.1 Fed batch cultivations at high cell density

First, a DO-stat cultivation system was set to keep nutrient availability during growth, while avoiding overdosing and substrate inhibition effects (Figure 2). Feeding of medium started at 44 h of cultivation, after initial glucose depletion. Maximum specific growth rate of 0.03 h^{-1} was observed in the first 39 hours of cultivation, after which growth rate declined to approximately 0.02 h^{-1} until 160 hours of cultivation. A linear growth phase followed the exponential phase and ended at 256 hours of cultivation, after which a sudden decrease in growth was observed. From 256 to 278 hours of cultivation, no change in optical density neither glucose concentration was observed. A sudden increase in DO followed these events, indicating reduction of respiratory activity and leading to the decision of terminating the cultivation. Stirring speed steeply decreased to 150 rpm as a cascade response to DO increase. Biomass concentration reached a maximum 73.7 g L^{-1} or 129 g of total biomass in a final volume of 1750 mL with overall biomass productivity of 6.3 g L^{-1} d^{-1} (Table 2). During the fed-batch stage, the addition of glucose rich medium caused an oscillating behaviour of DO, which varied between 35 and 0 %. The lowest levels of DO were observed right after feeding pulses and did not last more than a few seconds (Figure 2), as the cascade agitation system quickly reacted, by increasing agitation rate. .

To test the hypothesis that the oscillation of oxygen concentration and substrate availability observed in DO-stat cultivation was responsible for biomass growth deceleration, a second feeding strategy was applied, based on substrate off-line concentration control. For pulsed feeding, large volumes of medium were added in each feeding pulse leading to high glucose concentrations, right after addition of substrate. A total of six pulses were added to the culture increasing the glucose concentration up to 40-50 g L^{-1} each time. Glucose concentration was monitored and kept under 50 g L^{-1}, as preliminary data indicated substrate inhibition for higher glucose concentrations (Annex I). Maximum growth rate was observed from 0 to 48 h, reaching 0.03 h^{-1}, followed by a second phase with growth rate of 0.015 h^{-1}, which lasted from 48 to 144 h^{-1} and decreased to 0.005 h^{-1} until growth stopped at 285h. Biomass concentration reached a maximum 77 g L^{-1} or 144 g of total biomass in a final volume of 1870 mL reaching overall productivity of 6.5 g L^{-1} d^{-1}. Lipid content in final biomass was similar for both feeding strategies, with 20.9 % (m/m) in DO-stat fed-batch and 19 % (m/m) in pulsed feeding strategy. When errors are

accounted, no significant differences between feeding strategies was observed for biomass and lipid productivities (Table 2).

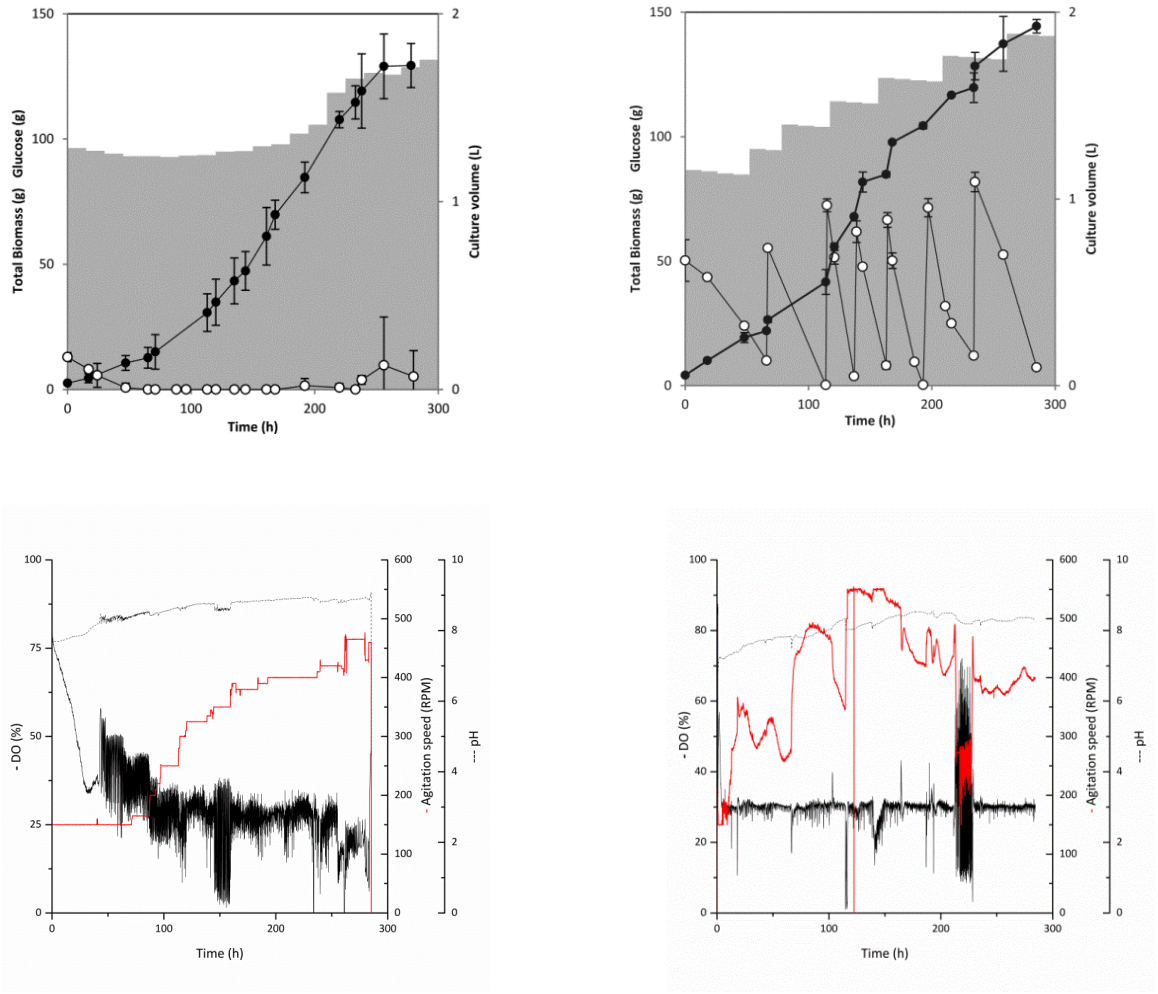

Figure 2. Experimental results of DO-stat (left) and pulsed (right) fed-batch cultivation. ● Averages of total biomass (dcw); ○ averages of total glucose; ▇ Culture volume; — Agitation rate; ▬ DO; ▪▪▪ pH.

For both DO-stat and pulsed feedings, dry cell weight measurements showed a different behaviour than optical density at the end of growth curve. In DO-stat fed batch, optical density decreased in the last hours of cultivation, while dry cell weight analysis of the same points showed that growth was still ongoing. Similar behaviour occurred in pulsed feeding, however in this case the difference was observed early in the process, around 150 h, and more intense than in DO-stat process (Annex II). Dry cell weight results take around 24 h to be confirmed and, therefore, are not suitable for real time monitoring, while optical density analysis only takes a few minutes to issue results. However, previous studies have shown that morphological alterations, such as changes in pigment content, can lead to significant errors over the course of growth cycles, due to changes in absorbance [45]. Pigment change could have been the cause for the difference observed between dry cell weight and optical density results, as a slight change in the colour of the culture was anecdotally observed, especially in the pulsed feeding process. For these reasons, the results presented in this study are based on dry cell weight measurements.

The analysis of substrate consumption profiles reveals that the DO-stat process consumed a lower total amount of glucose throughout the process. Figure 3 shows the accumulated consumption of glucose throughout the cultivation (-ΔS). While DO-stat cultivation had an overall substrate yield of 0.43 ± 0.02 g biomass dcw / g glucose, pulsed cultivation yielded 0.37 ± 0.01 g biomass dcw / g glucose. The higher consumption of substrate in pulsed feeding may have been caused by the availability of excess carbon source, which can lead to production and excretion of secondary products. Chlorella strains were shown to excrete a number of molecules including

polysaccharides, peptides, amino acids and fatty acids [46]. It was also reported that when high biomass concentrations were achieved, growth inhibitory substances such as FFAs were produced by algal cells [47].

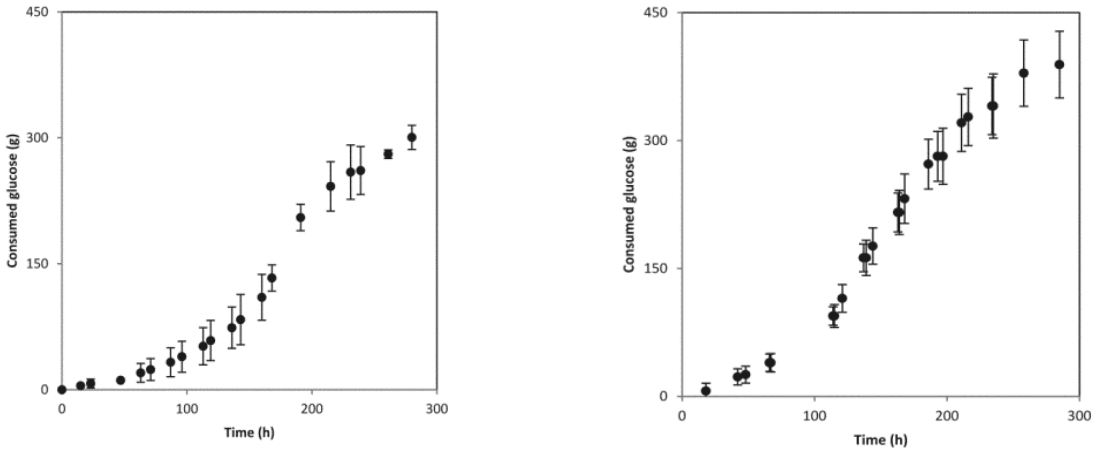

Figure 3. Accumulated substrate consumption for DO-stat fed batch (left) and pulsed feeding fed batch (right). Calculated as $\Delta S = S_{initial}+S_{feed}-S_{residual}$.

To assess the possibility that secondary metabolites were produced, samples of the pulsed feeding fed-batch were analysed for total organic carbon and total nitrogen dissolved in the medium (Annex III), apart from the fed carbon source (glucose concentration was analysed for each sample and the corresponding carbon content was subtracted from the TOC values). The results showed that up to 12 g L^{-1} of organic carbon accumulated in the culture medium, indicating that a considerable fraction of the carbon source was lost as unknown by-products. As a matter of comparison, this amount of carbon is the equivalent to 30 g L^{-1} of glucose or, approximately, 25 g L^{-1} of biomass (based on average carbon content of 50 % in biomass). The amount of total nitrogen in the supernatant was approximately 0.25 g L^{-1}, which shows that the accumulated by-products had average high carbon/nitrogen ratio, suggesting that high carbon-containing molecules such as fatty acids or polysaccharides could be among the molecules exuded. However, further studies are necessary in order to identify and quantify these metabolites.

Table 3. Final results for biomass and lipid production in DO-Stat and pulsed feeding fed-batch strategies

Result	Unit	DO-Stat	Pulsed Feeding
Duration	(h)	280	285
Final Volume	(mL)	1750	1870
Final biomass concentration	(g L^{-1})	74 ± 2	77 ± 3
Total biomass produced	(g)	129 ± 5	144 ± 5
Lipid content	(% m/m)	20.9 ± 0.6	19 ± 2
Lipid produced	(g)	27 ± 1	28 ± 3
Lipid-free biomass concentration	(g L^{-1})	51 ± 2	54 ± 3
Lipid-free Biomass produced	(g)	102 ± 5	116 ± 5
Total glucose consumed	(g)	300	389
$P_{totalbiomass}$	(g L^{-1} d^{-1})	6.3 ± 0.5	6.5 ± 0.5
P_{Lipid}	(g L^{-1} d^{-1})	1.3 ± 0.2	1.3 ± 0.1
$P_{deff\ biomass}$	(g L^{-1} d^{-1})	5.0 ± 0.5	5.2 ± 0.2
$Y_{lipid/S}$	(g_{Lip}^{-1} g_{gluc}^{-1})	0.09 ± 0.01	0.07 ± 0.01
$Y_{deff/S}$	(g_{biom}^{-1} g_{gluc}^{-1})	0.34 ± 0.02	0.30 ± 0.01

Lipid-free biomass: Total biomass – lipid mass; $P_{totalbiomass}$: volumetric productivity of whole biomass (lipid+lipid-free biomass); P_{Lipid}: volumetric productivity of lipids; $P_{deff\ biomass}$: volumetric productivity of lipid-free biomass; $Y_{lipid/S}$: yield of lipids on substrate (glucose); $Y_{deff/S}$: yield of lipid-free biomass on substrate (glucose). Values are averages of two independent cultivations.

Despite the nutrient and oxygen availability, biomass concentration did not exceed 77 g L^{-1}. Considering a biomass water content of 70-80 % [29] and that the maximum density of packed spheres is around 64 % (640 g L^{-1} if biomass density is the same as water) [48], the maximum theoretical expected dry cell weight would be around 127-190 g L^{-1} [10]. The only study that reported high biomass concentrations by a strain of *C. vulgaris* achieved 117 g L^{-1}. The authors used a pulsed fed-batch strategy (4 pulses) without nutrient limitations.

That shows that the fed-batch cultures in this study were far from being limited by lack of physical space. A possible explanation for the observed growth limitation is the existence of a self-inhibition mechanism that prevents the microalgae population to grow beyond a certain limit. Quorum sensing is a known process by which bacteria control cell density by producing and detecting signal molecules [49]. Some microalgae like *Chlamydomonas reinhardtii* can secrete compounds that mimic bacterial quorum sensing signals as a way to limit bacterial growth [50]. It is uncertain, however, if such molecules could have an inhibitory effect on the microalgae itself above a certain concentration. One of the few studies to investigate the growth limitation of *Chlorella vulgaris* showed that microalgae growth was inhibited when the culture reached biomass concentrations around 30 g L^{-1}. When exponentially growing cells were inoculated in fresh medium supplemented with spent medium from the high cell density culture, inhibitory effects were detected in comparison with cells grown in 100 % fresh medium [51]. A number of studies have shown that the re-use of spent medium in microalgae cultures can lead to growth inhibition due to accumulation of organic material and/or minerals, however no studies on nutrient recycling are found for heterotrophic conditions [52] The results from our TOC and TN analyses (annex III) reinforce the possibility that growth inhibiting substances were produced by the microalga at high cell densities, limiting the final biomass concentration. However, further studies are necessary to identify the secreted molecules and confirm their inhibition effect.

3.2 Growth model fitting

Growth models were fitted to the experimental results. The fitted curves are shown in Figure 4 .

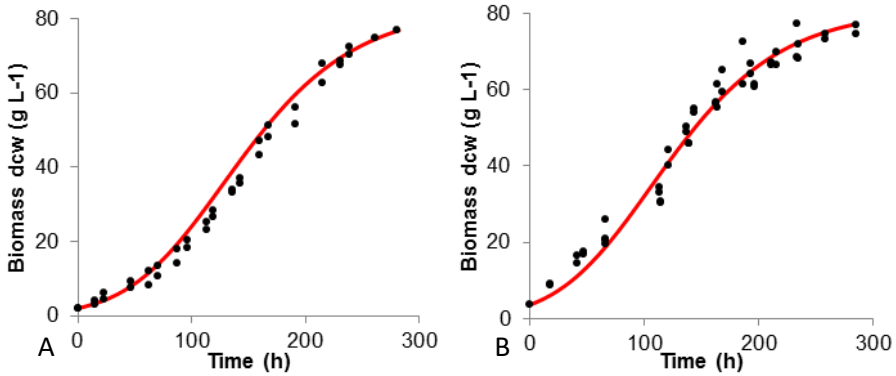

Figure 4. A: Mulchandani model fitting of experimental points. A: DO-stat cultivation; B: Pulsed feeding cultivation. • Experimental biomass concentration

The logistic model modified by Mulchandani had the best fitting compared with the two other models, with the estimated self-inhibition coefficient (α) of 0.37 and 0.39 (Table 3). The coefficient α represents the deviation of the growth behaviour from the exponential case. For very large values of α (α>>0), growth follows the classic exponential pattern; for α = 1 growth follows the self-inhibition pattern described by the logistic equation, in which the inhibition effect is linearly dependent to the biomass concentration; and α<0 indicates a higher degree of inhibition effect in relation to the logistic equation, with inhibition increasing exponentially with biomass concentration [43].

Table 4. Models parameters and validation parameters fitting comparison

Parameters	Mulchandani		Logistic		Modified Gompertz	
	D.S.	P.F.	D.S.	P.F.	D.S.	P.F.
μ_{max}	0.043	0.045	0.022	0.021	0.023	0.022
X_m	83.4	74.98	83.3	71.8	89.7	74.9
α	0.37	0.39	-	-	-	-
λ	-	-	0	0	0	0
Validation parameters						
R^2	0.98		0.95		0.96	
RMSE	3.24		4.55		4.25	
%SEP	9.72		13.2		12.40	
Af	0.98		0.91		0.94	
Bias	1.10		1.17		1.15	

Based on the validation parameters, the Mulchandani model was selected as the best for describing the high cell density growth and estimating growth parameters. Since the biomass concentration was correlated with negative effects over biomass growth, it is important to determine the conditions for maximum productivity. From the estimated values of biomass concentration, productivity was calculated and plotted against the corresponding value of biomass concentration (Figure 5). Analysis of the curve showed that the maximum productivity was obtained when the biomass concentration reached 62 ± 3 g L^{-1}. For a two stage cultivation, in which cell growth and product accumulation are separated, the biomass concentration stage should not exceed 60-65 g L^{-1}, as biomass productivity tend to decrease when higher concentrations of biomass are attained.

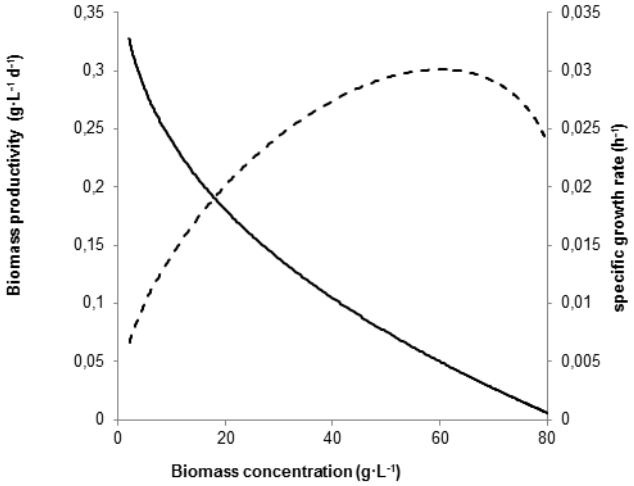

Figure 5. Estimated productivity and growth rate in relation to biomass concentration based on fitting of Mulchandani equation

3.3 Two stage cultivation

The study of growth limitation suggested that unknown factors interfere in the accumulation of biomass in the reactor for concentrations above 70 g L^{-1}; therefore attempts to increase biomass concentration above that range could be counterproductive. In addition, DO-stat culture had better yields on substrate, probably due to the production of secondary metabolites in the pulsed feeding culture. For the two stage cultivation, microalgal biomass was grown in a DO-stat batch under nutrient sufficient conditions up to the concentration correspondent to maximum biomass productivity (60-65 g L^{-1}). This was followed by a second stage in which nitrogen free medium was fed to the reactor in order to induce lipid accumulation. The results of the two stage cultivation are shown in Figure 6.

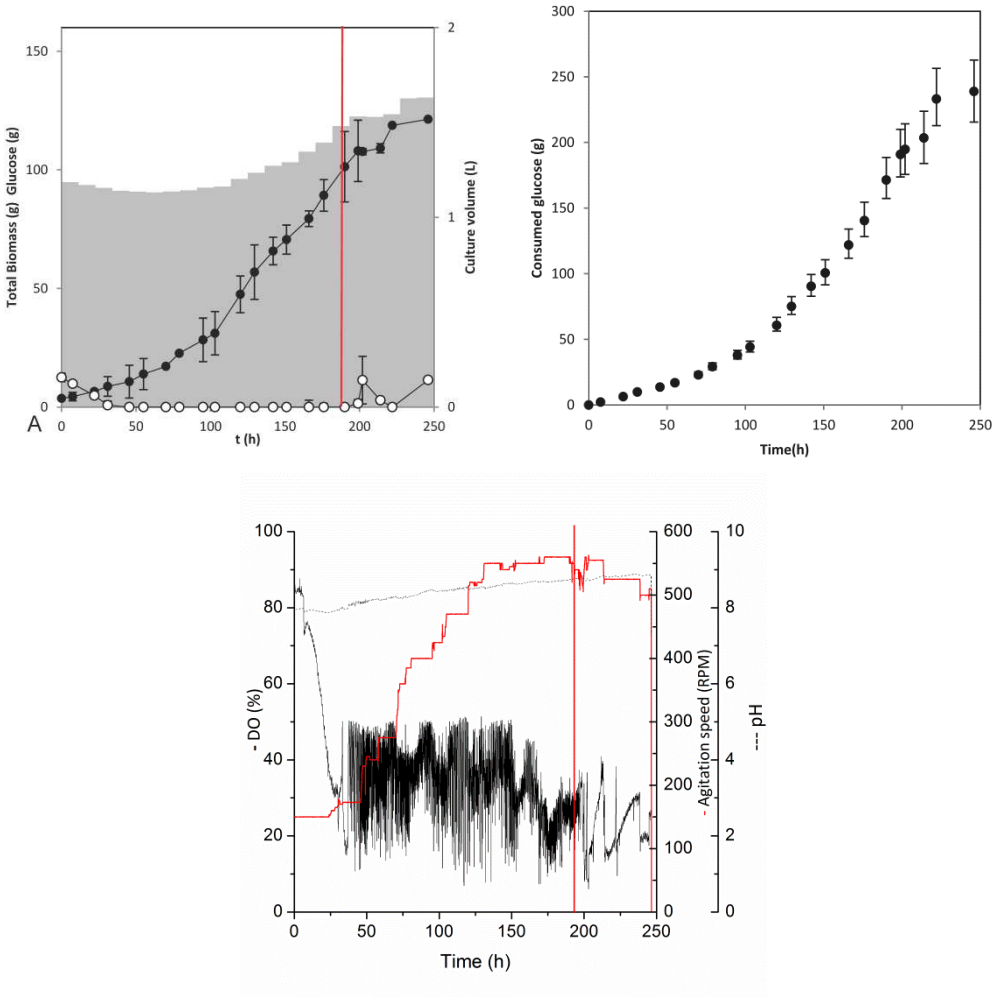

Figure 6. Experimental results of two Stage cultivation. Vertical red line represents the division between the nitrogen –sufficient and nitrogen-limited stages. A: ● Averages of total biomass (dcw); ○ Averages of total glucose; ▓ :Culture volume ; vertical red line: division between N-sufficient and N-limited stages; B: ● substrate consumption; C: — Agitation rate; ▬ DO; ▪▪▪ pH.

Biomass reached 64 g L^{-1} during the nitrogen sufficient stage after 199 h of DO-stat fed-batch cultivation and glucose concentration remained close to zero as a result of the carbon limited strategy. Feeding was then replaced by nitrogen-free medium in order to induce lipid accumulation. Biomass concentration (lipid-free biomass + lipids) stabilized after 246 h of cultivation at 74 ± 3 g L^{-1} or 121 g of total biomass in the bioreactor. From this point on, OD measurements did not show further increase in biomass concentration and glucose consumption slowed down, causing a sudden increase of the residual glucose concentration. Lipid content increased from 21 ± 1 % (w/w) to 27.4 ± 0.6 % (w/w) (statistically significant difference at 95 % confidence level; p-value: 0.0304) during nitrogen starvation stage. Although the feeding medium of second stage did not contain any nitrogen sources, lipid-free biomass increased from 51 to 54 g L^{-1}, probably due to the presence of residual nitrogen from the first feeding stage. Final biomass and lipid concentrations and productivities are described in Table 3.

Table 5. Lipid and biomass productivities for two stage cultivation

Parameter	Unit	First stage	Second stage*	Total/average
Duration	(h)	199	47	246
Final Volume	(mL)	1530 ± 20**	1632 ± 5***	1632 ± 5
Biomass concentration	(g L^{-1})	64 ± 6	74 ± 3	74 ± 3
Total biomass produced (lipid-free + lipids)	(g)	97 ± 4	23.1 ± 0.9	121 ± 5
Lipid content	(% m/m)	21 ± 1	27 ± 2	27 ± 2
Lipid produced	(g)	20 ± 3	12.4 ± 3	32 ± 4
Lipid-free biomass concentration	(g L^{-1})	51 ± 4	54 ± 3	54 ± 3
Lipid-free Biomass produced	(g)	77 ± 7	11.1 ± 0.9	89 ± 5
$P_{totalbiomass}$	(g L^{-1} d^{-1})	7.7 ± 0.3	7.4 ± 0.3	7.3 ± 0.3
P_{Lipid}	(g L^{-1} d^{-1})	1.6 ± 0.2	4 ± 1	2.0 ± 0.3
$P_{deff\ biomass}$	(g L^{-1} d^{-1})	6.1 ± 0.5	1.9 ± 0.2	5.3 ± 0.7
$Y_{lipid/S}$	(g^{-1} g^{-1})	0.12 ± 0.01	0.18 ± 0.1	0.14 ± 0.2
$Y_{deff/S}$	(g^{-1} g^{-1})	0.45 ± 0.02	0.09 ± 0.01	0.37 ± 0.03

Lipid-free biomass: Total biomass – lipid mass; $P_{totalbiomass}$: volumetric productivity of whole biomass (lipid+lipid-free biomass); P_{Lipid}: volumetric productivity of lipids; $P_{deff\ biomass}$: volumetric productivity of lipid-free biomass; $Y_{lipid/S}$: yield of lipids on substrate (glucose); $Y_{deff/S}$: yield of lipid-free biomass on substrate (glucose)

** Biomass and lipids produced in second stage were calculated by subtracting the results of first stage from final results.*

*** estimated by liquid height in the bioreactor*

**** total volume was removed from bioreactor and measured with calibrated glassware*

Total lipid productivity during the nitrogen limited stage was 115 % higher in comparison with the nitrogen sufficient stage, increasing from 1.6 to 3.9 g_{Lipids} L^{-1} d^{-1}. As expected, lipid-free biomass production decreased steeply, from 6.1 to 1.8 g_{Lipids} L^{-1} d^{-1} and lipid-free biomass concentration increased only 6% during second stage. The non-lipid portion of biomass increased slightly during the starved nitrogen stage, probably as a result of residual nitrogen from the nitrogen sufficient phase.

The yield of biomass and lipids over glucose changed considerably in the nitrogen limited stage. The yield of lipid-free biomass over glucose decreased from 0.45 to 0.09 $g_{biomass}$ / $g_{glucose}$, while the yield of lipids over glucose increased from 0.12 to 0.16 g_{lipids} / $g_{glucose}$. The overall yield of biomass decreased significantly, which suggests that carbon consumption is slower under nitrogen limitation.

Lipid productivity in the two stage strategy was 1.5 fold the obtained in simple DO-stat and pulsed feeding fed-batches. The effect of nitrogen limitation was significant for lipid accumulation even though it was maintained for only 20 % of the whole cultivation time (47 h). The results suggest that lipid accumulation could be improved if the nitrogen limitation phase was kept for longer periods. If nutrient limitation started in an early stage of the process, higher lipid contents could be achieved and the already mentioned inhibitory effects observed at high biomass concentrations could be avoided.

3.4 Biodiesel production

3.4.1 Biomass hydrolysis and lipid extraction

Extraction of lipids from the microalgae biomass produced in the two stage cultivation was performed for the production of biodiesel. After centrifugation and hydrolysis steps, hexane was added and the mixture was agitated. A very stable emulsion was formed after agitation, with no visible separation of aqueous and organic phase after 1h (Figure 7). Many different biological components found in the membrane or cytoplasm of most microorganisms can act as stabilizers of O/W emulsions such as phospholipids, proteins, polysaccharides, lipo and

glyco-proteins, and even whole cells, among others [53]. In order to break the emulsion, centrifugation at 2,000, 4,000 and 8,000 g was applied. The three intensities were successful in completely separating the organic and aqueous phases. Alternative separation methods were also tested as low cost and the corresponding results are described in Annex IV.

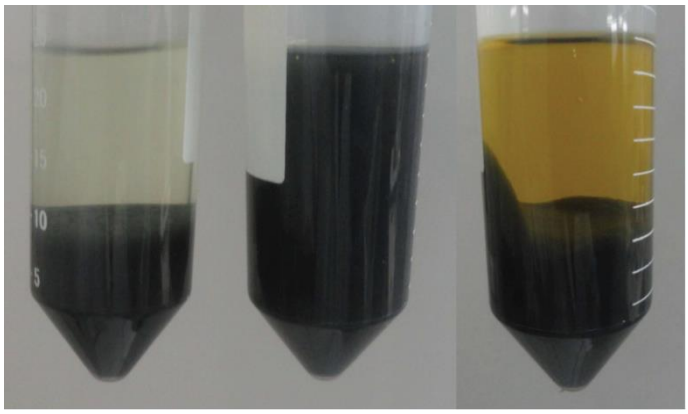

Figure 7. Emulsion formed during wet lipid extraction from microalgae biomass. Left: wet biomass with added solvent before agitation; Center: biomass and solvent emulsion after agitation; Right: Phase separation after centrifugation at 2.000 g.

After separation of phases, lipid extracts were dried under vacuum and oven at 50 ºC before weighing. The final results were compared with the analytical values obtained by the Bligh *et al.* [37] method. The final lipid content obtained from the two stage fed-batch biomass was 23 ± 1 % (m/m) resulting in 28 g of total lipids. The results were slightly lower than the obtained by the analytical method, with an average recovery of 87 ± 2 %. However, the analytical method is based on the use of a more polar solvent (chloroform) that can recover a higher fraction of polar lipids such as phospholipids, glycolipids and chlorophyll components [54]. Hexane extraction, on the other hand, tend to be more selective and recover a higher fraction of neutral lipids, which are more suitable for biodiesel production [55].

3.4.2 Pre-treatment of extracted lipids

After extraction of lipids, it was necessary to evaluate the content of FFAs in the extracted microalgae oil.

The acid value of the extracted oil, analysed by titration method described in ASTM D974 – 14, was 19.1 ± 0.1 mg KOH g^{-1}. Considering that the average molecular weight of the fatty acids in the sample is the same as oleic acid (286.5 g mol^{-1}) the percentage of FFAs in the sample was calculated as 9.7 ± 0.1 %. Very different contents of FFAs in *Chlorella* lipids are described in literature. While Chia *et al.* [56] found the FFA content in *Chlorella vulgaris* strains to be around 5% of total lipid content, Yao *et al.* [54] found FFA contents as high as 30 % of total lipids. However, the authors stated that such high content was likely the result of lipid degradation during storage and processing.

It is likely that the content of FFA found in the extracted oil is the result of hydrolysis of triacylglycerols (TAG) during extraction. Takisawa *et al.* [57] showed that the combination of H_2SO_4 and high temperatures is very effective in hydrolysing TAGs into FFAs. To confirm the FFA content, as well as quantify triacylglycerol (TAG) content, samples were analysed by size exclusion chromatography (Figure 8).

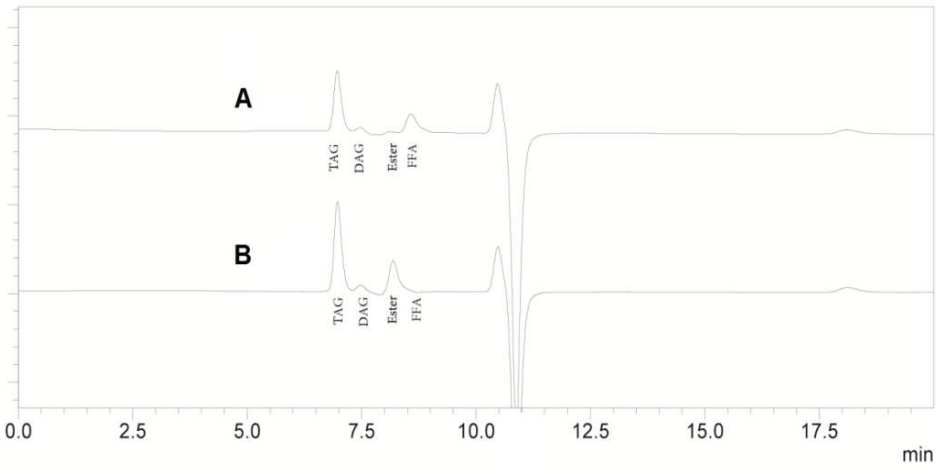

Figure 8. HPSEC chromatogram of extracted Chlorella vulgaris lipids. A: before acid esterification; B: after acid esterification

From the HPSEC chromatogram, TAG and FFA contents were calculated from integration of peak areas and compared with calibration curves. FFA and TAG contents were calculated as 9.6 % and 90.1 % of total lipid content, respectively. A small peak was identified as di-acylglycerol (DAG) but not quantified.

When the transesterification of fatty acids is catalysed by a base, the FFAs react with the catalyst forming soap and water. If the FFA content is higher than 5 %, the soap formed inhibits separation of esters and glycerol and contributes to emulsion formation. Therefore, acid catalysts may be used to esterify the FFAs into methyl esters prior to the alkali catalysed reaction [35].

The extracted lipids were esterified in the presence of methanol and H_2SO_4 as catalyst for one hour. The reaction was intended to esterify the FFAs but not the TAGs, as those would take a much longer time of reaction. While FFA esterification is a one-step reaction, TAG transesterification is a three-step reaction with an overall slower reaction rate [57].

The products of the reaction were analysed through HPSEC and FFA content was quantified through titration in order to evaluate the extent of reaction. Acid value was determined as 0.97 ± 0.01 mg KOH g^{-1}, equivalent to 0.48 ± 0.01 % of free fatty acids (oleic acid equivalent). In Figure 8 B is the HPSEC chromatogram of the products of esterification reaction. The FFA peak could not be identified, showing that the reaction was successful in eliminating FFAs from the extracted lipids. An ester peak was identified and quantified through comparison with an methyl oleate calibration curve. A DAG peak was also identified but not quantified. The results from the analysis of the extracted lipids before and after the acid esterification are shown in Table 4.

Table 6. HPSEC results of lipids before and after acid esterification (pre-treatment)

Component	Before esterification (%)	After acid esterification (%)
TAG	90.1 ± 0.5	81 ± 1 *
FFA	10.1 ± 0.5	0.48 ± 0.01
Esters	1.3 ± 0.5	17 ± 1

Ester content increased from 1.3 to 17 % of the total lipids. Although not the objective of this stage, part of the TAG content (around 7 %) was also esterified. For the total transesterification of fatty acids, acid catalysed reactions may take longer than 60 times the time needed in alkaline transesterification [33].

3.4.3 Transesterification reaction

The pre-treated oils were then trans-esterified through alkaline catalysis. After the end of the acid catalysed reaction, alkaline catalyst mixed with methanol were added to the mixture. The mass of sodium hydroxide

was calculated as 1% of total oil mass plus the enough to neutralize the H_2SO_4 added in pre-treatment. Methanol was added in the molar ratio of 10:1 (methanol:fatty acids) and the reaction was performed at 60 °C for 1 h.

The resulting material was left to settle for a few hours for phase separation. To encourage separation of glycerol and esters, 50 g of water at 50 °C were added to the mixture. After phase separation the bottom layer was discarded and the upper phase containing the fatty acid methyl esters was washed four times with warm distilled water and once with ascorbic acid 0.8 % solution. The FAME mixture was dried in oven at 60 ºC and through addition of anhydrous sodium sulphate, which was then removed through filtration.

Samples were analysed through HPSEC and CG-MS to evaluate the extent of reaction. The HPSEC chromatogram is depicted in Figure 9.

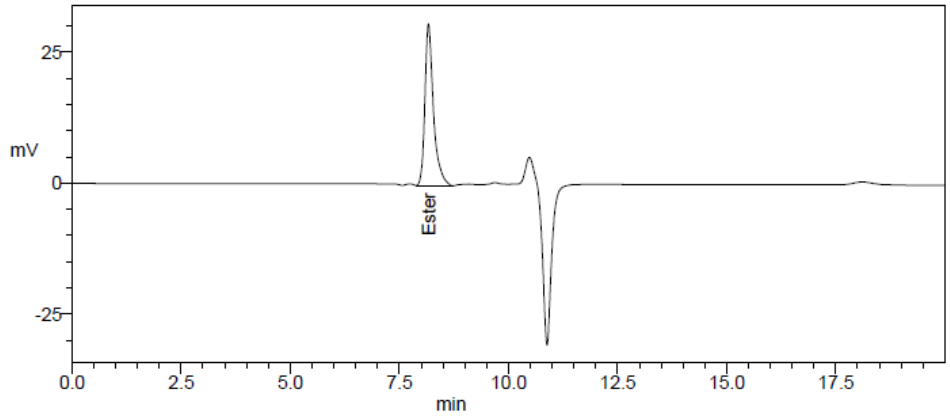

Figure 9. HPSEC chromatogram of transesterified Chlorella vulgaris lipids

The HPSEC chromatogram showed that the final product of the reaction had no detectable FFA, TAG, DAG or MAGs (mono-acylglycerol0. The only detectable peak was identified as the ester peak (retention time was compared to standard methyl oleate). Integration of the peak area was compared with a methyl oleate calibration curve resulting in the ester content of 101 %, which was obviously an overestimation (Figure 10). The reason for this result is probably due to the limitations of the estimation method, which considers that the mixture of fatty acid methyl esters has the same response factor as the calibration curve standard compound (methyl oleate). As demonstrated elsewhere, the degree of unsaturation of FAME significantly influences the response factor of HPSEC-RI results, for instance methyl linoleate (C18:2) was shown to have a response factor 20 % higher than methyl oleate (C18:1) and 59 % higher than methyl stearate (C18:0) [58].

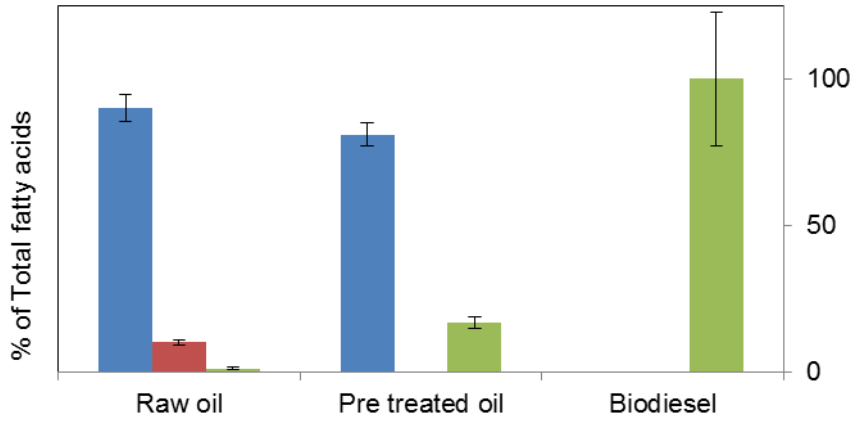

Figure 10. Summary of HPSEC analysis of conversion of raw oil of microalgae into fatty acid esters (biodiesel).

From CG-MS analysis, the fatty acid composition of lipids was quantified (Figure 11). Methyl oleate was by far the major fatty acid ester, with 52 % of total FAME content. Monounsaturated fatty acids, especially oleic acid,

were shown to possess the most balanced characteristics for biodiesel production based on the quality properties of the resulting fuel, such as cetane number, iodine value and cold filter plugging point [59].

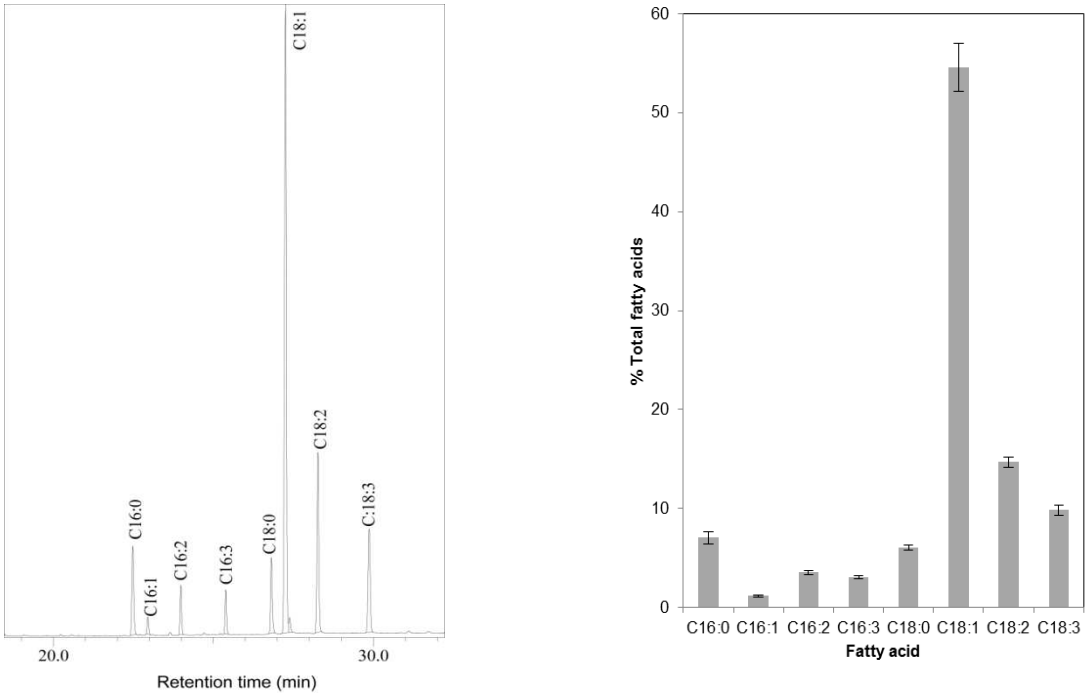

Figure 11. Chromatogram (left) and fatty acid composition of microalgae biodiesel (right), analysed by GC-MS analysis

The concentration of total esters was estimated by comparing the results of CG-MS with a previously established methyl oleate calibration curve as 77 ± 2 % of the total lipids extracted from the microalgae biomass, which is a far more realistic estimation than the obtained in HPSEC results. This shows that the lipid extraction was indeed selective, since previous studies have shown that neutral lipids compose around 50-60 % of total lipids in *Chlorella* strains. The combined yield of extraction and conversion of lipids into FAME was 68 %, based on total lipids. Although it was not the scope of this study to optimize the microalgal FAME conversion and recovery, the final yield is comparable to the obtained in recent studies on the subject (Table 7).

Table 7. Summary of FAME yields obtained from microalgae in recent literature

Feedstock	Method of extraction	Method of conversion	Fame yield (g_{FAME}/g_{Lipid})	Ref.
Wet biomass	Hydrolysis (H_2SO_4 60%; 100 °C; 240 min) and hexane extraction	Acid catalysed esterification	50 %	[60]
Lyophilized biomass	Hexane based extraction	Saponification (KOH) followed by acid catalysed esterification	32 %	[61]
Lyophilized biomass	DMC extraction	Lipase catalysed transesterification	75.5 %	[62]
Wet biomass	Hexane extraction (after transesterification)	Hydrolysis followed by direct transesterification	74.5 % (over total transesterifiable FAs)	[63]
Wet biomass	Hexane extraction (after transesterification)	In situ-transesterification (methanol, H2SO4	38.6 % (92.2 % conversion of 42% transesterifiable lipids in total lipids)	[64]

Considering the whole process (growth + extraction + biodiesel production), the final yield of the biodiesel production was calculated as 0.09 $g_{esters}\ g^{-1}_{glucose}$. A simple economic analysis shows that the contribution of sugar to

production cost of 1 L biodiesel would reach $2.76, more than two times the price of diesel (considering price of sugar $0.29 kg^{-1} and diesel $1.08 L^{-1} (Indexmundi, 2016) and biodiesel density = 860g L^{-1}). For the production of biodiesel from heterotrophic microalgae to become feasible, the yield over substrate must be significantly enhanced and/or low cost substrates must be employed. The theoretical yield of single cell oils from sugars is 32-33 % (m/m), however most experimental studies reach less than 20 % conversion [65]. Even if the maximum theoretical yield was achieved, the production cost of biodiesel from simple sugars (e.g. glucose or sucrose) would be close to its selling price. Thus, feasibility of the process depends on the development of new engineered metabolic routes for enhanced conversion of inexpensive substrates, such as waste streams, as well as valorisation of all bio-products from cell biomass [65, 66].

Table 8. Summary of product yields in biodiesel production from heterotrophic microalgae

Stage	Product mass (g)	Yield of product on sugar	
Lipid production	32 g	0.14 g/g	
	↓		
Lipids extraction	28 g	0.12 g/g	89 % recovery of total lipids
	↓		
Biodiesel (FAME) production	21.5 g	0.09 g/g	77 % conversion of total lipids

4. Conclusion

The study of growth of *C. vulgaris* CPCC 90 in fed-batch heterotrophic cultivation showed that biomass concentration could not be increased above concentrations around 70 g L^{-1}. Although medium nutrients were constantly fed and oxygen concentration was kept at relatively high levels, a strong inhibition of growth was observed, which could be caused by the accumulation of metabolites in the culture medium, as evidenced by organic carbon analysis.

Based on the results from the initial biomass growth evaluation, a two stage cultivation was performed in order to decouple biomass growth and lipid accumulation, since the latter occurs in growth limiting conditions. The two phase strategy was successful in reaching high cell densities (74-77 g L^{-1}) and then inducing lipid accumulation in the accumulated biomass, resulting in overall higher lipid productivity.

The lipids extracted from the microalgae biomass contained 10 % of FFA, thus requiring a pre-acid esterification step (first stage) prior to alkaline transesterification (second stage) in order to avoid soap formation. After the second stage, the resulting biodiesel contained no detectable amounts of FFA, MAG, DAG, TAG or methanol and the total ester content was estimated as 77 % through CG analysis. The overall yield of conversion of sugar into biodiesel was estimated as 9 % (m/m) or 0.09 $g_{biodiesel}$ g^{-1}glicose. These results show that heterotrophic cultivation at high cell densities can be a technically feasible way of producing lipids and biodiesel, however more studies are necessary in order to understand the limitations of biomass growth and improve yields of conversion.

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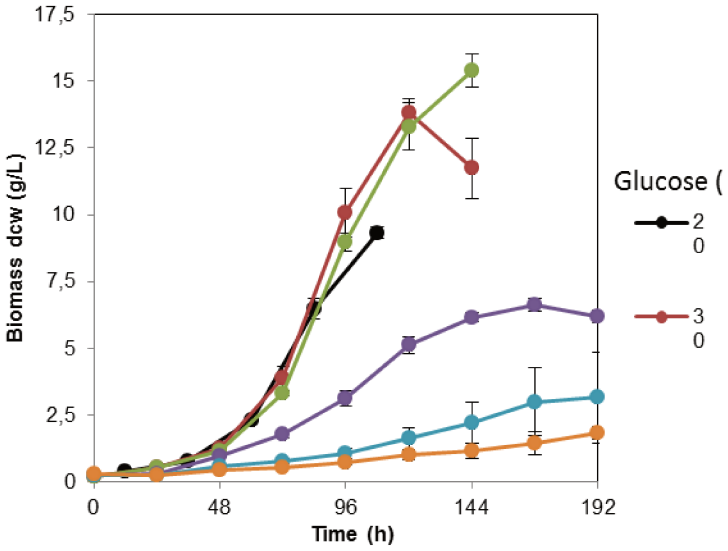
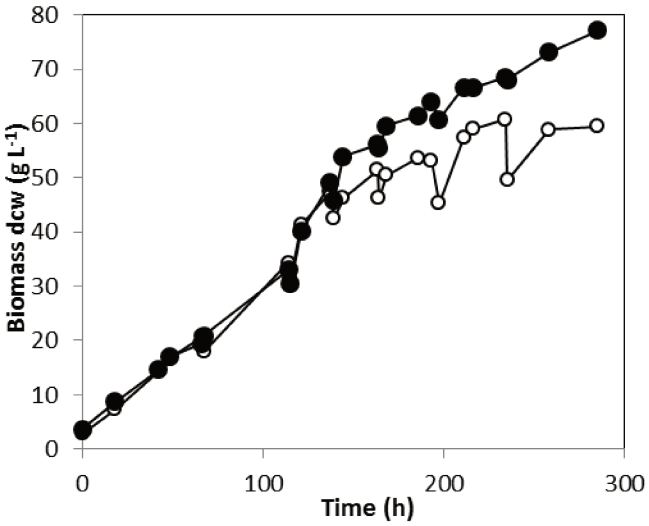
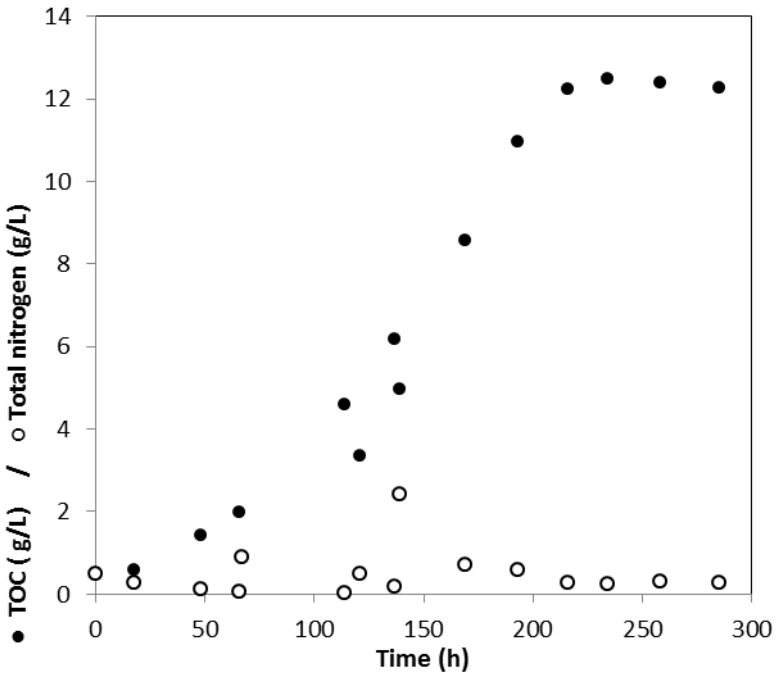
Supplementary data



Annex I. Substrate inhibition growth curves at different initial glucose concentrations



Annex II. Comparison of biomass concentration results between optical density analysis (○) and dry cell weight (●) for Pulsed-feeding cultivation



Annex III. Organic carbon concentration (excluding carbon from glucose) and total nitrogen concentration of Pulsed-feeding cultivation

Annex IV. Results of alternative separation methods during extraction of lipids.

As alternative methods to centrifugation, heating enhanced phase separation only at 60 ºC, while salt addition (Na_2SO_4 and NaCl) was completely ineffective in breaking the emulsion. The addition of the surfactant Tween 80 had a positive joint effect with temperature at the concentration of 5 mL per liter of biomass. The positive effect of the surfactant for breaking the emulsion may be due to a change in Hydrophilic Lipophilic Balance (HLB) of the emulsion and/or due to changes in viscosity of the liquids [67].

Chapter 4

High cell density cultures of microalgae under fed-batch and continuous growth

This chapter was published as:
Coelho, R.S., Vidotti, A.D.S., Reis, E.M., and Franco, T.T., **High cell density cultures of microalgae under fed-batch and continuous growth**, in Chemical Engineering Transactions 2014 p. 313-318

High cell density cultures of microalgae under fed-batch and continuous growth

Abstract

Microalgae are pointed as one of the best alternatives for biofuels production due to high lipid accumulation capacity. However, large scale production often results in low cell concentrations and low volumetric productivities. Heterotrophic cultures of microalgae can reach high biomass (over 100 g L^{-1} DCW - dry cell weight) and lipid (over 50 % w/w) concentrations reducing downstream costs and increasing volumetric productivities. In this work fed-batch and continuous systems were evaluated for heterotrophic high cell density (> 10 g L^{-1}) cultures of microalgae *Chlorella sp.* for biomass and lipids production. In fed-batch growth a two stage feeding strategy (nitrogen sufficient and nitrogen limited) was employed. For continuous cultures different dilution rates were evaluated. Fed-batch culture achieved maximum biomass concentration and productivity of 58.8 g L^{-1} DCW and 6.6 g L^{-1} d^{-1}, respectively. Under nitrogen limitation an increase of 38 % in lipid content (from 21 to 29 % w/w) and 26 % in overall lipid productivity (from 1.25 to 1.58 g L^{-1} d^{-1}) was observed. Continuous growth of microalgae started at a biomass concentration of 30 g L^{-1} DCW. At the dilution rate of 0.02 h^{-1} biomass concentration remained around 19 g · L^{-1} DCW with a lipid content of 18 % under steady state conditions, resulting in biomass and lipid productivities of 9.1 and 1.5 g L^{-1} d^{-1}, respectively. The results show that high biomass and lipid productivities can be attained in batch and continuous processes when high cell densities are applied.

1. Introduction

Most of the research on microalgae is concentrated on photosynthetic metabolism, which is limited by light irradiance and mutual shading of cells at high cell concentrations. Nevertheless, heterotrophic microalgae can be grown in the dark in the presence of an organic carbon source and potentially deliver higher concentrations of biomass and lipids [1].

Harvesting is considered to be the most costly and energy intensive stage in microalgae biomass production due to the low biomass concentrations achieved in traditional autotrophic cultivation [2]. Raceway ponds can typically achieve 0.5 - 1.0 g L^{-1} DCW (dry cell weight) while tubular photobioreactors will generally reach a maximum of 4 g L^{-1} DCW [3]. Higher concentrations produced under heterotrophic conditions can significantly reduce harvesting costs and energy use by reaching concentrations as high as 100 g L^{-1} DCW [4].

High cell density cultures are usually those with dry cell weight values from 10-20 g L^{-1} up to more than 100 g L^{-1}, however there is no clear definition for the term in literature [5, 6]. For microalgae, in recent literature the term has been used for microalgae concentrations as high as 20 g L^{-1} [7].

High cell densities of microorganisms are typically achieved with fed batch cultivation methods. These are based on feeding highly concentrated nutrient solutions in bioreactors where the biomass is retained. The highest microalgal concentrations found in literature were attained using fed batch heterotrophic processes [7].

An alternative to fed batch operation is the continuous process, which is usually highly productive due to the reduced process downtime for cleaning, sterilization and setup when compared with batch and fed batch processes. Also, since it operates in steady state conditions, control of the process can be more simple and precise. Most studies involving continuous microalgae cultivation are done in photobioreactors in autotrophic conditions. Few studies are found in literature for heterotrophic continuous cultures of microalgae and even fewer operate at high cell densities.

The objective of this study is to study both fed batch and continuous cultivation mode for obtaining high cell densities of microalgae and high productivities of biomass and lipids.

2. Materials and Methods

2.1 Microalgae strain and cultivation methods

The *Chlorella sp.* strain was obtained from Canadian Phycological Culture Centre. Stock cultures were maintained axenically on synthetic modified BBM medium with the following composition (mg L^{-1}): Na_2EDTA (50), KOH (3.1), $CaCl_2.2H_2O$ (25), $MgSO_4.7H_2O$ (75), K_2HPO_4 (75), KH_2PO_4 (175), NaCl (25), MoO_3 (0.71), $Fe_2SO_4.7H_2O$ (4.98), H_2SO_4 (1 µL/L), H_3BO_3 (11.42), $ZnSO_4.7H_2O$ (8.82), $MnCl_2.4H_2O$ (1.44), $CuSO_4.5H_2O$ (1.57), $Co(NO_3)_2.6H_2O$ (0.49) [8]. Glucose was added as carbon source at concentration of 10 $g.L^{-1}$. $NaNO_3$ was added as nitrogen source as a function of carbon source to achieve the C/N ratio of 20). pH was adjusted at 6.8.

2.2 Fed-batch cultivation

Fed-batch cultivation was carried out in 3 L bioreactor (BioFlo III - New Brunswick Scientific-Edison N.J. U.S.A.) with 2 L of culture medium. The culture was inoculated with an initial *C. vulgaris* biomass concentration of 2 g L^{-1} grown heterotrophically. The initial glucose concentration was 10 g L^{-1}. The experiments were carried at 26 ºC, aeration rate of 1 vvm (volume of gases per volume of culture per minute), in dark and at initial agitation rate of 150 rpm.

Whenever high oxygen intake was observed, the agitation rate varied between 150-500 rpm to ensure that DO (dissolved oxygen concentration) remained above 20 % of saturation.

The feeding strategy was divided in two phases. In the first phase (nitrogen sufficient) substrate concentrations were maintained within inhibition ranges (below 10 g L^{-1} glucose) by feeding medium with the same composition described in item 2.1, but with all components concentrated 30 fold to allow higher final biomass concentrations. An increase in DO over 40% saturation indicated substrate depletion. Feeding pump was then activated to provide substrate (DO-stat strategy). When DCW reached approximately 50 g L^{-1}, nitrogen limited feeding started. Feeding medium composition was changed to a glucose solution at concentration of 500 g L^{-1} without any other nutrients to induce a high carbon to nitrogen ratio.

2.3 Continuous cultivation

Continuous cultures started in a fed batch mode to concentrate biomass up to 30 g L^{-1} DCW following the process described in item 2.2. Once the culture reached this concentration, fresh sterile medium feeding started while broth containing biomass was harvested at the same flow rate to maintain constant volume. Feeding flow was adjusted according to the desired dilution rate. Feeding medium was prepared according to the composition described in item 2.1 but with all components concentrated 6 fold. Steady state was considered after 3 residence times. Four different dilution rates were evaluated: 0.005, 0.01, 0.015 and 0.02 h^{-1}. The range tested was based on

2.4 Analysis

Glucose content in the broth was analyzed using a commercial enzymatic glucose-oxidase assay kit by Bioliquid®.

Biomass dry cell weight was measured by filtering a 5 mL sample of the culture broth through a 0.22 µm porosity membrane and drying of the filter in an oven at 50 °C until constant weight.

For lipid content determination, cells were harvested by centrifugation at 8000 g for 10 min, frozen at -80 °C and freeze-dried at -30 °C and 50 mmHg. 400 mg of lyophilized biomass were digested in 5 mL of HCl 2 mol.L^{-1} at 80 °C during 1h. After digestion, extraction process started with the centrifugation (500 g for 2 min) of the digested biomass and addition of 4 mL of methanol, 2 mL of chloroform, and 3.6 mL of distilled water to the biomass pellet. The mixture was agitated (2 min) and centrifuged (500 g for 2 min). The lower phase containing chloroform and lipids was transferred to a test tube. Re-extraction was carried out in upper phase by addition of 4 mL of a 10% v/v methanol:chloroform solution, followed by agitation and centrifugation (500 g for 2 min). The lower phase was added to the test tube and the chloroform was removed in a rotary vacuum evaporator. The remaining material (total lipids) was left in the oven at 50 °C for 24 h, and the cooled down to room temperature and weighed. **2.5 Calculations**

Biomass productivity (P_X) is the relation of the variation of biomass concentration (X) in time:

$$P_X = \frac{X_{final} - X_{initial}}{t} \quad (1)$$

In continuous processes (substrate limited) where the growth is kept exponential at specific growth rate (μ) equal to the dilution rate (D), biomass productivity can also be expressed as:

$$P_X = \mu \cdot X = D \cdot X \quad (2)$$

The lipid content of the microalgae was calculated by the ratio between lipid weight (W_L) and dry biomass weight (W_X).

$$\%\text{Lipid} = \frac{W_L}{W_X} \quad (3)$$

Lipid concentration [Lipids] is calculated as the product of biomass concentration (X) and Lipid content (%Lipid):

$$[Lipids] = X \cdot \%Lipid \quad (4)$$

Lipid productivity (P_L) was determined as a function of biomass productivity and lipid content:

$$P_{Lipid} = P_X \cdot \%Lipid \quad (5)$$

Defatted biomass is calculated as the difference of X minus L:

$$DefX = X - L \quad (6)$$

3. Results and Discussion

3.1 Fed batch cultivation

Figure 1 shows biomass, substrate and lipids (during nitrogen limitation phase) profile during fed batch cultivation of *C. vulgaris*. Maximum specific growth rate (μmax) was observed during batch phase in the first 48 hours reaching the value of 0.035 h^{-1}. DO controlled feeding started after substrate depletion around 48 hours. During DO-stat mode the residual concentration of glucose inside the bioreactor remained at values lower than the analysis sensitivity. After 7 days of cultivation DCW reached 50 g L^{-1} and feeding medium was changed for diluted glucose (500 g L^{-1}) to induce nitrogen exhaustion. Two feeding pulses of glucose were applied in the lipid accumulation phase.

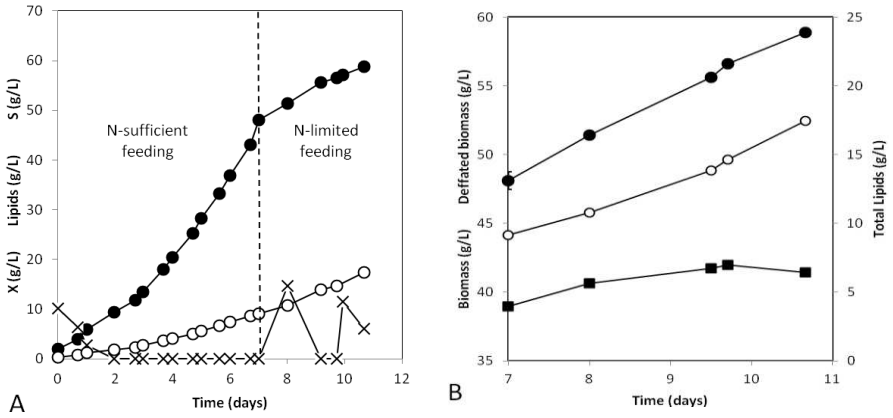


Figure 1. A : Biomass, Substrate and Lipid profiles under fed batch heterotrophic growth of C. vulgaris. -●- DCW; -○- Lipids; -x- Glucose. B: Composition of biomass under second feeding phase (Nitrogen limited). -●- Biomass; -○- Lipids; -■- Defatted biomass.

At the end of nitrogen sufficient feeding stage, DCW reached 48 g L^{-1} in 7 days cultivation with a global productivity of 6.9 ± g L^{-1} d^{-1}. Lipid content at this stage was determined as 18.9 % (w/w) with a global lipid productivity of 1.3 g L^{-1} d^{-1}.

Final lipid content at the end of the nitrogen limited stage reached 30 ± 1 % (w/w) of a final biomass of 58.9 ± 0.4 g L^{-1}. Data are summarized in Table 1. It is noteworthy that defatted biomass (Total biomass - Lipids) remained practically constant throughout nitrogen limited phase. This behavior was expected since nitrogen depleted conditions favours lipid accumulation and reduce amino acids and proteins synthesis [9], which constitute a large portion of lipid-free biomass. Lipid productivity and total lipid concentration almost doubled during second stage (1.8 and 1.9 fold, respectively).

Table 1. Summary of parameters from fed batch heterotrophic growth of C. vulgaris.

Parameter	Final N-sufficient stage	Final N-limited stage	Global
X (g L^{-1})	48.2 ± 0.5	58.9 ± 0.5	-
Defatted Biomass (g L^{-1})	38.9 ± 0.4	41.4 ± 0.3	-
Lipid concentration (g L^{-1})	9.1 ± 0.3	17.5 ± 0.6	-
P_x (g L^{-1} d^{-1})	6.9 ± 0.2	2.9 ± 0.1	5.5 ± 0.1
P_{Lipid} (g L^{-1} d^{-1})	1.3 ± 0.1	2.3 ± 0.1	1.6 ± 0.1

X = biomass concentration in dry cell weight, Defatted biomass = X - Lipid concentration, Px = biomass productivity, P_{Lipid} = Lipids productivity

Global productivity of biomass and lipids reached 5.5 and 1.6 g L^{-1} d^{-1}, respectively. The optimization of the duration of each phase for maximum biomass and lipid productivity may allow for even higher values.

3.2 Continuous cultivation

All experiments in continuous mode started at 30 g L^{-1} DCW as initial concentration. DCW at steady state decreased for higher dilution rates, as well as the lipid content. The average biomass concentration and lipid content are shown in Figure 2. Although biomass concentration decreased, the global productivity increased significantly.

Similar behavior was observed in literature for different microorganisms. When the microalgae *Schizochytrium limacinum* was grown in continuous cultures for DHA production both biomass and total fatty acids concentrations decreased with increasing dilution rates [10]. For the yeast *Pichia angusta* DL-1 a significant decrease in biomass concentration was observed with increasing dilution rates [11].

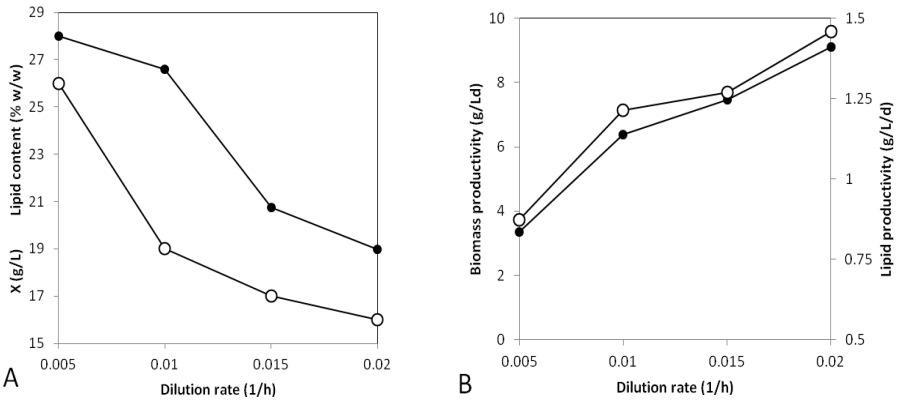


Figure 2. A: Biomass and lipid content profiles for different dilution rates during continuous heterotrophic growth of C. vulgaris -●- DCW; -○- %Lipids (w/w). B: Biomass and lipid productivities for different dilution rates. -●- Biomass productivity; -○- Lipid productivity.

Lipid content decreased significantly with increasing dilution rates. Data for continuous experiments are summarized in Table 2. Changes of lipid concentrations as function of dilution rate were observed for different microorganisms as well. Papanikolaou and Aggelis [12] observed a reduction in lipid content with increasing

dilution rates when growing the yeast *Yarrowia lipolytica* in chemostats for lipids production. The authors noted that the high growth rates forced by higher dilution rates may change the metabolic state of the cells favoring the flux of carbon to lipid free materials, such as amino acids and nucleic acids.

Table 2. Summary of steady state parameters of continuous heterotrophic cultivation of C. vulgaris.

	Dilution rate (h^{-1})			
Parameter	**0.005**	**0.01**	**0.015**	**0.02**
X (g L^{-1})	28 ± 1	26.6 ± 0.6	20.7 ± 0.5	18.9 ± 0.9
Lipids conc. (g L^{-1})	7.3 ± 0.2	5.1 ± 0.2	3.5 ± 0.1	3.0 ± 0.2
Px (g L^{-1} d^{-1})	3.4 ± 0.1	6.4 ± 0.1	7.5 ± 0.1	9.1 ± 0.1
P_{Lipid} (g L^{-1} d^{-1})	0.92 ± 0.03	1.22 ± 0.05	1.3 ± 0.1	1.5 ± 0.1

X = biomass concentration in dry cell weight, Px = biomass productivity, P_{Lipid} = Lipids productivity

Comparable productivities of lipids and even higher biomass productivities were achieved in continuous cultivation when compared with fed batch. However, the highest productivities were obtained at the condition of lowest biomass and lipid titers. This may seem contradictory but even at lower concentrations of biomass and lipids, the higher dilution rate results in an overall larger production. The downside of this condition is that, as cited before, lower concentrations lead to higher downstream costs and energy use. This shows how complex the selection of the production regimen may be.

4. Conclusion

Both fed batch and continuous cultivation methods could yield high productivities at high cell densities. Fed batch cultivation achieved the highest biomass and lipids concentrations (58.9 and 17.5 g L^{-1}, respectively). High biomass concentrations are desirable for reducing costs and energy use on cell harvesting. Similarly, higher lipid contents helps reducing downstream extraction costs.

Continuous growth, on the other hand, resulted in higher biomass productivity (9.1 g $L^{-1}\cdot d^{-1}$ at D = 0.02 h^{-1} against 6.9 g L^{-1} d^{-1} in fed batch) and similar lipid productivity (1.5 g $L^{-1}d^{-1}$ against 1.6 g $L^{-1}\cdot d^{-1}$) when compared with the fed batch experiment. Although the lower biomass titer and lipid content may increase downstream costs, continuous production usually results in lower upstream costs due to the reduced downtime for cleaning, sterilization and setup. Upstream and downstream conditions may also vary if other co-products besides lipids are recovered from the biomass, such as chemicals for pharmaceutical and nutraceutical industry [13]. If lipids are not the most valuable product, growth conditions can be designed to reduce lipid content favoring non lipidic material synthesis. From the economical point of view all these variables should be considered during process design.

5. Acknowledgements

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Chapter 5

Influence of C/N ratio and dilution rate on biomass productivity and composition of heterotrophic microalgae

This chapter will be submitted as:
Coelho, R. S.; Franco, T. T. **Influence of C/N ratio and dilution rate on biomass productivity and composition of heterotrophic microalgae**

Influence of C/N ratio and dilution rate on biomass productivity and composition of heterotrophic microalgae

Abstract

Microalgae have been considered for decades to be one the most promising feedstocks for biofuels due to their fast growth and a high oil content. However, production costs are still very high and scaling up of photobioreactors remains a major challenge. Differently from their photosynthetic counterparts, heterotrophic microalgae have been successfully explored for lipid and biomass production due to the high biomass concentrations and lipid productivities obtained. For biofuels, however, profit margins can be very low and thus production costs must be significantly reduced. Continuous cultivation at high cell densities allows for reduced downtime and high volumetric productivities, resulting in lower production costs. In addition, the steady state nature of continuous processes allows the accurate evaluation of process conditions effects over biomass production and composition. In this study, the continuous cultivation of a strain of *Chlorella vulgaris* at high cell densities was evaluated and optimized. Carbon to nitrogen ratio and dilution rate effects on biomass and lipid productivities and on fatty acid composition were evaluated through a central composite rotational experimental design (CCD) and response surface analysis. Based on the results obtained, predictive models were built for biomass and lipid productivity and composition. The fatty acid composition of microalgae was significantly altered by different cultivation conditions and biodiesel quality parameters were predicted from the experimental runs. All models were validated by comparing predicted and experimental runs performed at the optimized lipid productivity conditions, which reached 8.95 g L^{-1} d^{-1}. The models could accurately predict the validation results and the maximum relative error among all model predictions was 8.6 %.

1. Introduction

Microalgae are considered to be a promising alternative feedstock for biofuels, due to some advantageous characteristics such as high lipid yields, potential use of contaminated land and water for cultivation and integration with carbon dioxide sources [1]. Estimated potential equivalent oil productivities vary between 20,000 and 120,000 L ha^{-1} yr^{-1} of oil, which are significantly higher than for the most productive agricultural oleaginous crops such as palm oil, which yields up to 6,000 L ha^{-1} yr^{-1} [2, 3].

Yet, for the feasible production of biofuels from microalgae, production costs must still decrease by an order of magnitude [4]. Although microalgae can grow with doubling times as short as 3.5 h under optimal conditions, self-shading of the cells limits light penetration as biomass concentration increases, which leads to low biomass productivities and high production costs [5, 6]. Heterotrophic microalgae can grow in the dark in the presence of organic carbon sources and thus are not limited by light irradiation, which allow them to grow up to high biomass densities [7]. Although the need of carbon sources like glucose can represent high economic and environmental costs, the growing investment in cellulosic biomass hydrolysis and the potential use of wastewater as substrate indicates that there may be low cost alternatives for heterotrophic microalgae production [8]. Most of the studies about heterotrophic microalgae utilize fed-batch systems in order to improve biomass and lipids concentration, as the biomass is retained and accumulates in the bioreactor [9]. However, a precise and reliable evaluation of actual lipid productivity can also be obtained from steady state continuous cultivations, since all parameters remain constant, allowing a more accurately evaluation of single parameters effects [10]. In a previous study, chemostat cultivation at high cell densities and different dilution rates were compared with fed-batch cultivation of heterotrophic *Chlorella vulgaris* for biomass and lipid production. The results showed that comparable productivities were achieved for both cultivation modes, and suggested that continuous cultivation and lipid production could be optimized if a wider range dilution rates was tested and nitrogen limitation conditions were applied. [11].

It has been shown that the chemical composition of microalgae lipids, including fatty acids chain lengths and unsaturation degree, may vary with the age of the culture and nutrient availability conditions, such as the carbon to nitrogen (*C:N*) ratio [12, 13]. The manipulation of these variables may thus allow the targeted production of specific fatty acids and improve overall productivities.

In this study we present an evaluation of biomass and lipid productivities and fatty acid composition of microalgae *Chlorella vulgaris* CPCC 90 through response surface methodologies in chemostat cultivation. Steady-state operation in chemostat systems is used for the accurate evaluation of cell aging and nutrient availability effects over cell growth and compositional changes.

2. Material and Methods

2.1 Microorganism and inoculum preparation

The microalgae strain *Chlorella vulgaris* CPCC90 was purchased from the Canadian Phycological Culture Centre (CPCC). Stock cultures were maintained axenically on synthetic modified BBM medium with the following composition (mg.L^{-1}): Na_2EDTA (50), KOH (3,1), $CaCl_2.2H_2O$ (25), $MgSO_4.7H_2O$ (75), K_2HPO_4 (75), KH_2PO_4 (175), NaCl (25), MoO_3 (0.71), $Fe_2SO_4.7H_2O$ (4.98), H_2SO_4 (1 µL/L), H_3BO_3 (11.42), $ZnSO_4.7H_2O$ (8.82), $MnCl_2.4H_2O$ (1.44), $CuSO_4.5H_2O$ (1.57), $Co(NO_3)_2.6H_2O$ (0.49) (Stein-Taylor, 1973). The basal medium for maintenance and cultivation was the modified Bold's Basal Medium (BBM) prepared as described above but supplemented with 10 g L^{-1} glucose and 1.21g L^{-1} $NaNO_3$ to allow heterotrophic growth.

All feeding medium used in fed-batch and continuous cultivation was prepared with same composition described above, but concentrated 6 times, that is, the concentration of all nutrients was six times higher than in the maintenance medium, with the exception of $NaNO_3$. The nitial glucose concentration of feeding medium was 60 g L^{-1} and different *C:N* ratios were obtained by changing nitrogen source $NaNO_3$ concentration.

All experiments started with the same inoculum preparation procedure: cells were transferred from agar slants to shake flasks containing modified BBM medium with 10 g L^{-1} of glucose and 1.2 g L^{-1} of sodium nitrate (final *C:N* ratio of 20 g of carbon for 1 g of nitrogen) and incubated at 26 ºC and agitation rate of 150 rpm for 4 days. The inoculum culture was then transferred to the cultivation vessels (shake flasks or bioreactors). The transferred volume was based on the desired initial biomass concentration.

2.2. Preliminary experiments

Preliminary experiments in batch and continuous mode were performed for setting the initial operating conditions and for the estimation of kinetic parameters.

Batch experiments in shake flasks were used for a preliminary evaluation of the effect of *C:N* (w/w) ratio over biomass growth and lipid accumulation. The culture medium was prepared by mixing modified BBM with stock solution of glucose (500 g L^{-1}) and sodium nitrate (powdered) in order to reach varying *C:N* ratios: 20, 40 and 60. All batch experiments were performed in 250 mL shake flasks at 26 ºC and agitation rate of 150 rpm in triplicate. Initial biomass concentration was 0.4 ± 0.05 g L^{-1} dry cell weight for all flasks.

For preliminary chemostat cultivations additional dilution rates to those reported in our previous work (Chapter 4) were tested. In that work a range of dilution rates were tested up to 0.02 h^{-1}, with the latter resulting in the highest lipid productivity [14]. It was uncertain, however, if even higher dilution rates would result in higher productivities. Therefore, additional dilution rates of 0.03 and 0.04 h^{-1} were applied to the continuous culture. For each condition, samples were collected for biomass and lipid analysis until steady-state was reached (see section 2.3), after which flow rates were modified and the system was again allowed to stabilize.

2.3 High cell density continuous cultivation

Continuous cultures were initially grown in fed-batch mode to reach high cell density in a 5 liter New Brunswick Scientific Bioflo 115 fermentor. Dissolved oxygen tension (DO), pH and temperature were controlled with built-in sensors. Initial conditions were set as T = 25 ºC, pH 7.0 and stirring speed of 150 rpm. The vessel was sterilized at 121 ºC for 30 min prior to inoculation. Cultivation of *C. vulgaris* started with initial glucose concentration of 40 g L^{-1}. After glucose depletion, feed medium (same composition as described in section 2.1, but concentrated 30 times) was added automatically through a feedback DO-Stat system: whenever a DO increase over 40%, saturation was detected (indicating glucose depletion), the feeding pump was activated, causing a sudden decrease of DO, after which the pump stopped. To avoid DO decrease below 10 % saturation, a cascade loop control was used, increasing agitation rate up to 600 rpm. Biocommand® software was used for cultivation data processing and record as well as cascade loop control.

When biomass concentration reached 30 g L^{-1} dry cell weight, continuous feeding of medium through a peristaltic pump started.

Simultaneously, harvesting of the medium was done by a second peristaltic pump, activated by a height contact sensor at the top of the liquid surface (the height of the sensor was calculated for total liquid volume of 5 liters). For each condition steady state was considered after at least three residence times and when biomass and glucose concentrations were stable for 24 h.

2.4 Experimental design

Optimum *C:N* ratios are crucial for enhancing lipid productivity, since lipid accumulation usually occurs with limited nitrogen and excess carbon sources [15]. However, there is a trade-off involving growth rates and lipid accumulation since limited nitrogen availability obviously results in lower growth rates and biomass productivity [16].

Different studies have evaluated the effect of *C:N* ratio and cell age over biomass and lipid production in batch cultivation assays. It was shown that carbon and nitrogen intake occur in different rates and vary between and within species under different growth conditions [17-19]. Moreover, in batch studies it is sometimes difficult to separate the effects of cell age and nutritional deficiencies as they occur simultaneously. Cell age also strongly affects fatty acid composition, thus imparting the final product quality [20]

To evaluate the effect of dilution rate and *C:N* ratio over biomass and lipids production, a design of experiments (DOE) strategy was applied. In central composite designs (CCD) there are three types of design points: factorial, axial and center points. Factorial or two-level designs (2^k runs where k is the number of factors) are useful for describing linear models and center points are often added to test the linearity of the model. If the curvature of the design is significant, then a second order model is necessary a few more independent runs must be added. For CCDs, the number of extra runs is determined by 2k and are called axial points [21]. A two factors central compositional design (CCD) was created with eleven experiments (2^2 runs + 2 · 2 axial points + 3 central points). The coded value array of experiments was designed with Software Statistica® 7.0 and is shown in Table 1.

Table 131. CCD design with 11 runs (22 runs + 2·2 axial point and 3 central points) with coded levels

Runs	Levels (coded)	
	C:N	D
1	-1	-1
2	1	-1
3	-1	1
4	1	1
5	-1,41	0
6	1,41	0
7	0	-1,41
8	0	1,41
9	0	0
10	0	0
11	0	0

The factors levels used in the experimental design were determined based on previous experiments and literature data. In our previous work, the highest lipid productivities were obtained at the highest dilution rate tested (0.02 h^{-1}), but no higher dilution rates nor nitrogen limitation conditions were tested then [11]. For oleaginous yeasts, it was shown elsewhere that the highest lipid contents were obtained at dilution rates around one third of the maximum growth rate [22]. This observation was confirmed in more recent studies, however the same studies showed that higher lipid productivities were obtained at higher dilution rates [23, 24]. In batch preliminary experiments described in the Results section of this study, the maximum specific growth rates observed were between 0.037 and 0.045 h^{-1} for different *C:N* ratios. Therefore, the lower end of the range for the dilution rate was determined as 0.01 h^{-1} as -1 and 0.008 h^{-1} as -1.41, which are lower than one third of the lower μ_{max} observed. Preliminary experiments also showed that dilution rates around 0.03 h^{-1} were close to wash-out, thus causing a rapid decrease of biomass concentration and productivity. To avoid this condition, the higher end of the range was

established at lower values, with 0.02 at +1 and 0.022 as +1.41. The real values were calculated proportionally to the coded values and are shown in Table 2.

Table 2. Coded levels and real values for CCD factors

	Levels				
Factor	-1,41	-1	0	1	1,41
C:N (w/w)	11,7	20	40	60	68,3
D (h^{-1})	0,008	0,01	0,015	0,02	0,022

Dilution rates were changed by increasing or decreasing the feeding flow rate while keeping the culture volume constant. Feed flows for each dilution rate were calculated according to equation :

$$F = D \times V$$

Where F (L h^{-1}) is the feeding flow rate, *D* (h^{-1}) is the dilution rate and V (L) is the liquid volume (kept constant at 5 L).

Response surface methodology was used to evaluate the effects of the two variables (factors) over different responses: biomass and lipid productivities, lipid content, fatty acid composition, and biodiesel quality parameters estimates.

2.5 Analytical methods

Cell growth was measured by absorbance reading of cell suspensions at 680 nm wavelength. The biomass concentration (gL^{-1}) was calculated by a previously established calibration curve. For the construction of the calibration curve, biomass dry cell weight was gravimetrically determined by filtering a 5 mL sample of the culture broth through a 0.22 µm porosity membrane and drying of the filter in an oven at 50 °C until constant weight. Biomass dry cell weight absorbance showed linear behaviour from 0 to 1.2 g L^{-1}. More concentrated samples were diluted to fit in the linear range.

Glucose content in the fresh filtered cell-free broth was analyzed using a commercial enzymatic glucose-oxidase assay kit by Bioliquid®. Samples were diluted with distilled water in order to reduce glucose concentration to lower than 2 g L^{-1} (limit of linearity of calibration curve). Next 10 µL of the samples were to 2 mL of Bioliquid® glucose oxidase reagent. The mixture was incubated at 37 °C for 10 min and cooled down before absorbance reading at 505 nm. Nitrate concentration was determined according to Collos *et al.* [25]. The filtered cell broth was diluted 20 times and analysed in a UV/vis spectrophotometer (Genesys™-10UV) at a wavelength of 220 nm with deionized water as blank.

For lipid content determination, cells were harvested by centrifugation at 8000 x g for 10 min, frozen at -80 °C in ultra freezer SO-LOW PV 85 and freeze-dried at -30 °C and 50 mmHg in lyophilizer LIOBRAS L101. 400 mg of lyophilized biomass were hydrolyzed in 5 mL of HCl 2 mol L^{-1} at 80 °C during 1h. After hydrolysis, extraction process started with the centrifugation (500 g for 2 min) of the digested biomass and addition of 4 mL of methanol, 2 mL of chloroform, and 3.6 mL of distilled water to the biomass pellet. The mixture was agitated (2 min) and centrifuged (500 g for 2 min). The lower phase containing chloroform and lipids was pippeted to a test tube. Re-extraction was carried out in upper phase by addition of 4 mL of a 10% v/v methanol:chloroform solution, followed by agitation and centrifugation (500 x g for 2 min). Chloroform was removed in a rotary vacuum evaporator and the remaining material (total lipids) was left in the oven at 50 °C for 24 h, cooled down to room temperature and weighed.

Fatty acid composition was determined through GC-MS analysis. Lipids were converted into fatty acid methyl ester (FAME) through in situ transesterification according to Lewis *et al.* [26] and then analysed using a GC/MS gas chromatography (Shimadzu, Japan) system according to Tapia *et al.* [27]

2.6 Estimation of biodiesel quality parameters

Experimental determination of fuel properties demand expensive and time-consuming procedures, as is the case of cetane number analysis [28]. Therefore the prediction of biodiesel quality based on the properties of its individual fatty acids alkyl esters has been widely studied [29]. Some of the most important biodiesel quality parameters that could be explained through predictive models are shown In Table 3.

Table 3. Estimation equation for biodiesel quality parameters from fatty acid alkyl ester composition

Property	Equation	Reference
Cetane Number	$CN = \sum \frac{X_{ME} \times CN_{ME}}{100}$	[30]
Oxidation stability (OSI)	$OSI = \Sigma X_{Me}\ (wt.\%) \cdot OS_{Me}$	[31]
Cold filter plugging point (º C)	$CFPP = 3.1417 \cdot (0.1 \cdot C16{:}0(wt.\%) + 0.5 \cdot C18{:}0\ (wt.\%) + 1 \cdot C20{:}0\ (wt.\%) + 1.5 \cdot C22{:}0\ (wt.\%) + 2 \cdot C24{:}0\ (wt.\%)) - 16.477$	[32]
Lubricity	$Lub = \sum \frac{X_{ME} \times Lub_{ME}}{100}$	[30]
Kinematic viscosity	$\ln(V) = \sum \frac{X_{ME} \times \ln(V_{ME})}{100}$	[33]
Heating value	$Hv = \sum \frac{X_{ME} \times HV_{ME}}{100}$	[32]

X_{Me} (wt.%) is the percentage of the fatty acid alkyl ester in the mixture, CN_{Me} is the measured cetane number of the fatty acids, OS_{Me} is the measured oxidation stability of the fatty acids; D_{Me} is the number of double bonds in each fatty acid; MW is the molecular weight of fatty acids. Lub_{Me} is the measured lubricity of each fatty acid; ln(v) is the logarithm of mixture viscosity; ln(v) is the logarithm of each fatty acids measured mixture viscosity Hv_{Me} is the measured heating values of each fatty acid.

Cetane number (*CN*) is one of the most important fuel quality properties and is related to the capacity of ignition of the fuel and combustion quality for diesel engines. Cetane numbers increase with increasing chains lengths and decreasing saturation. [34]. Oxidative stability is defined as the time necessary to degrade fatty acids up a certain extent under standard conditions [30]. Algae based biodiesel often present very poor oxidation stability due to high double bonds content found in microalgae fatty acids, and this is considered to be one of the most significant challenges facing algal biodiesel [35]. This property is influenced by a number of factors such as presence of air, light, traces of metal and storage conditions, however the most important factor for the auto-oxidation of biodiesel is the number and position of double bonds in the structure of the fatty esters [36]. Heating value or heat of combustion is defined as the energy liberated by the complete combustion of the fuel. It increases with the chain length and decrease with the degree of unsaturation of fatty acids esters in biodiesel [37]. Cold filter flow plugging point is used as low temperature filterability test for fuels and it is especially important for cold weather countries, as low temperatures may lead to the formation of wax crystals and cause filter blockage [38]. Lubricity is defined as the ability to reduce friction between moving parts of the engine. It is usually measured as the wear scar diameter created by rubbing a steel ball of 6 mm against a flat steel disc immersed in the fluid sample under standard conditions [39]. It is enhanced with longer chain lengths and higher degree of unsaturation, although other factors like the presence of free fatty acids or monoglycerides also have a strong impact over it [37]. Kinematic viscosity is of major importance for diesel fuels specification as it affects atomization of the fuel upon

injection in the combustion chamber of the engine, and the formation of engine deposits. Viscosity increases with chain length and increasing degree of unsaturation of fatty acids in the mixture [40].

For the calculation of quality parameters of fatty acids mixtures, properties of individual fatty acids were taken from literature and are described in Table 4.

Table 4. Fuel properties of individual fatty acids

Property	Fatty acid									
	C5	C16:0	C16:1	C16:2	C16:3	C17:0	C18:0	C18:1	C18:2	C18:3
CN	17.8	74.5	51	33.1	20.7	87.75	101	59.3	42.2	22.7
OSI (h)	40	24	2.1	0.25	0.07	24	24	2.79	0.94	0.2
Lub ws1.4 (µm)	357	357	246	211	168	339.5	322	290	236	183
v (mm^2/s)	0.526	4.32	3.67	4.08	2.59	4.53	4.74	4.51	3.27	3.14
Hv (kJ/kg)	28.12	39.47	39.32	39.14	38.86	39.785	40.1	39.93	39.72	39.37

Experimental values taken from Knothe [41], Knothe [42] and Gopinath et al. [37]. CN: cetane number; CFPP: Cold flow plugging point; OSI: oxidative stability index; dH: heating value; Lub: Lubricity; v: kinematic viscosity. See Table 3 for more information.

3. Results and Discussion

3.1 Preliminary experiments

3.1.1 Carbon nitrogen ratio preliminary tests

Preliminary *C:N* ratio tests were performed in shake flask batch cultures. Biomass composition was evaluated only at the end of the experiments, as samples collected during growth contained insufficient amounts of biomass for accurate analysis. Growth and residual substrate curves are displayed in Figure 2.

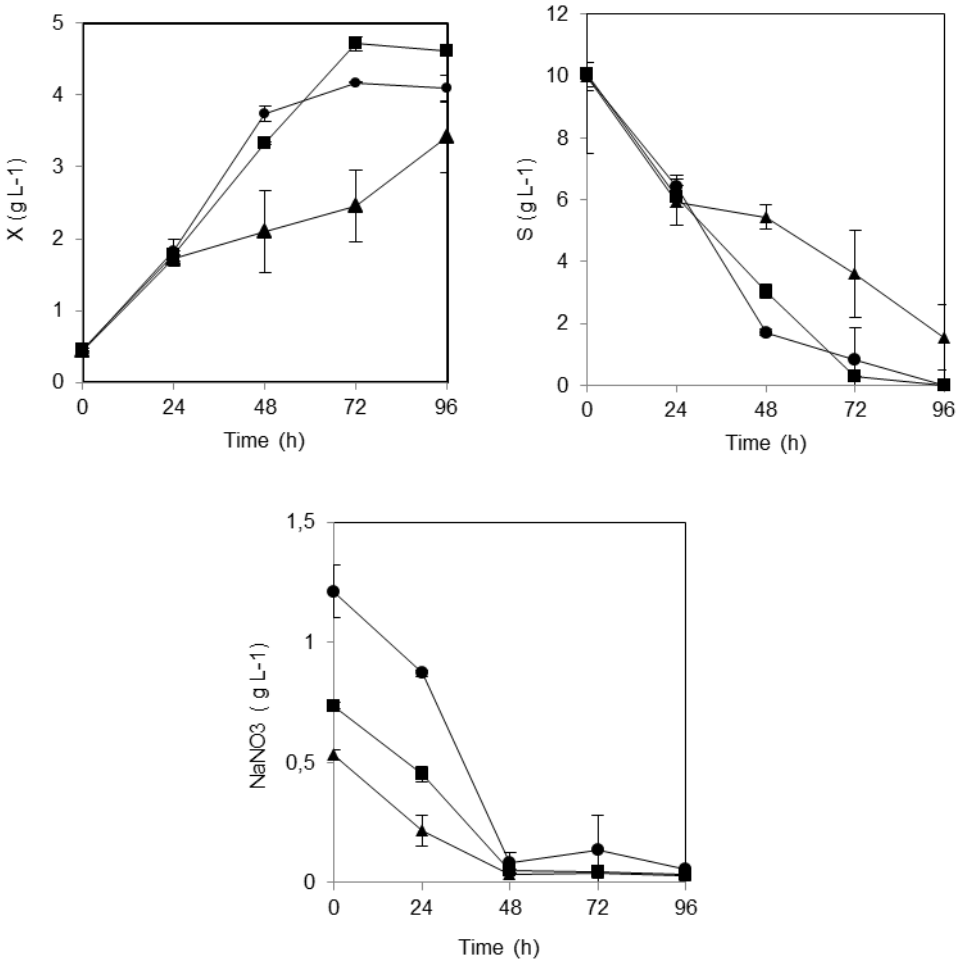


Figure 1. Biomass growth and substrate consumption profiles of preliminary batch experiments with varying C:N ratios (average values of triplicates). —● C:N 20; ■ C:N 40; —▲ C:N 60

Growth at *C:N* ratios 20 and 40 (w/w) showed similar behaviour, reaching maximum biomass concentrations of 4.2 and 4.7 g L^{-1} after 3 days of cultivation. Growth at *C:N* 60 (w/w) had specific growth rate similar to *C:N* 20 and 40 until the first day of cultivation, after which growth slowed down. After 4 days of cultivation glucose was not completely exhausted and the maximum biomass concentration was 3.4 g L^{-1}.

Table 5. Preliminary batch experiments results with different C:N ratios

C:N (w/w)	μ_{max} (h^{-1})	*X* (g L^{-1})	*Lipid* (%)	*Px* (g L^{-1} h^{-1})	P_{Lipid} (g L^{-1} h^{-1})
20	0.045 ± 0.001	4.1 ± 0.2	19.5 ± 1.9	0.038 ± 0.002	0.007 ± 0.004
40	0.041 ± 0.001	4.63 ± 0.07	32.6 ± 0.8	0.043 ± 0.001	0.014 ± 0.002
60	0.037 ± 0.007	3.4 ± 0.6	33.6 ± 0.4	0.031 ± 0.008	0.010 ± 0.008

Lipid content for *C:N* 40 and 60 was around 70 % higher than for *C:N* 20. Highest biomass and lipid productivities were highest for *C:N* ratio 40. Although the highest lipid content was observed for *C:N* ratio 60, the lower final biomass concentration resulted in lower lipid productivity.

Overall lipid productivity depends on the balance of lipid accumulation and cell growth. Nitrogen limitation conditions affect amino acid synthesis increasing the metabolic carbon flux towards lipid metabolism [43]. While this

effect can result in lipid contents as high as 65 % of dry cell weight [44], the declining protein synthesis slows down cell division rates and reduces overall biomass productivity [45].

The preliminary results showed that the optimum *C:N* ratio for lipid production must be somewhere around 40 (w/w). Biomass grown at *C:N* ratio 60 contained a slightly higher amount of lipids, however biomass growth was much slower in that condition.

3.1.2 High cell density chemostat cultures

The capacity of the microalgal cultures to maintain high cell densities under continuous mode was evaluated at varying dilution rates. The cultures started in fed-batch mode in order to reach the biomass density of 30 g L^{-1}, after which the cultures were cultivated continuously. Cultures reached steady-state after 3-5 residence times. An example of the fed-batch growth profile is shown in Figure 2.

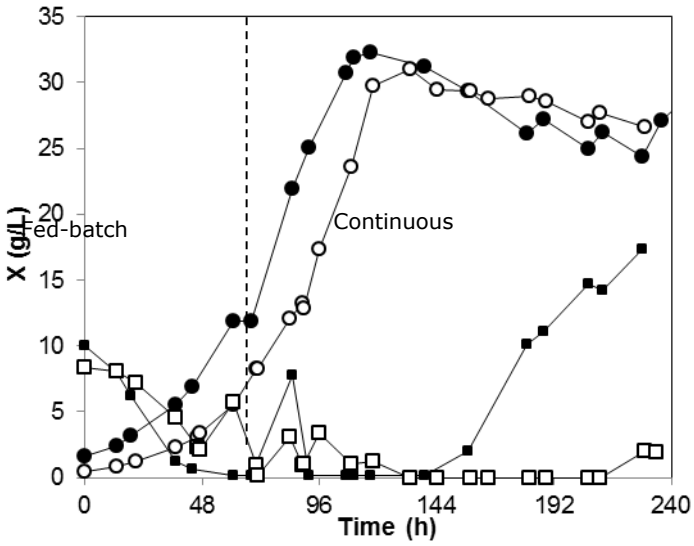


Figure 2. Growth and substrate profile of initial fed-batch and continuous process. Black circles biomass for D = 0.04 h^{-1}; black squares: glucose for D = 0.04 h^{-1}; White circles biomass for D = 0.03 h^{-1}; white squares: glucose for D = 0.03 h^{-1};

Washout of the culture was observed for D = 0.04 h^{-1}. Washout occurs whenever the rate of cell removal is greater than the rate of cell generation inside the bioreactor, causing the cell concentration inside the reactor drop to zero [46]. This occurs for a critical value of *D,* which is usually close to the maximum specific growth rate of the microorganism [47]. For D = 0.03 h^{-1}, the biomass concentration was considerably lower than the obtained for lower dilution rates. This was probably due to the fact that near washout conditions, continuous cultures are very sensitive to small changes in *D*, which can cause large variations in biomass concentration [48]. Moreover, the increasing substrate concentration may have caused inhibition effect over the microalgae. Strains of *Chlorella vulgaris* were found to be inhibited at glucose concentrations higher than 40 g L^{-1}, which was observed dilution rates 0.03 and 0.04 h^{-1} [49]

Table 6. Results of preliminary continuous cultivations with varying dilution rates and fixed C:N ratio

Dilution rate	Results			
D (h^{-1})	X (g L^{-1})	S (g L^{-1})	Lipid (%)	P_{Lipid} (g L^{-1} d^{-1})
0.01	26.6 ± 0.6	0	19.1	1.22 ± 0.05
0.02	15.4	27.6	15.8	1.5 ± 0.1
0.03	6.19	42.0	14.3	0.64 ± 0.2
0.04	Washout	60	-	-

Lipid content in the biomass decreased from 19.1 to 15.8 % (w/w) when dilution rate was increased from 0.01 to 0.02 h^{-1}, which corresponds to 17 % reduction (Table 6). A further increase to 0.03 h^{-1} resulted in a lower decrease of lipid content, from 15.8 to 14.3 % (w/w), which corresponds to 9 % less lipids. Basal lipid levels of *Chlorella* strains under nitrogen replete conditions are usually around 10-15 % (w/w) and may explain why the lipid content was not further decreased [44, 50]. An inverse relation between dilution rate and lipid content of cells was observed in different studies regarding oleaginous microorganisms [51, 52]. In chemostats, the culture is kept constantly in exponential growth and its specific growth rate is determined by the dilution rate applied. In favourable conditions for growth, cells tend to utilize carbon sources more efficiently for cell replication and, consequently, less carbon is accumulated as energy reserve [23].

3.2 Experimental design

3.2.1 Biomass and lipids production

The results of preliminary experiments were used for setting the ranges for the experimental design. Batch experiments showed that, for *C:N* 20, lipid accumulation was much lower than for *C:N* 40 and 60, while *C:N* 60 showed slower growth rates than *C:N* 20 and 40. Preliminary continuous experiments showed that dilution rates over 0.03 h^{-1} result in low biomass concentration and productivity. An example of the continuous growth profile of run number 10 (*C:N* = 40 and *D* = 0.015 h^{-1}) is shown in Figure 3.

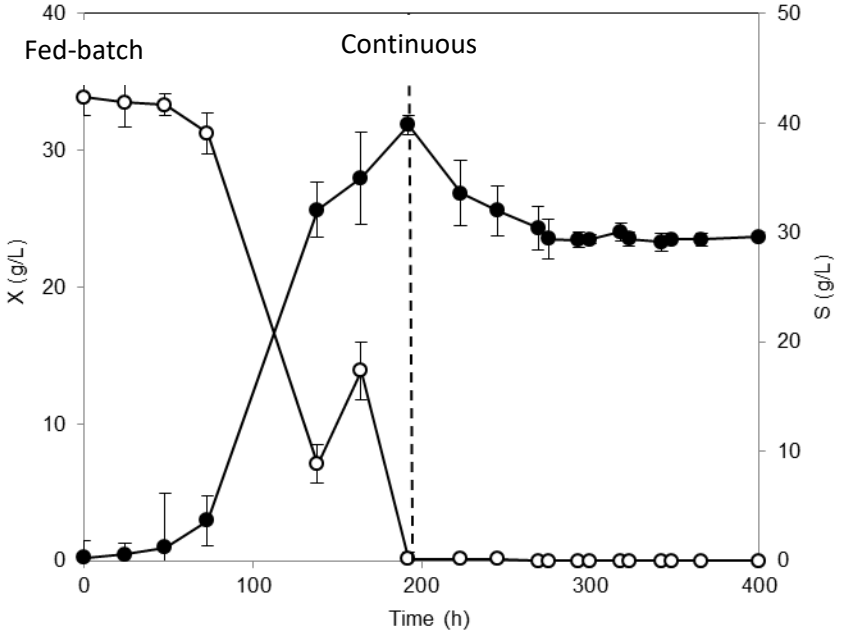


Figure 3. Continuous cultivation profile of Run 1 from CCD experimental design (C:N = 40 and D = 0.015 h^{-1}). —●— Biomass dry cell weight;—○—: residual glucose

The results of the CCD experimental runs are shown in Table 7. In none of the experimental conditions initial glucose content was completely exhausted. This was intended, as carbon source limitation would interfere in the variable effect analysis.

The highest glucose consumption and biomass concentration were observed in run 1 (*C:N* ratio = 20 and *D* = 0,01 h^{-1}), with *X* = 26.6 g L^{-1}. The lowest value of *X* was observed in run 6, when the culture was fed with the highest *C:N* ratio among the experimental runs. At the same time, the first condition reached 19% (w/w) lipid content, whereas the second reached 28.2% (w/w). These results are in agreement with the theory that, under nitrogen limitation, lower biomass concentrations are attained while greater accumulation of lipids occurs. Moreover, the first condition showed higher biomass productivity, while the second had higher lipid productivity.

Table 7. Biomass and lipid production experimental results

Runs	*C:N* (w/w)	*D* (h^{-1})	*X* (g L^{-1})	*S* (g L^{-1})	P_X (g L^{-1} d^{-1})	*Lipid* (%)	P_{Lipid} (g L^{-1} d^{-1})	*YLipid/S* ($g_{Lipid}/g_{glucose}$)
1	20	0.01	26.40 ± 1.0	4.8 ± 0.3	6.4 ± 0.3	19 ± 1	1.2 ± 0.3	0,09 ± 0.01
2	60	0.01	18 ± 2	19.9 ± 0.2	4.3 ± 0.5	29.7 ± 0.7	1.3 ± 0.5	0,13 ± 0.01
3	20	0.02	19 ± 1	9.6 ± 0.3	9.1 ± 0.5	16.0 ±	1.5 ± 0.5	0,06 ± 0.01
4	60	0.02	15.2 ± 0.3	29.5 ± 0.8	7.2 ± 0.2	19.0 ± 0.6	1.4 ± 0.2	0,09 ± 0.01
5	11.72	0.015	20.10 ± 0.25	17.8 ± 0.4	7.2 ± 0.2	16.7 ± 1.3	1.2 ± 0.2	0,08 ± 0.01
6	68.28	0.015	14.94 ± 0.21	25.2 ± 0.6	5.4 ± 0.1	28.2 ± 0.8	1.5 ± 0.1	0,12 ± 0.01
7	40	0.008	24.1 ± 0.4	14.3 ± 0.5	4.6 ± 0.1	19.3 ± 0.6	0.9 ± 0.1	0,10 ± 0.01
8	40	0.022	15 ± 1	26.0 ± 0.5	7.9 ± 0.5	15.6 ± 0.9	1.2 ± 0.5	0,06 ± 0.01
9	40	0.015	24.8 ± 0.6	7.65 ± 0.2	8.9 ± 0.3	22.0 ± 0.9	2.0 ± 0.3	0,10 ± 0.01
10	40	0.015	24.1 ± 0.6	7.1 ± 0.2	8.7 ± 0.3	22 ± 1	2.0 ± 0.3	0,10 ± 0.01
11	40	0.015	25.2 ± 0.6	8.1 ± 0.3	9.1 ± 0.3	22 ± 1	2.0 ± 0.3	0,11 ± 0.02

Comparing runs 1 and 2 with runs 3 and 4, we observe that the increase in dilution rate from 0.01 h^{-1} to 0.02 h^{-1} caused the decrease of both biomass and lipid concentrations but increase of biomass and lipid productivities.

The highest biomass productivity (9.1 g L d^{-1}) was observed for run 3 (*C:N* 20 and *D* 0.02 h^{-1}), while the highest lipid productivity was observed for the central point condition (*C:N* 40 and *D* = 0.015 h^{-1}).

Statistical effect analysis was applied to the experimental results and is shown in Table 8. The statistically significant effects (90%) confidence level are shown in bold letters, as calculated Student's t-test (significant when p-value < 0.10) (Rao & Iemma, 2005).

Table 8. Effects analysis of CCD experimental design

		Factors				
Response	Mean	*C:N*	$(C:N)^2$	D	$(D)^2$	*C:N* * D
Px	**0.371**	**-0.068**	**-0.098**	**0.109**	**-0.099**	0.005
p-value		**0.003**	**0.001**	**0.000**	**0.001**	0.773
Plipid	**0.082**	0.004	**-0.023**	0.008	**-0.036**	-0.003
p-value		0.241	**0.002**	0.058	**0.000**	0.556
X	**24.72**	**-4.94**	**-6.63**	**-5.82**	**-4.58**	2.43
p-value		**0.002**	**0.001**	**0.001**	**0.007**	0.102
Lipid	**22.22**	**7.48**	0.79	**-4.95**	**-4.52**	**-3.88**
p-value		**0.001**	0.554	**0.005**	**0.015**	**0.047**

The effect analysis indicates trends that occur in response to changes in factors' levels. Significant first order or linear effects can show positive or negative trends caused by increments of the factors. When second order or quadratic effects ($C:N^2$ and D^2) are significant, response behavior may be described by a second order model, and thus a maximum or a minimum value may exist within the variation ranges. The *C:N* x *D* interaction factor is related to the interdependence of the two variables and may indicate interaction effects, such as synergy or antagonism between them [53, 54].

For biomass concentration (X), the effects of the two linear variables were found to be negative and significant at 90 % confidence level, pointing to a negative trend of *X* with the increase of *C:N* and *D.* Lower *C:N* ratios were However, the second order effects of both variables were also negative and significant, which points to the existence of a maximum point for *X* (peak) within the ranges tested.

For the lipid content or P (%), there was a positive linear effect of *C:N* and a negative effect of linear *D,* confirming that lower dilution rates favor the accumulation of lipids. Also there was a significant effect of interaction between the two variables (*C:N* x D). It's not always clear to identify the physical meaning of interaction effects, but it might indicate that the effect of one factor depends on the level of another factor or, in other words, that there may exist synergy or antagonism between the variables [54].

The optimization of both biomass concentration and lipid content play an important role in downstream process, as biomass dewatering and lipid extraction can represent up to one third of the total production costs [55].

For the biomass productivity Px, the positive effect of linear factor *D* is expected, since, as already mentioned, biomass productivity is directly proportional to the dilution rate. The negative linear effect of *C:N*, on the other hand, is consistent with the theory that the lower availability of nitrogen reduces cell division and biomass growth.

For P_{Lipid}, in turn, the positive linear effect of *D,* significant at almost 95% confidence, confirms that lipid productivity tends to increase with increasing dilution rates. Interestingly, although important for lipid accumulation, the linear factor *C:N* had no significant effect over lipid productivity. However, both 2nd order effects of *C:N* and *D* showed significant effects which indicate the existence of a maximum point within the factors tested range.

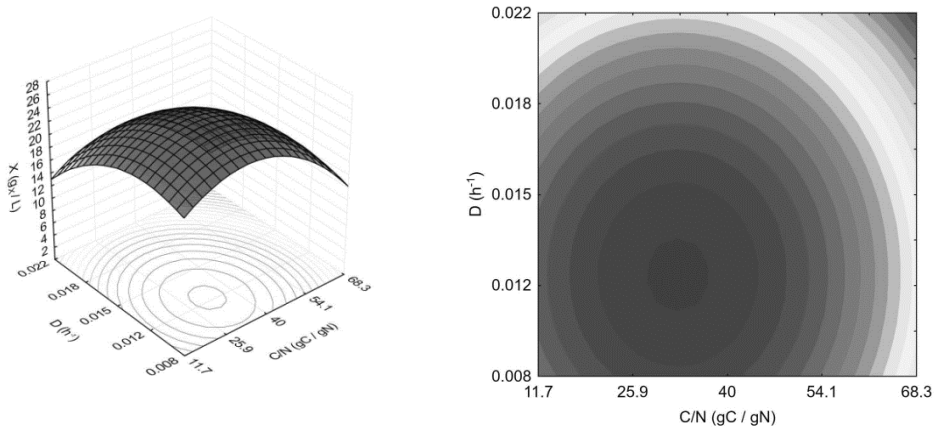
From the analysis of the response variables, the significant factors were selected for the design of statistical models through multiple regression of the data using the Statistica analysis software. The statistical significance of the models was calculated through the F-test for ANOVA (Table). The four models designed were statistically significant at 95 % confidence level, since the probabilities (p-value) of the regression were lower than 0.05. The coefficient of determination was higher than 0.93 for all models, which means that at least 93 % of the experimental results were explained by the variables.

There is no consensus about the value of the coefficient of determination (R^2) that indicates if a model is actory or not, but some authors state that a minimum of 0.7-0.85 should be considered [56]. The coefficient of determination alone is usually not sufficient to evaluate the adequacy of the fitted model and thus F-statistics in Anova table is usually performed to test for significance of the slope [57]. In our study both criteria were used to evaluate the fitted models, with a minimum value of 0.7 for R^2 and significance of model regression at minimum 90 % confidence level.

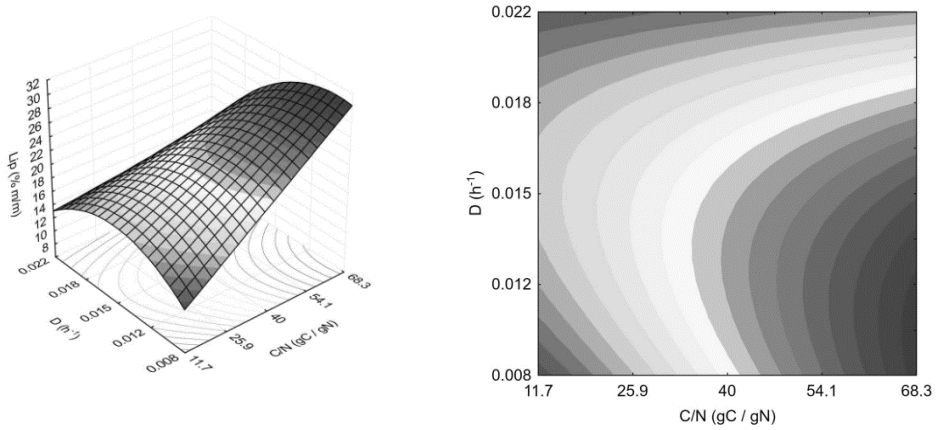
Table 9. Results of Anova evaluation of models

Factor	p-value Regression	p-value Lack of fit	R^2
X	0.001073	0.092926	0.934407
Lipid	0.000573	0.0124	0.946945
Px	8.422E-05	0.175142	0.972192
P_{Lipid}	0.000170	0.077629	0.933668

With the formulated models shown in Figure 3, response surfaces were built for each variable to illustrate the responses' behavior. The analysis of these surfaces allows the identification tendencies and optimum operation points for each response.

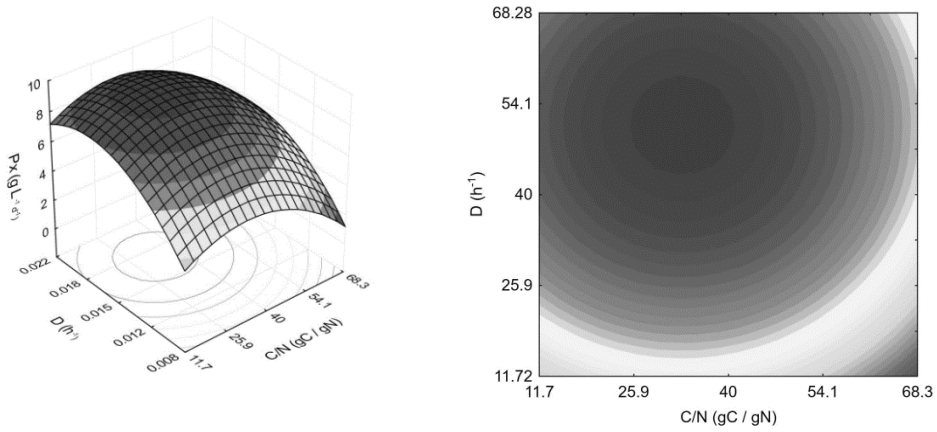


$$X = 24.718 - 2.470 \times C/N - 2.910 \times D - 3.315 \times C/N^2 - 2.290 \times D^2$$

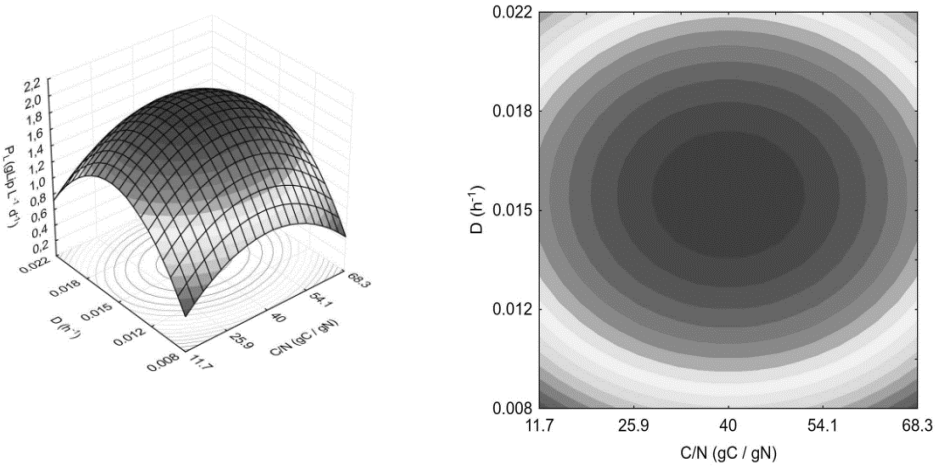


$$Lipid = 22.595 + 3.738 \times C/N - 2.474 \times D - 4{,}505 \times D^2 - 3{,}881 \times C/N \times D$$

Figure 4a. Response surfaces of biomass and lipids concentrations

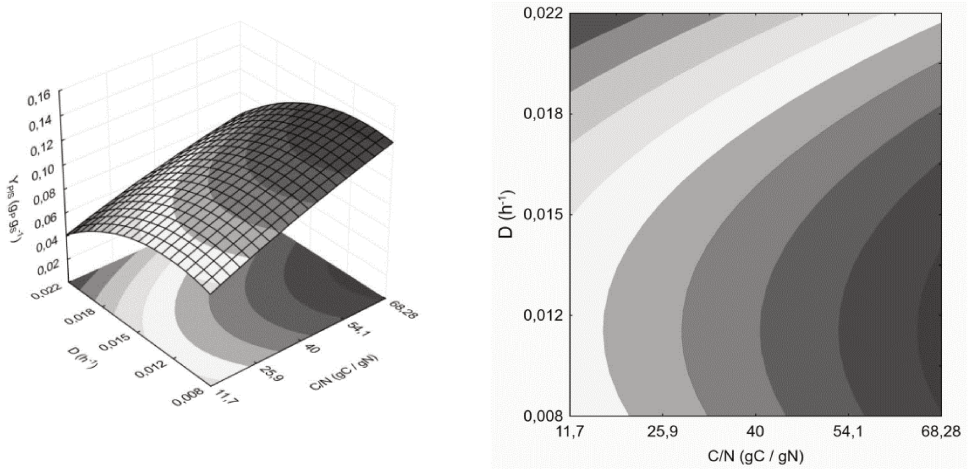


$$Px = 0.371 - 0.034 \times C/N + 0.054 \times D - 0.049 \times C/N^2 - 0.050 \times D^2$$



$$P_{Lipid} = 0.082 + 0.004 \times D - 0.011 \times C/N^2 - 0{,}018 \times D^2$$

Figure 4b. Response surfaces of biomass and lipids productivities



$$Y_{Lipid/S} = 0.103 + 0.017 \times C/N - 0.015 \times D - 0.009 \times D^2$$

Figure 34c. Response surfaces yield of lipids over substrate

For biomass concentration an optimal area was identified at levels of *C:N* ratios dilution rate lower than the central points (*C:N* 40 and 0.015 h^{-1}). This is in accordance with the fact that non-limiting nitrogen conditions (lower *C:N* ratios) and longer residence times (lower dilution rates) tend to favor the accumulation of biomass.

Maximum lipid productivity is in turn observed at lower dilution rates and higher values of C/N. These results are similar to the observed for other oleaginous heterotrophic organisms such as yeasts [24, 52]. Only the first order factor of the *C:N* ratio was significant at 90 % confidence level, and no maximum point was observed. This suggests that the lipid content could be increased if higher *C:N* ratios were utilized. Lipid productivity, however, reaches its maximum close to the central point of the experimental design. Since biomass productivity and lipid content are maximized in different conditions (higher *D,* lower *C:N* vs. lower *D* and higher *C:N*), it makes sense that the maximum lipid productivity is reached in a midpoint between the two conditions.

$$P_{Lipid} = P_x\left(\uparrow D; \downarrow \frac{C}{N}\right) x\, L\left(\downarrow D; \uparrow \frac{C}{N}\right)$$

For the lipid yield on substrate, C:N ratio had a strong positive linear effect with no maximum within the tested range, indicating that higher ratios could result in even higher yields. The dilution rate had a negative effect with a point of maximum yield around 0.011 h^{-1}. The maximum predicted yield based on the model described in Figure 4 was calculated as 0.13 $g_{Lipid}\ g_{glucose}^{-1}$. The predicted maximum yield, however, did not exceed the experimental results shown in Table 7. Although the lipid yield could probably be enhanced by increasing the C:N ratio, a large increment is unlikely and could probably be offset by the resulting lower lipid productivity. The maximum theoretical yield of lipids from sugars based on the energy content of product and substrate, is 37 % w/w, however experimental yields are usually around 2-17 % w/w [58]. This difference is caused by the use of substrate for cell mass growth and maintenance and production of extracellular compounds. Significant increase of the lipid yields is likely to be achieved with the use of metabolic engineering strategies, through overexpression of lipid and fatty acid synthesis related genes or repression of competing pathways, such as synthesis of phospholipids and starch [59].

The predicted optimum levels of *D* and *C:N* for each response were calculated by regression analysis and are shown in Table 10.

Table 10. Optimal conditions for CCD responses

Response	Maximum value	C/N (gC / gN)	D (h^{-1})
X (g L^{-1})	26,2	32,7	0,012
Lipid (%)	30,7	68,2	0,009
P_x (g L^{-1} d^{-1})	9,44	33,2	0,018
P_{Lipid} (g_{Lipid} L^{-1} d^{-1})	1,99	40	0,0175
$Y_{Lipid/S}$ (g_{Lipid} $g_{glucose}^{-1}$)	0.13	68.2	0.109

Figure shows the difference between the different maximum points for each response. The focus of this study is to determine optimal conditions for enhanced lipid productivity, as this is usually the most important parameter for process feasibility. However, it is known that a major component of biofuels process costs lies on downstream processing of lipids, which depends directly on biomass and lipid concentration [60]. In addition, lipid-free biomass is a protein-rich product with added value and potential to become an important nutritional source[61]. Therefore, high markets prices for single cell protein may stimulate the increase of low-lipid biomass productivity. Hence, the setting of operational conditions should depend on a more detailed cost analysis and our results show that *C:N* ratio and dilution rate can be manipulated to fit the best configuration possible.

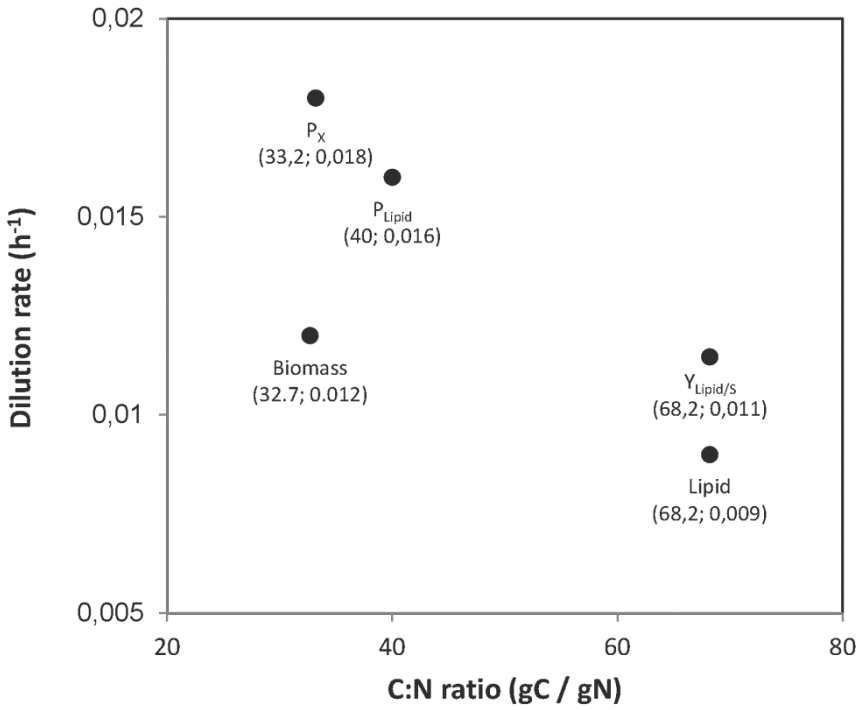


Figure 5. Optimal conditions for biomass and lipid concentrations and productivities.

3.2.2 Fatty acid composition

Fatty acid methyl ester composition was analysed for all CCD runs in order to evaluate the compositional changes with different *C:N* ratios and dilution rates. Tableshows the fatty acid compositions from *C. vulgaris* for all CCD runs. Lipid analysis showed the presence of ten different fatty acids, being four saturated (C5:0, C16:0, C17:0 and C18:0), two monounsaturated (C16:1 and C18:1), two di-unsaturated (C16:2 and C18:2) and two tri-unsaturated (C16:3 and C18:3). The predominant component for all runs was C18:1 (oleic acid) with an average of 43 ± 3 % of all fatty acids, followed by C18:2 (linoleic acid) with 15 ± 2 % and C16:0 (palmitic acid) with 12 ± 1 %. Lipid composition can greatly vary among strains of the same species. Some studies have found that the fatty acid profile of some strains of *C. vulgaris* can be composed of up to 82 % of saturated fatty acids [62], while other studies have found up to 68 % of polyunsaturated fatty acids in different strains of the same species [63]. This only goes to show the wide genetic variability and capacity for adaptation of these microorganisms and how growth conditions may affect lipid metabolism.

Table 11. Fatty acid composition of Chlorella vulgaris biomass produced in experimental runs of the CCD experimental design

	Fatty acids (%)									
Runs	C5:0	C16:0	C16:1	C16:2	C16:3	C17:0	C18:0	C18:1	C18:2	C18:3
1	1.5	13.1	1.6	0.2	2.1	4.2	7.6	46.2	16.1	7.5
2	0.8	11.0	1.5	0.2	4.2	3.5	8.1	45.8	13.0	11.9
3	1.5	13.6	1.1	0.3	1.6	5.8	8.3	42.3	18.9	6.6
4	1.7	14.7	0.9	0.3	4.2	5.0	7.2	36.9	15.3	13.8
5	2.3	11.9	1.0	0.3	1.6	4.5	6.8	46.8	18.4	6.4
6	1.2	12.7	1.0	0.3	4.4	4.0	8.4	41.8	13.2	13.0
7	1.3	13.6	1.8	0.0	1.6	3.7	12.6	47.2	12.3	6.0
8	2.0	11.0	1.3	0.2	1.9	4.0	12.2	47.3	13.9	6.2
9	1.6	12.6	1.2	0.3	4.1	4.7	6.8	41.3	14.7	12.7
10	1.5	13.0	1.2	0.3	4.1	4.7	7.1	41.0	14.5	12.6
11	1.6	12.1	1.3	0.3	4.1	4.8	6.5	41.6	14.9	12.7

Biodiesel quality parameters are basically affected by a) fatty acid chain lengths and b) saturation levels. In order to evaluate the variation of fatty acid composition of microalgae lipids, the percentages of saturated (*SFA*), mono (*MUFA*), di- (*DUFA*), tri- (*TUFA*) and total poly-unsaturated fatty acids (*PUFA*) were calculated, as well as the average fatty acids chain lengths (*AvgCL*) for all experimental runs (

Table).

It is usually considered that the presence of long chain polyunsaturated fatty acids (*PUFA*) is undesirable for biodiesel quality, as they tend to reduce the ignition capacity and oxidation stability of the fuel . Saturated fatty acids may be undesirable as well, as they have higher melting points and crystallize at low temperatures. Longer chain monounsaturated fatty acids such as palmitoleic (C16:1) and oleic (C18:1) acid have the most balanced characteristics for biodiesel quality and, therefore, it is preferred that biodiesel feedstocks contain predominantly this class of fatty acids.

Table 12. Percentages of fatty acids classes for CCD experimental runs.

Runs	*SFA* (%)	*MUFA* (%)	*DUFA* (%)	*TUFA* (%)	*PUFA* (%)	*AvgCL* (C-number)
1	26.3	47.8	16.3	9.6	25.9	17.43
2	23.4	47.3	13.2	16.1	29.4	17.52
3	29.2	43.3	19.2	8.3	27.5	17.42
4	28.6	37.8	15.6	18.0	33.6	17.33
5	25.5	47.8	18.7	8.0	26.7	17.36
6	26.3	42.7	13.5	17.5	30.9	17.44
7	31.2	49.0	12.3	7.6	19.9	17.46
8	29.1	48.7	14.1	8.1	22.2	17.42
9	25.7	42.5	15.0	16.8	31.8	17.39
10	26.3	42.2	14.8	16.7	31.5	17.39
11	25.1	42.9	15.2	16.9	32.1	17.38

Polyunsaturated fatty acid content varied from 19.9 (Run 7) to 33.6 % (Run 4) among the runs. Saturated fatty acids varied up to 33 % (from 23.4 to 31.2 %). Monounsaturated fatty acids were the major portion in all runs with a minimum of 37.8 % and a maximum of 49 %. Tri-unsaturated fatty acids seemed to be the most affected by the factors between the runs from 7.6 to 18 %. The average chain length of fatty acids seemed to change little among the experimental runs, with a minimum of 17.328 (Run 4) carbon atoms and a maximum of 17.520 (Run 2) carbon atoms.

From the experimental results, effects estimates were calculated in order to quantify the effect of dilution rate and *C:N* ratio over fatty acids unsaturation and chain length and check which factor were statistically significant for the changes observed (Table).

Table 13. Means and effect estimates analysis of fatty acid classes of CCD experimental design.

		Factors				
Response	Mean	X_1 (C:N)	X_1^2 ($C:N^2$)	X_2 (D)	X_2^2 (D^2)	$X_1 * X_2$ (C:N * D)
SFA (%)	**25.694**	-0.581	-0.376	1.301	**3.882**	1.190
p-value		0.706	0.837	0.412	**0.075**	0.588
MUFA (%)	**42.522**	-3.318	1.254	-3.590	4.803	-2.514
p-value		0.163	0.626	0.137	0.104	0.421
DUFA (%)	**14.996**	**-3.512**	**1.819**	**1.980**	-1.096	-0.281
p-value		**0.005**	**0.091**	**0.042**	0.263	0.797
TUFA (%)	**16.787**	**7.411**	-2.698	0.308	**-7.588**	1.604
p-value		**0.002**	0.130	0.815	**0.004**	0.406
PUFA (%)	**31.783**	**3.899**	-0.878	2.289	**-8.685**	1.324
p-value		**0.099**	0.718	0.289	**0.013**	0.648
Avg CL (C-number)	**17.385**	0.029	0.016	**-0.063**	**0.055**	**-0.094**
p-value		0.209	0.537	**0.026**	**0.072**	**0.022**

*In **bold letters**: significant effects at 90 % confidence*

No significant effects for monounsaturated fatty acids were found. The only significant effect for saturated fatty acids at 90 % confidence level was D^2, which indicates the existence of a point of minimum *SFA* content. The percentage of total polyunsaturated fatty acids was positively affected by the *C:N* ratio, as demonstrated by effect analysis. While a positive effect of *C:N* ratio was observed for *TUFA*s content, *DUFA*s content was negatively affected by it (Fig. 6). This might be explained by the fact that tri-unsaturated fatty acids such as C16:3 and C18:3 are synthesized from desaturation of di-unsaturated C16:2 and C18:2 [20], and this reaction was probably favoured under higher *C:N* ratios. A positive effect of dilution rate was also observed for di-unsaturated fatty acids indicating that there may be a cell age dependence of the conversion of di to tri-unsaturated fatty acids. In this case, shorter residence times or lower cell age would favour the accumulation of di-unsaturated fatty acids.

After the multiple regression of experimental data, the significant factors were selected for the design of models. The statistical significance of the models was calculated through the F-test for ANOVA (Table). All models were statistically significant at 95 % confidence level, since the probabilities (p-value) of the regression were lower than 0.05. However the coefficient of determination for *SFA* model was 0.48, which shows that the regression explains only 48 % of the experimental data. For the other models, R^2 varied from 0.74 to 0.86

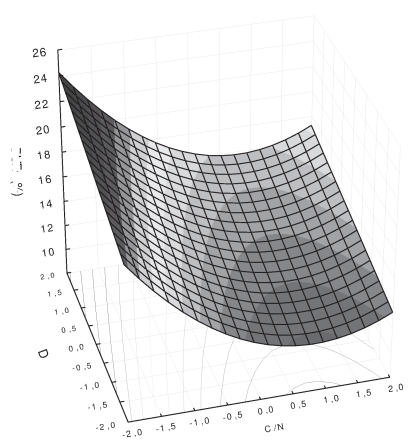
Table 14. Results of Anova evaluation of models of fatty acid composition

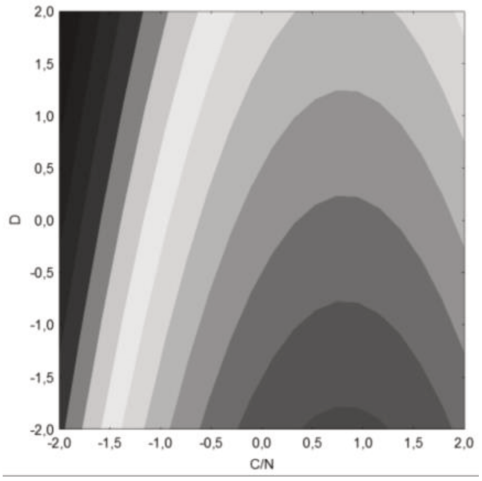
Factor	p-value Regression	p-value Lack of fit	R^2
TUFA	0.0004	0.001	0.86
DUFA	0.003	0.032	0.85
SFA	0.018	0.098	0.48
PUFA	0.005	0.010	0.74
AvgCL	0.012	0.001	0.77

Fitting of models for fatty acid composition resulted in lower R^2 values compared to the biomass and lipid production models. This means that the variability in fatty acid composition could not be totally explained by *D* and *C:N*, and other non-studied variables may also have a significant effect over the results. However, the R^2 values indicate that *D* and *C:N* could explain at least 74 % of the responses, with the exception of *SFA*. The physiological

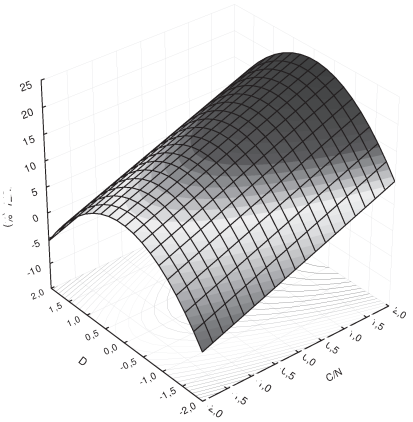
effect of *C:N* over unsaturation and chain length of fatty acids has been discussed in different studies with oleaginous microorganisms, reaching significantly different conclusions. According to Chen *et al.* [64], unsaturated fatty acid content decreased with increasing *C:N* ratios for heterotrophic cultivation of *Chlorella sorokiniana.* Similar behaviour was observed in a number of studies using oleaginous yeasts [65, 66]. Zheng *et al.* [67], on the other hand, found that the use of culture medium with low *C:N* inhibited the production of fatty acids. Microalgae may contain polar lipids, such as glycolipids and phospholipids, and neutral lipids, e.g. triacylglycerol's (TAGs). The first have structural functions, being usually confined to cell membranes and having a high content of polyunsaturated fatty acids [68]. TAGs are usually described as storage materials and are largely composed of saturated and monounsaturated fatty acids, which are accumulated under stress conditions such as nitrogen starvation. This may explain why high *C:N* ratios usually result in higher contents of saturated and monounsaturated fatty acids for some strains, as nitrogen limitation conditions are known to induce TAG accumulation. However, this may not always be the case as some strains were found to accumulate high levels of polyunsaturated fatty acids as TAGs, in which case nitrogen limitation increased the content of *PUFA*s [69].

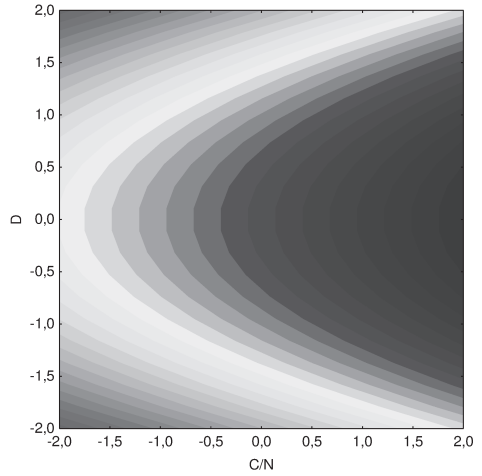
Since *SFA* model fitting was not sufficiently good and no significant factors were identified for the *MUFA* model, response surfaces were built for *DUFA*,*TUFA*, *PUFA* and Avg CL models only (Figure).





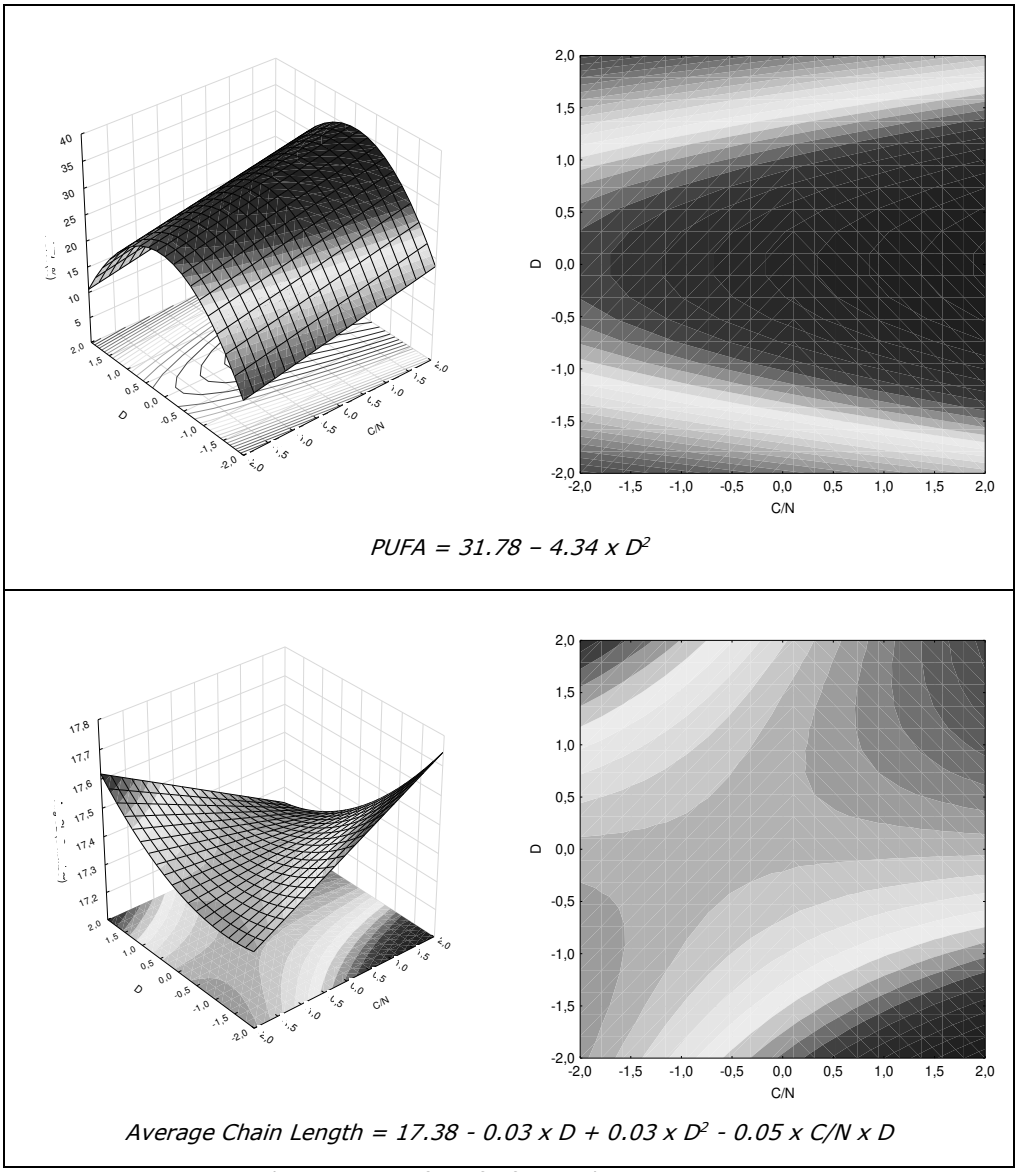
DUFA (%) = 15.00 - 1.76 x C/N + 0.91 x C/N^2 + 0.99 x D





TUFA (%) = 16.79 + 3.71 x C/N – 3.79 x D^2

Figure 6a. Response surfaces for fatty acid composition parameters



$PUFA = 31.78 - 4.34 \times D^2$

$Average\ Chain\ Length = 17.38 - 0.03 \times D + 0.03 \times D^2 - 0.05 \times C/N \times D$

Figure 6b. Response surfaces for fatty acid composition parameters

The effect of *C:N* ratio over *DUFA*s and *TUFA*s is shown in Figure 7 (areas representing the prediction by the models limited by the error range) . While *DUFA*s are maximised in the lower range of *C:N* ratios tested, *TUFA*s reach their maximum at the highest values of *C:N* ratios. The *TUFA*s response surface suggests that even higher content of *TUFA*s could be reached if *C:N* ratios above the tested range were used. For *DUFA*s, maximum percentages were obtained at higher values of *D,* indicating that shorter residence times and therefore younger cells tend to contain higher percentages of them. *TUFA*s were maximised around the central point range of dilution rate. Total *PUFA*s surface behaviour was similar to *TUFA*s surface, but with a less pronounced effect of *C:N* ratio, as *PUFA*s are the sum of *DUFA*s and *TUFA*s percentages.

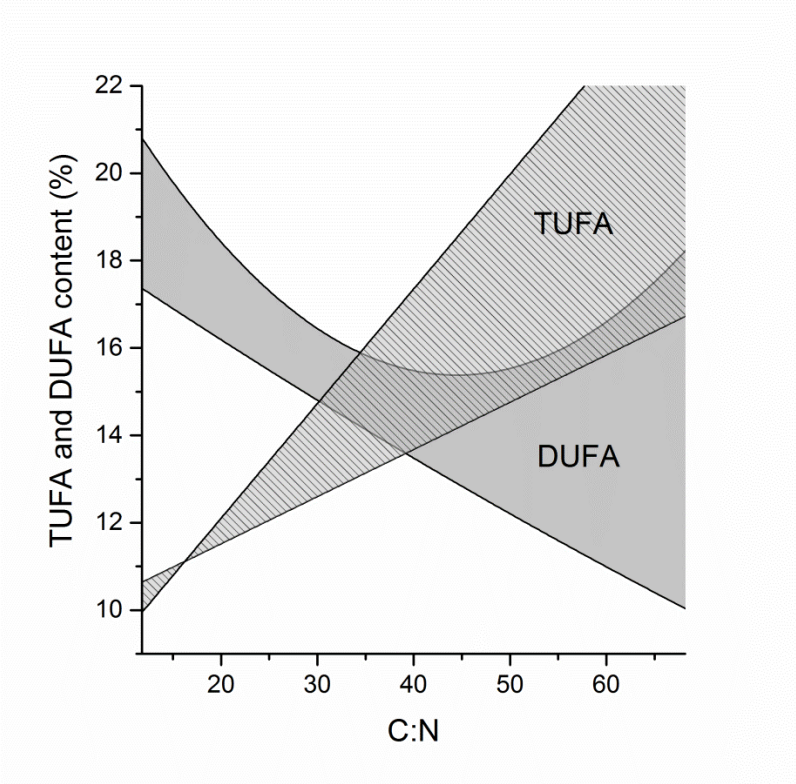


Figure 7. Tri and Di-unsaturated fatty acid model curves at D = 0.015. Solid gray: di-unsaturated fatty acids; Crosshatch gray: tri-unsaturated fatty acids.

While less unsaturated fatty acids are beneficial for biofuel production, polyunsaturated fatty acids can be high value added products for the food and nutraceutical sector, such as omega-3 fatty acids [70]. Our results show that the fatty acid composition can be, to some extent, adjusted in order to favour the desired product.

Average chain length model showed an interesting behaviour as the highest responses were observed at two different conditions: high values of *C:N* ratio combined with low values of *D* and low values of *C:N* ratio combined with high values of *D.* The combination of high values of both *C:N* and *D* resulted in the lowest chain lengths.

3.2.3 Biodiesel Quality Parameters

The analysis of fatty acid composition shows that both *C:N* ratio and dilution rate significantly influenced the degree of unsaturation and chain length and of the fatty acids from microalgae biomass. These characteristics are known to affect the physicochemical properties of lipids and, therefore, of the biodiesel produced out of them [30]. Some of the most common biodiesel quality parameters were shown to be directly affected by fatty acid composition, and thus predictive models based on fatty acids properties and structural configuration are often described in literature. In fact, for most of the experimental microalgal biofuels there is a lack of actual measured data, due to the fact that large amounts of material are required for performing the biodiesel evaluation tests [71].

By using prediction models described in literature, the quality parameters of the biodiesel were estimated and are shown in Table 15. Some of these parameters are defined by International Standards such as ASTM and ISO and, in some cases, minimum and/or maximum limits are determined. For some of them, such as cold filter plugging point (*CFPP*), the limits are determined in each region, due to different weather conditions [72].

Table 15. Biodiesel quality parameters predicted from fatty acid composition.

Runs	*CN*	*CFPP* (°C)	*OSI* (h)	*Hv* (kJ kg^{-1})	*Lub* (Ws1.4-µm)	*v* (mm^2 s^{-1})
1	58.1	3.5	8.1	39580	284.7	4.08
2	56.0	2.9	7.2	39627	277.3	4.05
3	59.1	6.4	8.6	39590	286.1	4.07
4	55.5	4.2	8.4	39498	277.3	3.96
5	57.7	2.1	8.0	39499	284.9	4.05
6	55.9	4.5	7.9	39569	277.7	4.01
7	60.9	11.1	9.2	39616	291.6	4.16
8	59.8	9.9	8.8	39543	288.8	4.12
9	55.6	2.6	7.8	39530	277.1	3.99
10	55.8	3.2	7.9	39536	277.7	3.99
11	55.3	2.1	7.6	39523	276.5	3.98
Limits						
ASTM D6751-08	47 (min)	-	3 (min)	-	-	1.9-6.0
EN 14214/14213	51 (min)	5 (máx)	6 (min)	35000 (min)	-	3.5-5.0

CN: cetane number; CFPP: Cold flow plugging point; OSI: oxidative stability index; dH: heating value; Lub: Lubricity; v: kinematic viscosity. See Table 3 for more information.

Cetane numbers increase with increasing chains lengths and decreasing saturation. [34]. For all runs, the estimated cetane numbers were considerably higher than both ASTM and EN minimum value (47 and 51, respectively) varying from 55.3 (run 11) to 60.9 (run 7). The estimated oxidative stability was higher than the ASTM and EN minimum value for all runs, which can be explained by the relatively low content of *PUFA*s. For instance, soybean oil is one of the most used oils for biodiesel production and may contain up to 60 % of *PUFA*s in its fatty acid composition [30]. Among the runs, little variation was observed for heating value, with a minimum of 39,498 kJ/kg (run 4) and a maximum of 39,627 kJ/kg (run 2). These results are lower than the observed for diesel fuel (42,600 kJ/kg) but higher than the observed for soybean biodiesel (37,200 kJ/kg). All the estimated heating values were higher than the minimum limit established by EN 14214 (35,000 kJ/kg). However, estimates based on fatty acid composition may be higher than the actual measured heating values, due to the presence of contaminants in the biodiesel that may considerably reduce the energy content of the fuel [38]. The estimated values of *CFPP* for

the experimental runs varied from 2.1 to 11.1 ºC. As mentioned before, the limits for this parameter are usually locally determined. For instance, Brazil sets regional limits, as weather conditions can vary significantly between locations. For the most southern states of Brazil, where winter temperatures are lower, the *CFPP* limit was established a 8 ºC [73]. Lower wear scar diameters indicate higher lubricity of the fuel and the European and American standards set maximum limits of 460 and 520 µm, respectively [39]. For all the experimental runs, estimated wear scar diameters were considerably lower than the official limits, with a maximum wear scar of 292 µm (run 7). This is not surprising as biodiesel usually have much better lubricity than diesel, and can even be used as an additive to diesel fuel in order to reach the standard limits [74]. In the experimental design runs the estimated kinematic viscosity of the fatty acid mixture varied from 3.98 to 4.16. These values are within the ranges established in the ASTM and EN standards (1.9 – 6.0 and 3.5 -5.0 $mm^2 s^{-1}$, respectively)

From the estimated values of biodiesel quality parameters, regression analysis was performed and effect estimates for each factor were calculated and are shown in Table 16.

Table 1614. Effect analysis of biodiesel quality parameters

		Factors				
Response	Mean	X_1 (C:N)	X_1^2 ($C:N^2$)	X_2 (D)	X_2^2 (D^2)	$X_1 * X_2$ (C:N * D)
CN	55.56	**-2.04**	0.54	-0.27	**4.08**	-0.75
p-value		**0.04**	0.58	0.74	**0.01**	0.52
Lub	277.07	**-6.59**	2.01	-0.65	**10.94**	-0.63
p-value		**0.03**	0.47	0.78	**0.01**	0.84
OSI	7.76	-0.33	-0.04	0.31	**1.03**	0.29
p-value		0.36	0.93	0.40	**0.05**	0.56
Hv	39.53	0.01	0.01	**-0.06**	**0.06**	**-0.07**
p-value		0.49	0.58	**0.03**	**0.04**	**0.04**
CFPP	2.64	0.17	-0.67	0.61	**6.52**	-0.82
p-value		0.92	0.73	0.71	**0.02**	0.72
v	3.99	**-0.05**	0.02	-0.04	**0.13**	-0.04
p-value	0.00	**0.09**	0.52	0.15	**0.01**	0.27

*In **bold letters**: significant effects at 90 % confidence*

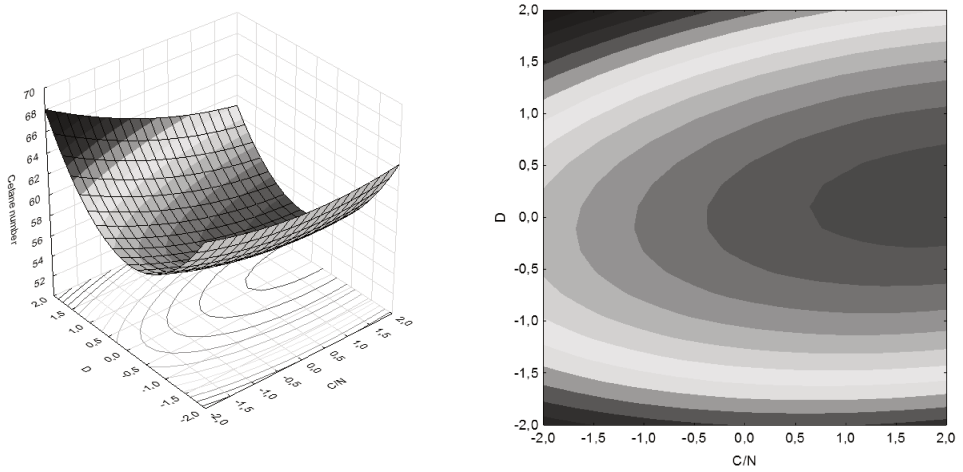
The analysis of effect estimates showed that *C:N* ratio had a negative linear significant effect and *D* had a second order negative effect over cetane number, lubricity and kinematic viscosity. This combination of effects is the opposite as the observed for total polyunsaturated fatty acids. This observation is explained by the fact that, as mentioned before, both and cetane number, wear scar diameter (inversely proportional to lubricity) and viscosity decrease with increasing unsaturation.

For *OSI* and *CFPP* were only significantly affected by dilution rate in a second order, however *CFPP* was the only response whose mean/intercept factor was not significant, meaning that the model was composed by the quadratic term of dilution rate only. Heating value showed a different behavior, with significant linear (negative) and quadratic (positive) factors of dilution rate and a negative interaction factor between *C:N* ratio and dilution rate. As mentioned before, this term indicates the interference of one factor over the other in a synergy or antagonism relationship.

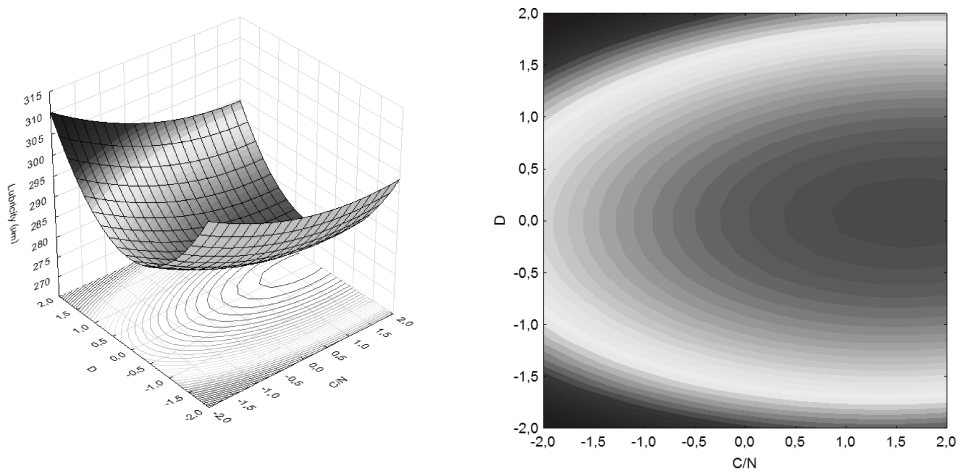
Table 17. Results of Anova evaluation of models for biodiesel quality parameters

Factor	p-value Regression	p-value Lack of fit	R^2
CN	0.001	0.043	0.823
OSI	0.013	0.071	0.513
Lub	0.001	0.039	0.824
Hv	0.008	0.053	0.801
CFPP	0.001	0.084	0.729
visc	0.006	0.017	0.722

The regression was statistically significant for all quality parameters models at 95 % confidence level (p-value of regression < 0.05) (Table 17). For *OSI* the coefficient of determination R^2 indicated that the regression only explained 51 % of the experimental data. Therefore, this model was not considered statistically valid (validation criteria: $R^2 > 0.7$ and p-value of regression < 0.05). The surface responses for all the other quality parameters estimations are depicted in Figure 7.

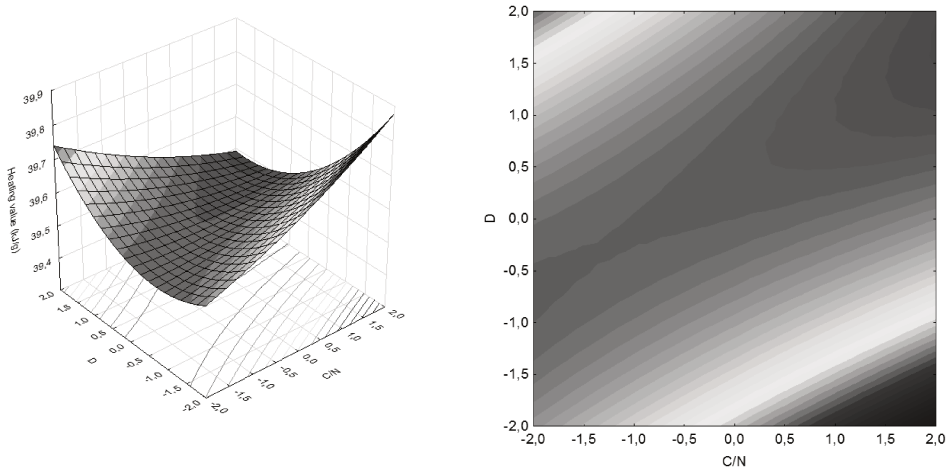


$CN = 55.56 - 1.019 \times C/N + 2.0395 \times D^2$

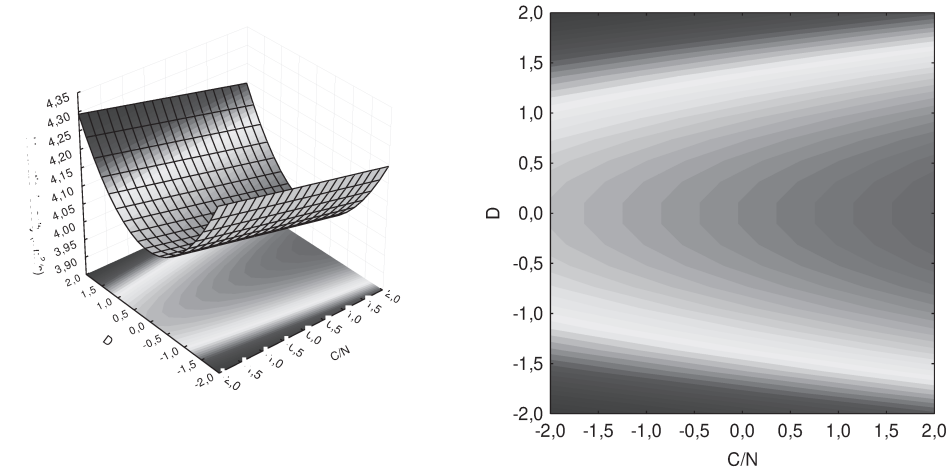


$Lubricity = 277.07 - 3.2938 \times C/N + 5.4697 \times D^2$

Figure 8a. Response surfaces of biodiesel quality parameters



Heating value = 39.5298 – 0.0278 x D + 0.029 x D^2- 0.0345 x C/N x D



Kinematic viscosity = 3.99 – 0.024 x C/N + 0.061 x D^2

Figure 8b. Response surfaces of biodiesel quality parameters

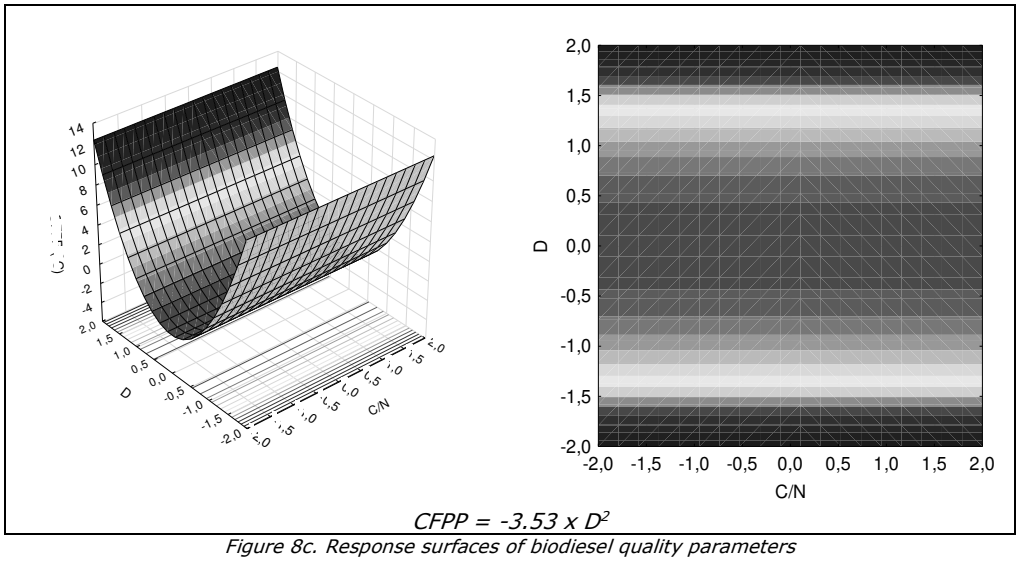


Figure 8c. Response surfaces of biodiesel quality parameters

It can be seen in Figure 7 that all models had a similar profile with minimum point around the central values of the dilution range tested range. From the analysis of fatty acid composition in section 3.2.2, we observed that the percentage of total polyunsaturated fatty acids was highest at dilution rates close to the central point (D = 0.015 h^{-1}), while the total average chain length was minimum at the same conditions. This affected all quality parameters in a similar way, however this effect can be desirable for some of the properties, such as *CFPP*, viscosity and but undesirable for Cetane number, lubricity and heating value. In fact there is a trade-off between this parameters, and the design of an optimal fuel should result in a balanced composition, so that all parameters fit the standard limits.

3.2.4 Validation of models

To check whether prediction models were valid, tests were performed under the optimal conditions calculated from the lipid productivity model, as this is one of the most important parameter for lipid production process. A continuous cultivation was repeated three times at the optimal conditions (D = 0.0175 h^{-1} and *C:N* ratio 40). The tests were performed in triplicate and are presented in the Table 18, along with values predicted by the models of biomass and lipid concentrations and productivities.

Table 20 Experimental and model predicted values of biomass and lipid production in validation runs

	X (g L^{-1})	Lipid (% dw)	*Px* (g L^{-1} d^{-1})	PLipid (g L^{-1} d^{-1})
Experimental	21.3 ± 0.5	21.1 ± 0.7	8.9 ± 0.6	1.88 ± 0.14
Predicted	22.7	20.7	9.25	1.99
Relative error of prediction (%)	6.6	1.9	3.4	5.9

Relative error of prediction calculated as: $REP = \frac{Experimental - Predicted}{Experimental} \times 100$

The relative errors for biomass and lipid concentrations and productivities were all lower than 10 %, indicating a good fitting of the data to the models. Experimental lipid productivity was 5.9 % lower than the predicted. However, it must be taken into account that the relative standard deviation for biomass and lipids were 2.3 and 3.3 %, respectively, which reduces the significance of such differences.

Fatty acid composition of biomass produced during validation runs was also analyzed for validation of fatty acid composition and biodiesel quality parameters models. The average fatty acid composition of the validation runs is described in Table 19.

Table 21.. Fatty acid composition of validation runs and experimental and predicted percentages of DUFA, TUFA, PUFA and Average chain length.

Fatty acid composition (%)	Experimental (%)	Predicted (%)	Relative error (%)
C16:0	11.37	-	-
C16:1	1.75	-	-
C16:3	4.26	-	-
C18:0	8.54	-	-
C18:1	44.76	-	-
C18:2	16.83	-	-
C18:3	12.49	-	-
DUFA (%)	16.83	15.49	7.9
TUFA (%)	16.75	15.84	5.4
PUFA (%)	33.58	30.69	8.6
AvgCL (C atoms)	17.65	17.37	1.6

The results of fatty acid analysis of validation runs showed a slightly different composition from the experimental design, as no C5:0, C16:2 and C17:0 fatty acids were identified in these runs. This could be due to an error in sample preparation or CG analysis. These two components, however, accounted for only 5% of the total fatty composition in the experimental design runs and their absence or presence would not significantly alter the biodiesel properties.

In order to validate the fatty acid compositional models, fatty acid classes percentages were calculated and confronted with predicted values of *DUFA*, *TUFA*, *PUFA* and average chain length. The calculated experimental values are described in Table 19 along with the predicted values from the statistically significant models. Saturated

and mono-unsaturated fatty acid percentages were not compared with models predictions, as those models were not statistically valid, as shown in section 3.2.2.

The models for fatty acid composition could predict the final percentages of di, tri and poly-unsaturated fatty acids as well as average chain length with reasonable accuracy. The highest relative error between the experimental and the predicted values was 8.6%, calculated for polyunsaturated fatty acids.

Biodiesel quality parameters were accurately predicted by the models, as shown by the relative error in Table 21, except for *CFPP*, for which the relative error was much higher than for the other parameters. This difference, however, can be explained by the fact that the experimental and predicted values of *CFPP* are close to 0, which results in a high relative error, even for a small absolute difference. For a better evaluation of the prediction performance of the model, the absolute difference between experimental and predicted error (0.3 ºC) was compared with the range of prediction of the model, within the tested ranges. After solving the model for the minimum and maximum points of *CFPP* (in the range of -1.41 to +1.41 for both factors), the total range of prediction was calculated as 13.2 °C (minimum predicted value: -3.3 ºC and maximum predicted value: 9.9 ºC). The relative error was then calculated as the ratio between the absolute prediction error (0.3 ºC) over the total range of prediction (13.2 ºC), which resulted in 2.3 % relative error.

Table 22. Experimental and predicted biodiesel quality parameters in validation runs

	Predicted quality parameters				
	CN	*Lub* (Ws1.4-µm)	*Hv* (kJ kg^{-1})	v (mm^2 s^{-1})	*CFPP* (ºC)
Experimental	56.07	278.44	39520	4.01	0.84
Predicted	54.22	273.42	39700	4.01	0.51
Relative error (%)	3.4	1.8	0.5	0.2	64.5

According to the estimation of quality properties, the biodiesel obtained met all the specifications of ASTM and EN. However, it must be taken into account that the models based on the fatty acid composition consider the absence of contaminants, that could interfere in the quality properties of the fuel. It was shown that the presence of contaminants produced by some microalgae can significantly modify the biodiesel properties from those predicted by fatty acid based models [71]. It is important, thus, that the predicted values are validated through physicochemical tests. In addition, if these contaminants are identified, their production could be studied as a response parameter, and optimal growth conditions could be determined in order to minimize its production.

4. Conclusion

Heterotrophic production of Chlorella vulgaris in continuous process proved to be a highly productive and easily manipulated process in order to obtain optimal lipid and biomass productivities. The proposed experimental design demonstrated the influence of *C:N* ratio and dilution rate on the productivity of the system, as well as over the concentration and composition of biomass. It has been shown that the variation of only two variables allowed the production of lipids with different fatty acid compositions and properties. The developed prediction models developed from the experimental design could accurately (maximum relative error of 8.6 %) predict biomass and lipid as well as lipid composition and potential microalgal biodiesel quality properties. These models could be used to set process conditions according to the desired result, tailoring process outputs such as lipid content as well as final products properties such as fatty acids unsaturation level and biodiesel cetane number.

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Chapter 6

Techno economic assessment of heterotrophic microalgae biodiesel production integrated with a sugarcane biorefinery

This chapter will be submitted as:
R. S. Coelho, M. C. Cuellar, L. A. M. van der Wielen, T. T. Franco, **Techno economic assessment of heterotrophic microalgae biodiesel production integrated with a sugarcane biorefinery**

Techno economic assessment of heterotrophic microalgae biodiesel production integrated with a sugarcane biorefinery

Abstract

One third of sugarcane biorefineries carbon emissions are the result of large quantities of diesel fuel still used in crop and transportation operations. Heterotrophic microalgae can accumulate lipids and grow at high cell densities with productivities orders of magnitude higher than autotrophic microalgae. In this study, a heterotrophic microalgal biodiesel plant, both stand-alone and integrated with a typical Brazilian sugarcane biorefinery were designed and evaluated. Molasses, steam and electricity from sugarcane processing were used as inputs for microalgae production. The cost of the microalgal biodiesel was evaluated for both continuous and fed-batch cultivation and the differences between the two cultivation modes were discussed. For a non-integrated plant, the cost of the microalgae biodiesel was estimated at 2.51 and 2.27 $/litre for fed-batch and continuous processes, respectively. Equipment purchase costs related to the fermentation section and carbon source costs were the most important factors affecting the financial feasibility of the proposed design.

Integration with a first generation sugarcane biorefinery can reduce biodiesel production costs by using molasses and surplus energy from sugarcane processing for microalgae production. However, at present ethanol and biodiesel selling prices, the profitability of a microalgae integrated plant would be lower than a first generation sugarcane biorefinery using fossil diesel fuel for its operations.

1. Introduction

Continuous improvement of the sugarcane harvesting process resulted in large reduction of greenhouse gas emissions (GHG) and fossil fuels consumption [1]. However, crop and harvesting operations still depend heavily on diesel for crop machinery, mechanical harvesters and sugarcane transportation [2].

The average diesel consumption in a sugarcane biorefinery was estimated to be around 200-300 L ha^{-1} yr^{-1} [2]. Considering the emissions potential estimated at 2160kg CO2 eq ha^{-1} yr^{-1} (Green harvested with reduced soil tillage, crop rotation and 100 % mechanized harvesting), and the average production of 80 ton of sugarcane ha^{-1}, this corresponds to 27 kg CO2eq / ton of sugarcane [3]. For a sugar mill with capacity for processing 5.000.000 tons of sugarcane per year, around 19 million liters of diesel oil are consumed. Considering the potential emission of 2.63 kg of CO_2eq per litre of diesel [4], the resulting emissions may reach almost 50 million kilograms of CO2eq, which is equivalent to up to one third of all the emissions produced during the sugarcane process [3]. If a renewable substitute for diesel was utilized it could lead to a significant reduction in GHG emissions and fossil fuel consumption, improve the renewability of sugarcane processing and reduce the dependency of production costs to oil price fluctuations.

A large number of technologies have been proposed for obtaining drop-in fuels for diesel engines from renewable sources. These include gasification and pyrolysis of biomass and hydrogenation and cracking of plant oils [5]. However, transesterification of fatty acids from triacylglycerols to produce biodiesel is already well established in industrial scale worldwide [6], corresponding to 17.9 % of global biofuel production in the period of 2010-2012 [7].

It is frequently argued that the use of agricultural commodities, such as corn and soybean, for biofuels production may increase food prices worldwide [8]. However, vegetable edible oils still correspond to, approximately, 95 % of the feedstock for biodiesel production, with soybean and rapeseed as the most common oil sources. In addition, these crops are far from being the most productive among oleaginous plants. Palm, *Calophyllum inophyllum* and Jatropha are regarded as the most productive oil crops, reaching up to 6, 5 and 2 tons ha^{-1} yr^{-1} of oil respectively but can only grow in tropical latitudes (Table 1). For non-tropical areas, oleaginous crops will typically yield from 0,2 to 1 ton ha^{-1} yr^{-1} of oil. [9]. Total biomass productivities (leaves, fronds, truncs, empty

fruit bunches etc) of oleaginous crops are generally not considered, but these can be up to 8 times (oil palm) larger then the oil productivity (e.g. around 50 ton/ha/yr or more for oil palm).

Table 1. Oleaginous feedstocks and oil productivities (Ong et al., 2011)

Feedstock	Productivity (litres / ha)
Palm	6000
Soybean	300
Rapeseed	1000
Sunflower	800
Jatropha	2000
C. Inophyllum	5000

On the other hand, grasses such as sugarcane can yield up to 150 ton ha^{-1} yr^{-1} of plant biomass [10]. These crops can grow well in temperate climates and are currently being used to produce first and second generation bioethanol, from sugars and lignocellulosic biomass. If these high productive crops could be converted to oils, high productivities of oil per area could be obtained. In São Paulo, Brazil's leading cane-producing state, biomass productivity reached 85 tons ha^{-1} in 2010, with an average sugar content of 140 total sugars per ton of sugarcane [11, 12]. Oleaginous microorganisms can convert sugar and other carbon sources into lipids with a maximum theoretical yield of 33% [13]. If all the sugars produced in 2010 from sugarcane were converted to lipids by oleaginous microorganisms, the maximum oil yields would be almost 4 ton ha^{-1} of oil.

Microalgae are regarded as the most productive culture for oil and biomass production, with theoretical yields of up to 40 tons of oil per hectare per year [14]. In practice, however, the productivities achieved are still far from this value and the equipment and energy costs associated with bioreactor construction and dewatering of cultures are still very high [15]. These organisms can be cultivated under autotrophic or heterotrophic conditions. In the former, light, CO_2 and nutrients must be provided for photosynthesis to occur. This process is usually run in either open raceway ponds or closed photobioreactors [16, 17]. Raceway ponds are closed loop oval channels reactors, usually agitated by a paddlewheel system with liquid depths not exceeding 0.4 m [18]. These systems have usually low costs of installation and operation but can result in low biomass concentrations (0.1 - 1.0 g L^{-1}) and, consequently, low productivities [19]. Photobioreactors, on the other hand, can achieve higher biomass concentrations (1 - 5 g L^{-1}), reduce contamination risk and harvest time and optimize light absorption due to the higher surface-to-volume ratio [20]. Nonetheless, this efficiency comes at high prices. Despite their obvious advantages over open systems, photobioreactors have much higher capital and operation costs, higher risks of overheating and high oxygen levels, that may be harmful for many algae species. Studies have shown that the final selling price of algal biodiesel cultivated in photobioreactors could be more than twice the price from open ponds cultivation [21]. In addition, the average energy input necessary for photobioreactor operation was calculated to be up to 350 % higher compared to raceway ponds [22].

Harvesting of the microalgae biomass is usually a very costly, and energy intensive operation. Due to the small cell diameter (typically < 15 μm) and low biomass concentrations typically achieved in traditional autotrophic systems (0.1 - 0.5 g L^{-1}) , this stage may be responsible for up to 25 % of total production costs [23]. Centrifugation is a well-established efficient operation for concentrating biomass up to 30 % total solids, however the energy necessary for concentrating diluted microalgae cultures makes it unfeasible at larger scales. For low cell concentrations, flocculation with inorganic compounds followed by gravity settling is frequently used for total volume reducing before centrifugation. Nonetheless, the addition of multivalent salts such as aluminium chlorides may contaminate the biomass, add to costsand have adverse effects on the final product quality [22]. Sun drying is probably the cheapest technology available for biomass dewatering and/or drying. Despite still being considered as

an alternative in recent techno-economic studies [24], this method demands large drying periods, huge drying surfaces and presents high risk of degradation of reactive products [25]. The solution for reducing harvesting costs may not be on harvesting technologies after all. Instead, increasing biomass density and lipid contents in the cultivation stage may reduce harvesting costs more efficiently. Dassey *et al.* [26] showed that increasing biomass concentration from 50 to 500 mg L^{-1} and lipid content from 10 to 65 % may reduce energy consumption in centrifugation by 98%.

Heterotrophic cultivation is an alternate approach for microalgae cultivation. A few species of microalgae can grow in the absence of light and CO_2 if an organic carbon source is provided. Since no light is needed to support growth, heterotrophic organisms can grow in standard closed fermenters, such as those extensively utilized for yeast and bacteria fermentations [27]. As cell concentration is inversely proportional to light irradiance in autotrophic growth, biomass yields tend to be very low, limited by the mutual shading of cells. In heterotrophic growth there is no influence of light on cell concentration, thus allowing increased cell densities and productivity [28]. Heterotrophic cultures can achieve biomass concentrations more than 100 times higher than those obtained in autotrophic open ponds. Such difference suggests that harvesting costs will be much less relevant in heterotrophic systems. Another advantage of using closed fermenters is the possibility of steady conditions throughout the whole year, without influence from environmental conditions [27]. Some of the best results for both autotrophic and heterotrophic processes are shown in Table 2.

Table 2. Highest productivities found in literature for autotrophic and heterotrophic cultivation

Metabolism	Microorganism	Biomass dry weight (g L^{-1})	Final Lipid productivity (g L^{-1} d^{-1})	Mode of operation	Reference
2 stages: Auto / Heterotrophic	*Auxenochlorella protothecoides*	111.6	14.6*	Fed-Batch	[29]
Heterotrophic	*Chlorella sorokiniana*	103.8	4.2	Fed-Batch	[30]
Heterotrophic	*Chlorella vulgaris*	117.18	2.37	Fed-Batch	[31]
Heterotrophic	*Auxenochlorella protothecoides*	144	8.2	Fed-Batch	[32]
Mixotrophic	*Auxenochlorella protothecoides*	123	11.8	Fed-Batch	[33]
Autotrophic	*C. vulgaris*	4.3	0.212	Batch	[34]
Autotrophic	*C. vulgaris*	0.87	0.144	Continuous	[35]
Autotrophic	*C. vulgaris* CCALA 256	7.59	1.425	Batch	[36]
Autotrophic	*C. zofingiensis* ASU 2	6.5	0.312	Batch	[37]
Autotrophic	*Neochloris oleoabundans*	3.2	0.133	Batch	[38]

**Heterotrophic cultivation started with initial biomass concentration of 23 g L^{-1}, obtained from centrifuged autotrophic cells grown in open ponds.*

Despite the much higher productivity of microalgae biomass and lipids in heterotrophic processes, the need of an external carbon source adds a relevant feedstock cost that is practically inexistent in autotrophic cultivation. Typical feedstocks found in literature are glucose, acetate and glycerol, with the first being by far the most successful [39]. To reduce feedstock costs, some lower cost substrates have been successfully utilized by heterotrophic microalgae, such as sugar cane molasses [40], crude glycerol from biodiesel production [17], rice straw hydrolysate [41] and cassava starch hydrolysate [42].

Recently, a number of companies started alternative microbial biofuels operations in Brazil associated with sugarcane biorefineries for substrate supply. Solazyme, Inc., is one of the first companies to utilize heterotrophic microalgae for oil production and is currently installing a 100,000 metric ton of oil capacity plant in Moema, Brazil, associated with Bünge Bonsucro® sugarcane mill [43]. As a non-algal benchmark, the company Amyris is currently

expanding the capacity of their Brazilian farnesene plant in Brotas, SP. Unlike Solazyme, Amyris utilizes yeasts to convert sugars into an isoprenoid product, which can also be converted to biofuels and chemicals. Their plant is associated in a joint venture with sugar and bioethanol producer Usina São Martinho (SMA) for sucrose supply [44].

As an alternative for total or partial substitution of diesel oil used by sugarcane biorefineries, heterotrophic microalgae biodiesel for an on-site renewable fuel production is proposed (see Figure 1). We present a techno-economic assessment of an integrated biodiesel production plant based on the conversion of part of the sugars extracted from sugarcane into microalgae lipids. The oil produced is then extracted and converted to biodiesel through alcoholic transesterification. In this analysis, we excluded valuable byproducts such as carotenoids, PUFA, and animal feed, but strictly focused on the biodiesel integration.

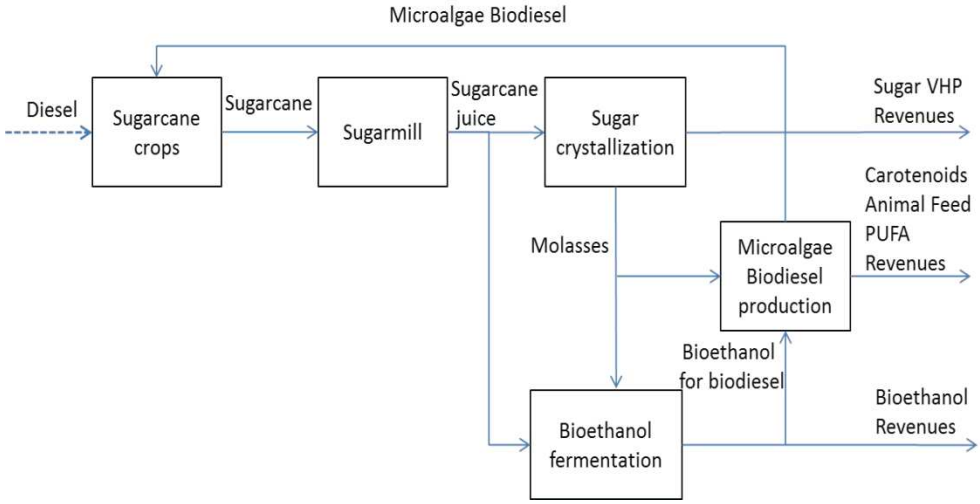

Figure 1. Block flow diagram of sugarcane refinery – microalgae biodiesel integration proposal

2. Material and Methods

2.1 Process simulation software

This study employed Superpro Designer 9.0 software for all process design and simulation.

2.2 Basic Assumptions

The proposed microalgae plant was designed to provide all the fuel consumed by a large first generation sugarcane biorefinery in Brazil, with a capacity of processing 5 million tonnes of sugarcane per year. According to recent estimates, the diesel consumption during sugarcane crop and processing operations is 4 L ton^{-1} sugarcane on average [45], which results in a yearly demand of 20 million litres of diesel for the bio-refinery considered in this study.

The sugarcane mill process was considered as a black box with inputs and outputs as described by Cavalett *et al.* [46]. That study considered different configurations and different sugarcane processing operations for economic and environmental analysis using the Virtual Sugarcane Biorefinery tool, developed by the Brazilian Bioethanol Science and Technology Laboratory (CTBE). The main inputs and outputs taken from that study are summarized in Figure 2.

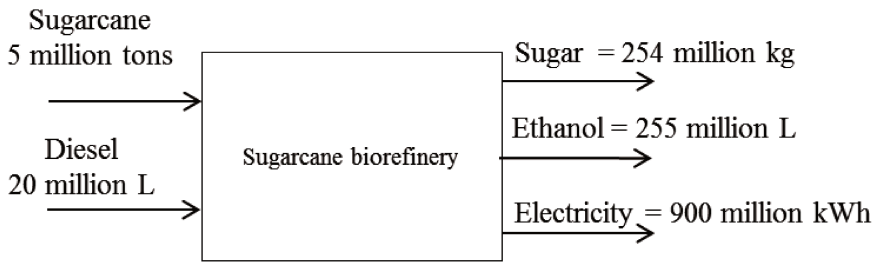

Figure 2. Annual input and output streams of the base case sugarcane biorefinery

The base case sugar mill considered used 50 % of the sugarcane juice to sugar production and 50 % to bioethanol production from a total 5.000.000 tons of sugarcane per year. A few modern flexible sugarcane refineries can operate in different product ratios (usually from 30:70 to 70:30 sugar:ethanol), but a 50:50 traditional refinery will be considered [47]. The main assumptions for the base case refinery are described in Table 3.

Table 3. Main assumptions for the base case sugarcane biorefinery

Parameter	Value	Reference
Diesel consumption	4 L / ton of sugarcane	[45]
Total sugars in sugarcane	150 g / ton sugarcane	[48]
Ethanol production from sugarcane (50-50 sugar/ethanol ratio)	40.4 kg / ton sugarcane	[47]
Sugar production from sugarcane (50-50 sugar/ethanol ratio)	50.9 kg / ton sugarcane	[47]
Electricity from co-generation	182 kwh / ton sugarcane	[47]
Sugar selling price	0.43	[47]
Anhydrous ethanol selling price	$ 0.6 / L	[47]
Diesel selling price	$ 1.06 / L	[49]
Electricity selling price	$ 0.058 / kWh	[48]
Algae Meal	0.45	[45]
Sugar production cost	0.25	[47]
Ethanol production cost	0.45	[47]
Electricity production cost	0.052	[50]
Molasses sugar content	55 %	[51]

In the proposed integrated plant, heterotrophic microalgae use the molasses generated during sugar crystallization as substrate for growth and lipid accumulation. The accumulated lipids are extracted and converted to biodiesel for sugarcane processing consumption, while the remaining cell debris is dried and sold as protein rich meal for animal feed. Other potentially high-value byproducts such as carotenoids and PUFA are not considered, but offer additional revenue potential.

For the biomass and lipids production stage, the main assumptions are taken from experimental data from Siegler *et al.* [32]. The following parameters were considered: specific growth rate: 0.04 h^{-1}; yield of biomass from sugars: 0.5 g g^{-1}; yield of lipids from sugars 0.25 g g^{-1}; maximum cell density: 144 g L^{-1}; final lipid content: 50 %.

Molasses is the concentrated solution obtained after sugar crystallization. It has high concentration of sugars (around 50-55% total reducing sugars per litre), minerals, suspended colloids and nitrogenous compounds [51]. It has been shown that molasses is a superior substrate for heterotrophic microalgae biomass and lipid production than glucose, due to its complex nutrient content [52]. Besides molasses, steam and electricity

generated during bagasse co-generation process are used as inputs for the microalgae plant. The main inputs and out puts for the integrated process are depicted in Figure 3.

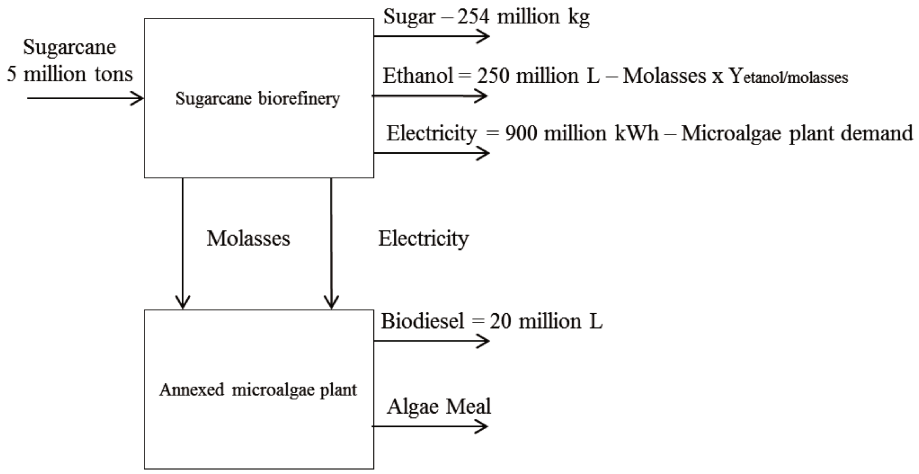

Figure 3. Inputs and outputs of the integrated microalgae plant.

Although it is an integrated process, the goal of this study is to evaluate the feasibility of the microalgae biodiesel production. Therefore, only the microalgae processing will be evaluated in detail. The process boundaries for the process are shown in Figure 4.

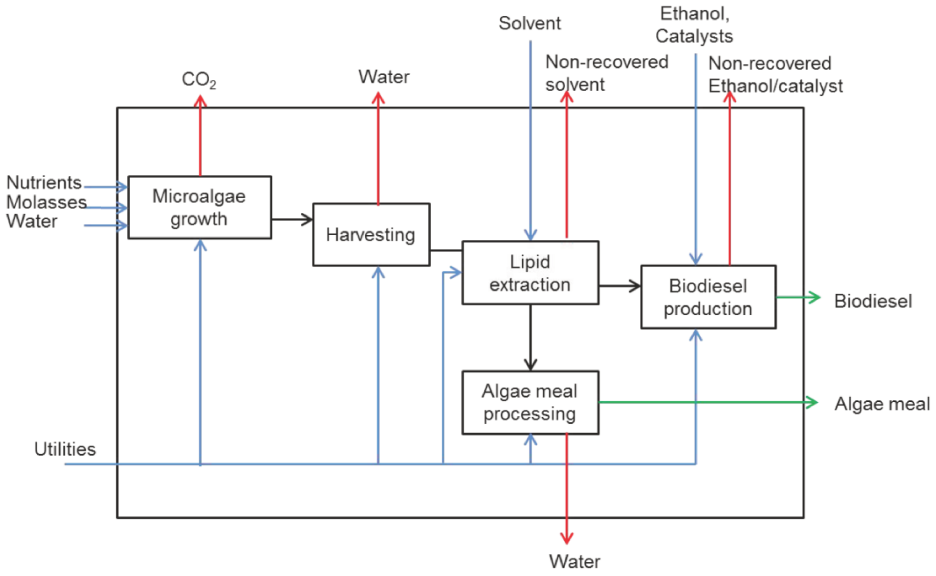

Figure 4. Process boundaries for the microalgae biodiesel plant Blue: Inputs; Green: outputs; Red: waste/emissions

2.2 Process Description

2.2.1 Medium preparation and sterilization

The culture medium is prepared in two separate mixing tanks. In one molasses is diluted in water and sent to a continuous sterilizer. In the second tank the salt based medium (Table 4) is diluted and sent to a second continuous sterilizer.

The main fermentation section starts with the input of molasses from sugar crystallization at 50 ºC (Figure 5). Although it has been reported that some *Chlorella* strains may be able to directly utilize sucrose or molasses for

heterotrophic growth [53], many studies showed that strains of *C. protothecoides* lack the enzymes required for hydrolysis and intake of sucrose [28, 52, 54]. Therefore, pre-hydrolysis of the sucrose contained in molasses into glucose and fructose is a necessary step. The molasses stream, containing circa 40 % m/m of sucrose, is hydrolysed in a reactor after addition of HCl at a 0.25% for 1 h at 85 ºC [55]. The acidification also induces precipitation of impurities and minerals from raw molasses that can later be removed by centrifugation [56]. After acidification the mixture is neutralized by addition of lime, forming $CaSO_4$ and increasing the content of precipitate in the molasses. The precipitated salts are then removed in a rotary vacuum filter and the clarified molasses is sent to sterilization. Other culture medium components (Table 4) are blended in a separate tank and sterilized in a continuous sterilizer before mixing with molasses stream in the fermentors. The feeding of medium salts and molasses is done separately, so the carbon / nitrogen ratio can be adjusted during different phases of growth to enhance cell multiplication or lipid acumulation.

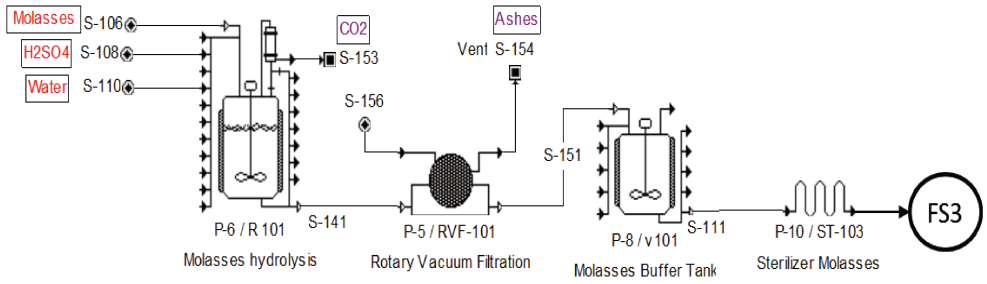

Figure 45. FS1 - Process flow design of molasses hydrolysis section

In addition to the sugars from molasses, a stream of glycerol, byproduct from oil transesterification, is also sent to the fermentation to be utilized as carbon source. *C. protothecoides* can successfully utilize glycerol, reaching biomass and lipid yields similar or larger than those obtained from glucose [17, 57, 58]. The glycerol stream, containing 84 % (m/v) of glycerol and around 18 % (m/v) of water, is mixed with water and salt medium components in the salt medium blending tank before sterilization.

Table 4. Culture Medium composition[32]

Nutrient	Concentration (g L^{-1})
Glicine	0.5
KH_2PO_4	0.25
$MgSO_4$	0.075
$CaCl_2$	0.025

2.2.2 Seed Culture train

For the inoculum of the bioreactors, a strain of *Auxenochlorella protothecoides* is used as the production organism. The seed cultures start with the transfer of axenic cells of *Chlorella* to shake flasks containing sterile medium. These are incubated and grown up to OD 600 and then transferred to a sterile bench scale bioreactor for a new growth phase. After reaching the specified OD value, the culture is transferred to a bioreactor with a 10 times larger capacity. This process is repeated through sequential seed fermenters of increasing capacities until the seed culture reaches 10 % of the process bioreactor volume.

The purchase and operation costs of seed fermenters and auxiliary equipment is often ignored in techno-economic studies, however they may represent a large part of the total capital costs associated with the fermentation section. The inoculation volume of the production fermenters is 10 % of the total working volume (2

production fermenters of 500,000 Liters). For the design purposes, only three stages of inoculum preparation were considered (100,000 L, 10,000 L an 1,000 L capacity fermenters) (Figure 6).

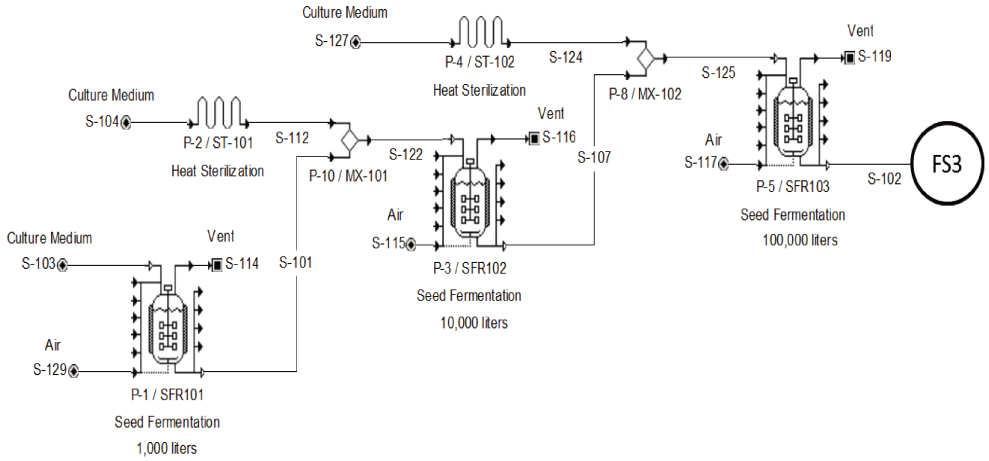

Figure 6. FS2 - Inoculum production configuration

For two 500 m^3 fermenters (450 m^3 of working volume) a seed culture of 90,000 litres is prepared in a 100.000 litres capacity fermenter. The inoculum is cultivated during 96 hours before the transfer [32]. Considering a total downtime of 24 hours (loading, discharge, cleaning and sterilization), five staggered seed fermenters are necessary to feed the production fermenters (Figure 7). The same criteria was applied for the previous inoculum cultivation stages (10,000 and 1,000 litres seed reactors).

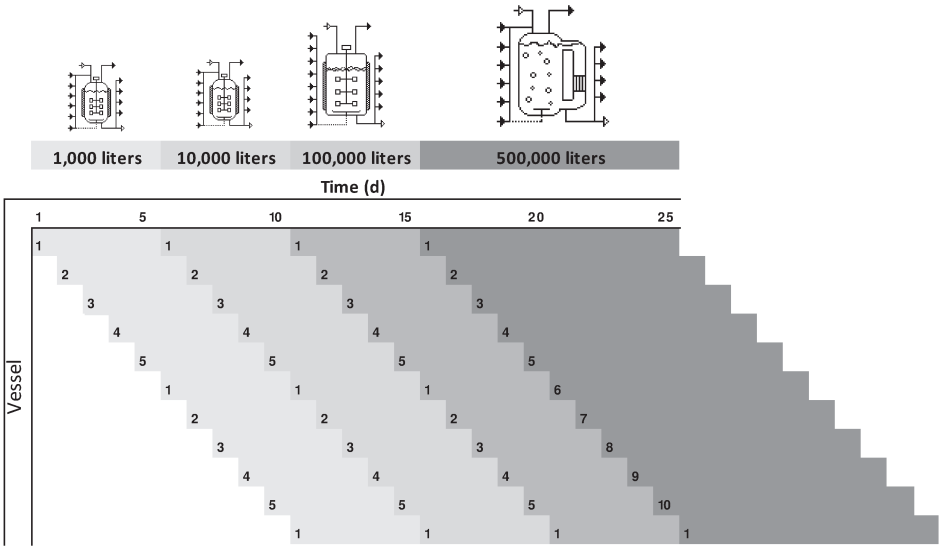

Figure 7. Scheduling of inoculation operations

Two continuous culture medium sterilizers were designed for the 10,000 and 100,000 liters fermenters. For the 1,000 liters fermenter, it was considered that the culture media was sterilized inside the equipment, since this is a common feature for smaller scale fermenters.

2.3 Biomass and lipids production (Fermentation section)

The bioreactor configuration consists of airlift fermenters, operating either in fed-batch or continuous culture. In fed-batch system the culture reaches higher cell concentrations but requires a residence time of 6 days.

2.3.1 Fed- Batch cultivation

Airlift bioreactors of 500 m^3 are used for the algae cultivation (Figure 8). The feasibility of operating large capacity fermenters has been validated by the leading heterotrophic algae based companies, Solazyme Inc., who utilizes 500 m^3 fermenters in its Iowa, US, facility since 2012 and has recently started operation of 625 m^3 fermenters in Moema, Brazil [59, 60]. These fermenters are more easily scaled up to high volumes due to their simple design and demand less power input for agitation. The vessels are aerated at a rate of 0.5 v.v.m. with centrifugal compressors. The energy consumption due to air pumping is relatively high for this type of fermenter, since the bubbling motion is responsible for both mixing and oxygenation of the culture. To allow maximum capacity utilization, 20 bioreactors operate in 10 staggered racks of two bioreactors each. Downstream processing of culture broth is run continuously from the bioreactor discharge (two fermenters a day).

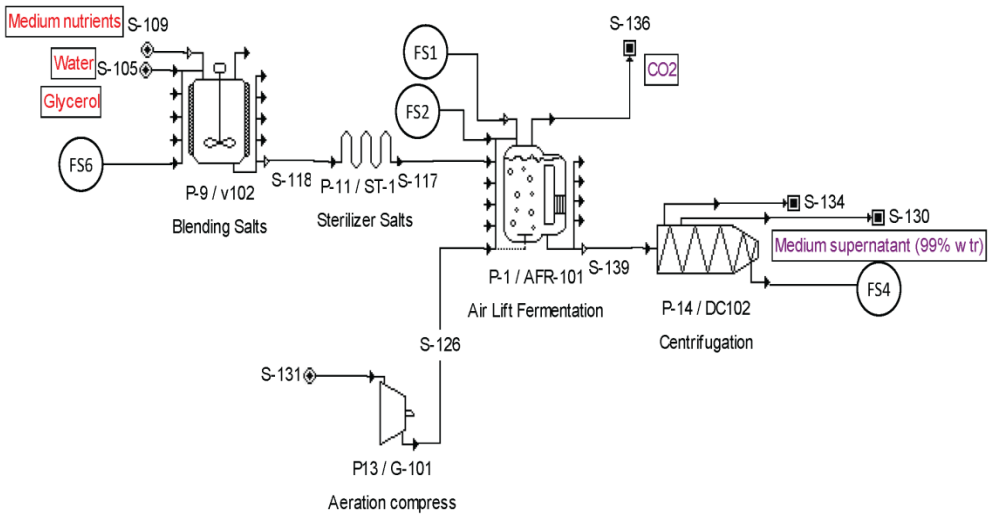

Figure 8. FS3 - Process flow diagram of microalgal biomass production section

The fed-batch process takes 210 hours with 12 hours for charge, CIP and sterilization procedures. The discharge of the fermentor takes 24 hours, during which all downstream operations are performed. The scheduling of the fermenters is done in a way that there is a constant discharge stream from the fermentation section. This way the downstream operations can operate continuously at a rate of one fermentor rack (2) per day (Figure 9).

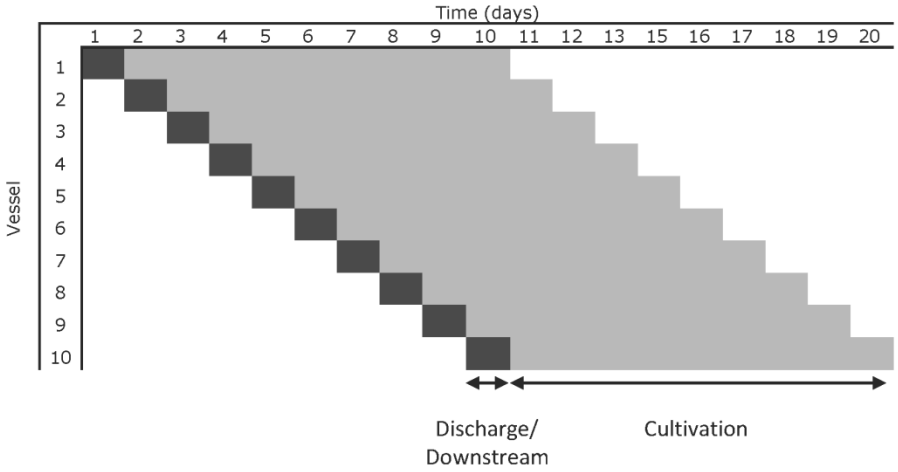

Figure 9. Gantt Chart of scheduling of production fermenters

2.3.2 Continuous cultivation

Continuous cultivations can usually reach higher productivities for biomass production, since non-productive periods are very reduced in comparison with batch process [61]. This assumption, however, is dependent on the characteristics of each process. Fed-batch processes are often preferred over continuous process in industrial bioprocessing due to the following disadvantages of the latter: 1. Difficult maintenance of sterility and homogeneity inside the bioreactor; 2. Poor flexibility to handle multiple products; 3. High costs and long times for development of complex continuous processes; and 4. Genetic instability of cells [62]. However, these constraints may not be critical for the biodiesel production process since 1. For biofuels and bulk chemicals the sterility of the process is not critical as long as the productivity of the system is not significantly affected; 2. The products are obtained in a single product plant; 3. Biomass and lipid production are obtained in single stage continuous cultivation; and 4. The use of wild strains reduce the risks of strain instability.

Few studies exist about continuous cultures of heterotrophic microalgae and even fewer regarding high cell density cultures, but the existing ones show that higher productivities are obtained for lower cell densities than the observed for fed-batch cultivation. This may be attributed to the fact that higher cell concentrations are obtained at very low dilution rates, at which a greater proportion of substrate is required for maintenance [61]. When grown on glycerol, continuous cultures of *C. prototheco*ides attained lower biomass concentrations (43.3 g L^{-1} against 65 g L^{-1}) but higher biomass productivities than fed-batch cultures with 9.4 g L^{-1} d^{-1} against 8.7 g L^{-1} d^{-1}. The highest lipid productivity was obtained in continuous culture at 0.2 d^{-1} dilution rate with 4.3 g L^{-1} d^{-1} against 1.18 g L^{-1} d^{-1} in fed-batch culture [17]. Similar results were obtained for *C. vulgaris* cultures grown on glucose, with maximum biomass concentration of 58.8 g l^{-1} and biomass productivity of 5.5 g L^{-1} d^{-1} for fed-batch cultivation against 18.9 g L^{-1} and 9.1 g L^{-1} d^{-1} for continuous cultivation at 0.5 d^{-1} dilution rate. In that study, however, the maximum lipid productivities were similar for both cultivation modes [63]. The same behaviour was observed for oleaginous yeast *L. starkeyi,* with similar lipid productivities but different biomass concentrations in fed-batch (6.72 g L^{-1} d^{-1} and 82.4 g L^{-1} CDW) and continuous cultivation (6.91 g L^{-1} d^{-1} and 10 g L^{-1} CDW) [64].

For continuous cultivation process design, we assumed, from the results found in the studies above cited, that the system can obtain the same lipid productivity obtained in fed-batch cultures, but with lower biomass concentration. From this assumption it is expected that the capital and operational costs of the upstream section will

benefit from the reduced downtime operations, but the downstream costs will likely be higher due to lower product concentration. When considering continuous cultures such as chemostats, biomass productivity may be defined as

$$P_L = D \times L$$

where D is the dilution rate, and L is the concentration of lipids inside the reactor. We considered that a continuous cultivation system would obtain the same lipid productivity of 8.2 g L^{-1} d^{-1} obtained by Siegler *et al.* [32], but running in steady state with a biomass concentration of 35 g L^{-1} CDW and 50 % (m/m) lipid content. From the lipid productivity equation a dilution rate of 0.02 h^{-1} or 0.48 d^{-1} is calculated.

From these assumptions, a total of 15 continuous fermenters of 500 m^3 are required, operating with a residence time of 50 hours. An important difference from the fed-batch process is the lower frequency of inoculation operations. Since it is assumed that the fermenters will run uninterrupted for long periods, a reduced number of seed vessels is necessary. It was considered that only one seed vessel of each volume (1,000, 10,000 and 100,000 litres) will be sufficient for the whole continuous operation, as the inoculation operations occur in low frequency

2.4 Harvesting – Biomass dewatering

Disk stack centrifuges are usually preferred for algae processing over decanter centrifuges, because they can process very dilute algal slurries (as low as 0.02 % solids). On the other hand, decanter centrifuges can produce much higher dense cakes (up to 40 % solids) but require higher feed concentrations (around 10 % solids), such as those achieved in heterotrophic cultivations [65]. In addition, decanter centrifuges can operate at higher throughputs than other types of centrifuges and are ideal for continuous operation [66]. Since the bioreactors operate at relative high densities and high throughputs, the decanter centrifuges were chosen for biomass harvesting. This operation concentrates the biomass stream up to a solids concentration 30 % (w/w). Algal slurries are usually easily pumped with solids concentrations as high as 35% wt [67], so a 30 % slurry will probably have no problems during pumping operations.

The total throughput of medium for the harvesting section was calculated as 35 m^3 h^{-1}. The cell limiting size for separation was considered to be 1 µm, since *Chlorella* cell size ranges from 2 to 5 µm [68]. The centrifuge operates continuously for both batch and continuous fermentation. In batch case, the discharge of each fermenter takes approximately 24 hours.

2.5 Oil extraction

The concentrated algal slurry is transferred to a high pressure homogenizer for cell disruption (Figure 8). High pressure homogenization (HPH) is an easily scalable technology and is highly effective to process wet biomass, eliminating the need of a preliminary drying stage [69]. Yap *et al.* [70] showed that disruption performance is independent of solids concentration between 0.25 and 25 % w/w, so HPH can be applied to slurries with high solids content. Moreover, the authors calculated that the energy consumed by high pressure homogenization for microalgae cell disruption may use only 6% of the energy density of the resulting biodiesel, from a 25 % solids and 30% TAG (dry weight) content feed. In the homogenizer, cells are ruptured at a pressure drop of 800 bar [69]. The resulting slurry contains 95 % [71] of disrupted cell material with 50 % lipid content [32] .

Lipids are extracted from the microalgal biomass through conventional liquid-liquid solvent extraction according to [72]. Differently from the other downstream operations, this stage is performed in batches. The disrupted microalgae slurry is transferred to stirred tank in which hexane at 60 ºC is added and mixed for five hours to solubilize the lipids at a yield of 95 % (Figure 10). The mixture containing lipids and hexane is then transferred to a decanter centrifuge for phase separation. The lighter phase, containing hexane and lipids, is sent to a single

stage evaporator for removal of the solvent. The evaporated solvent is recovered in a condenser and recirculated in the extraction system.

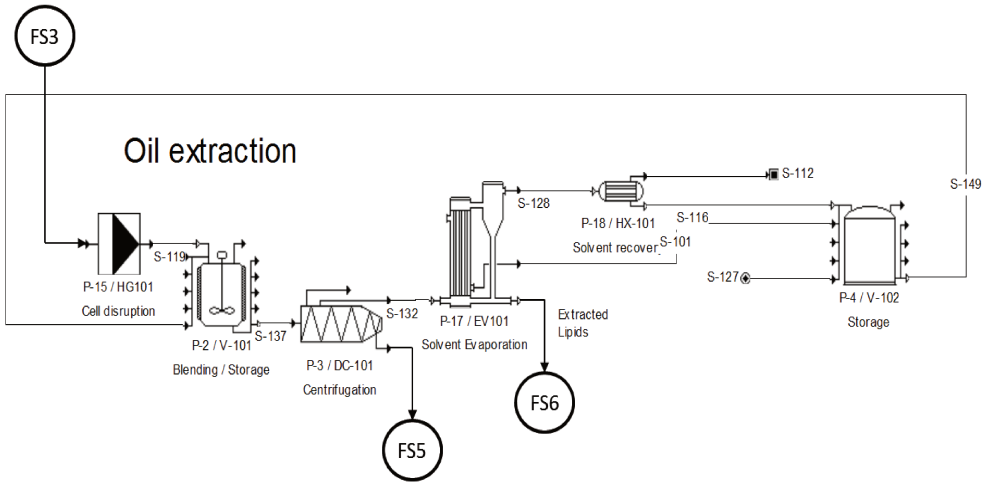

Figure 510. FS4 - PFD of oil extraction section

The extracted lipids free from solvent are transferred to the transesterification reactor for biodiesel synthesis. The aqueous phase, containing water and cell debris, is transferred to the algae meal section.

2.6 Biodiesel production

Biodiesel is usually synthesized through transesterification of fatty acids with methanol in presence of an acid or alkaline catalyst [73] . However, in our integration proposal it makes sense to use ethanol as feedstock for transesterification of microalgal fatty acids. Additionally, ethanol presents advantages over methanol such as renewability, less toxicity and higher cetane numbers of the ethyl esters compared to methyl esters [74]. On the other hand, stable emulsions may occur during the ethanol based transesterification reaction, making difficult the gravitational separation of the upper ester rich layer and the lower glycerol rich layer [75]. As described by Haas *et al.* [76] esterified fatty acids are force separated from the ethanol/glycerol phase using decanter centrifuges.

Microalgae free fatty acids content may be as high as 84% (m/m)[77], although contents of FFA above 5 % may be the result of TAG hydrolysis during sample preparation [78]. Transesterification of oils containing high content of free fatty acids using alkali catalyst usually leads to low yields due to the formation of soap, which inhibits separation of alkyl esters and glycerol [79]. We considered that the extracted oil contains a maximum FFA content of 10% (w/w) and, therefore, a pre-esterification of the oil with an acid catalyst is necessary to reduce the acid value before the addition of alkaline catalyst.

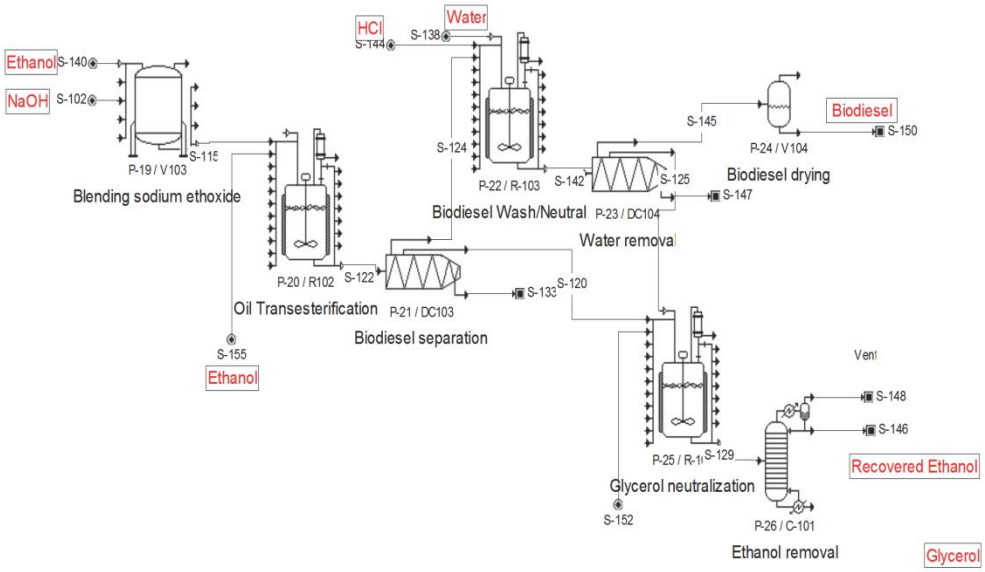

Figure 11. FS6 - PFD of biodiesel production section

To avoid soap formation during base catalysed transesterification, biodiesel synthesis is performed in a two-step reaction batch in the same reactor (Figure 11). In the first stage, sulphuric acid (1% of total FFA mass) and ethanol (6:1 molar ratio alcohol:FFA) are mixed with the extracted oil and reacted for 1 h at 60 ºC for esterification of free fatty acids. The resulting mixture contains ethanol, sulphuric acid, esterified fatty acids (10 % of total fatty acids), glycerol and the remaining lipids (circa 90% of fatty acid content).

In the second stage, sodium hydroxide (1% of total lipid mass) is mixed with ethanol (6:1 molar ratio alcohol:lipid) to form sodium ethoxide, before being added to the lipid mixture free from fatty acids. The basic transesterification reaction is run at 60 ºC for 1 hour, after which 95% of the total fatty acids are esterified. The reacted mixture is sent to a decanter centrifuge to separate the mixture in two streams: one heavy phase containing mainly glycerol and ethanol and one light phase rich in biodiesel. The biodiesel output stream is sent to a second reactor for neutralization of the remaining catalyst by addition of hydrochloric acid, and washing with water at 50 ºC to remove remaining glycerol, soap, catalyst and unreacted fatty acids. The neutralized mixture is centrifuged once more to remove most of the remaining water, glycerol and ethanol. The oil phase output of the centrifuge is then heated up to 220 ºC and flash evaporated to remove the last remainders of water and ethanol as the last stage of purification [76, 79].

2.7 Algae meal processing (residual biomass)

Algae meal or defatted microalgal biomass has been successfully tested as substitute of corn or soybean meal in animal feed for chicken, pigs and aquaculture [80-82]. In the proposed design, the remaining biomass from oil extraction is dried and sold as protein-rich supplement for animal feed.

The bottom phase of the lipid extractor contains 17 % (m/m) de-fatted biomass, 80 % (m/m) water and the remaining fraction composed by ash, soluble salts and 0.8 % (m/m) non-extracted lipids. Ultrafiltration was preferred over centrifugation because of the small diameter of the cell debris particulates, that may require long times of centrifugation [26] (Figure 12). Drum drying is a mature drying industrial technology and is often used in the food industry [83].

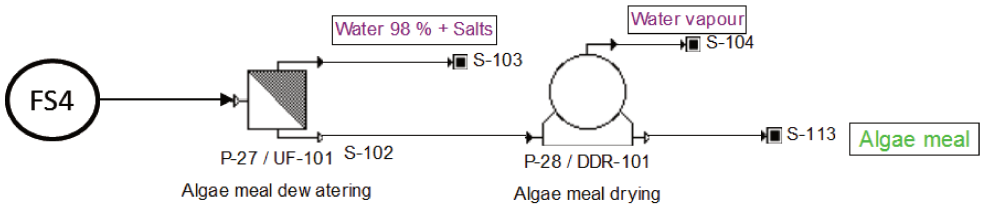

Figure 612. FS5 - PFD of algae meal processing section

2.8 Cost and Economic analysis

2.8.1 Direct Fixed Capital Cost Estimation

For the economic analysis of the proposed design, it is necessary to estimate the total Direct Fixed Capital cost of the plant. This includes a) purchase cost of all equipment; b) bulk items, such as pipes, valves wiring, instruments, and others; c) construction costs of buildings, roads and auxiliary facilities; d) engineering costs and e) contingency costs, to deal with uncertainties about the project cost estimates [84].

Purchase costs of equipment were estimated through built-in cost models from Superpro Designer database or estimated from literature sources such as Peters *et al.* [85], Towler *et al.* [86], and adjusted through the Chemical Engineering Index from the source period to the 2014 as year of reference. Costs and specifications of equipment for biodiesel processing and algae processing were also based on published data from Haas *et al.* [87] and Klein-Marcuschamer *et al.* [72] . Auxiliary equipment that is not itemized in the equipment list, such as pumps, valves and auxiliary tanks were estimated as 20% of the EPC. The choice of the multiplier depends on the level of detail of equipment listing and may vary from 5 to 25 % of the EPC [88].

There are different methods to estimate Direct Fixed Capital Costs, with different degrees of accuracy. For our purposes it is convenient to use an estimation method based on percentages of the equipment purchase costs. To estimate the Direct Fixed Capital Costs, we considered the factorial method described in Towler *et al.* [86] and Peters *et al.* [85]. Some modifications were made to adjust the method to the proposed design. Since this study proposes the design of a biodiesel plant annexed to a pre-established sugarcane biorefinery, items such as "Auxiliary Facilities Construction" were ignored, since we assumed that the existing infra-structure is sufficient for the operation. The factors used are summarized in Table 5.

Table 5. Capital Costs estimation parameters

Item	Contribution	Typical ranges [85, 86]
a. Equipment Installation	0.5	0.3-0.6
b. Piping	0.6	0.2-0.8
c. Instrumentation	0.15	0.2-0.3
d. Electrical	0.1	0.1-0.2
e. Buildings	0.5	0.3-0.5
f. Yard improvement	0.05	0.02-0.05
g. Auxiliary Facilities construction	0	0.4
	DC = Direct Capital Cost = EPC x (1+(a+b+c+d+e+f+g))	
h. Design and Engineering	0.1	0.1-0.3
	TPC = Total Plant Cost = DC x (1+h)	
i. Contractor's fee	0.05	0.02-0.06
j. Contingency	0.1	0.05-0.15
	DFC = Direct Fixed Capital = TPC x (1+h+i)	

2.8.2 Working capital

Working capital was calculated as the amount necessary for 30 days of raw materials, labor utilities and waste treatment costs.

$$30 \text{ days cost} = 30 \times (\text{Annual cost} / 330)$$

2.8.3 Operating costs

The operating costs OPC were estimated as

$$OPC = MC + UC + WC + LC + FC$$

Where MC = raw materials cost; UC = utilities cost (electricity, steam, high pressure steam, cooling and chilled water); LC = labor cost; WC = waste treatment cost; and FC = facility related costs. Operating costs are described in different sections as annual total costs and cost per litre of biodiesel. The latter is simply calculated by dividing the total costs by the total production of biodiesel and this analysis ignores other sources of revenues.

Materials cost were estimated from ICIS pricing database [89, 90], previous works [45], and Superpro built-in database.

Utilities costs were estimated from published studies on sugarcane biorefineries [45, 47, 50] and built-in costs from Superpro Designer.

For the labor costs estimation, the number of operators was estimated for each equipment described in the process design. This number was then multiplied by the total number of annual work hours and by $11.50 per work-hour [91]

The cost of the waste streams treatment (circa 98 % water) was estimated as $ 1.5 per metric ton [86]. This estimate is valid for waste streams with low dissolved solids content, non-toxic or corrosive. It was considered that the treated water can be later reused in the process. Solid wastes (up to 50 % water content) were disposed in landfills.

The facility related costs are defined as:

FC = Depreciation costs + Maintenance costs + Insurance (1 % DFC) + Taxes (2 % of DFC) + Miscelaneous factory expenses (1 % DFC)

Depreciation costs were calculated based on the total equipment purchase cost to be depreciated over a period of 10 years. For the estimate of the annual depreciation cost, a linear calculation was used:

Depreciation = (DFC + Startup and Validation Costs (5 % DFC)) / 10 years

2.8.4 Financial analysis

In order to assess the financial feasibility of the process with different configurations, the 30-year net present value (NPV) and the internal rate of return (IRR) of the project were assessed. Sensitivity analysis were used to determine the most important factors for the plant profitability and 30 year- NPVs assessments were applied to determine the minimum selling price of the biodiesel for profitability under different conditions.

2.8.5 Microalgae plant as independent business vs integration with sugarcane biorefinery

The financial analysis of the microalgae biodiesel plant was performed for a stand-alone facility and an integrated facility. The stand-alone microalgae plant was considered as an independent business. In this model, raw materials and utilities are purchased from external suppliers at market prices and biodiesel and algae meal are sold as commercial products. The second business model is a microalgae biodiesel plant annexed to a pre-existing sugarcane biorefinery.In this integrated model, the microalgae plant uses molasses generated during sugar crystallization as raw material and electricity and steam from bagasse burning and co-generation. Unlike the stand-alone model, the substrate is directly transferred from the crystallization process to the microalgae production plant, resulting in less energy usage for purification and transportation of the sugar. Apart from utilities, the equipment costs for microalgae-based biodiesel production are assumed to be identical in both set-ups.

3. Results and Discussion

3.1 Capital costs

3.1.1 Equipment purchase costs (EPC)

The equipment list and purchase costs for batch and continuous production is shown in Table 6 and Figure 13. The fermentation section is by far the most costly for the capital estimation with more than 64 % and 55.5 % of total equipment purchase cost for batch and continuous process, respectively.

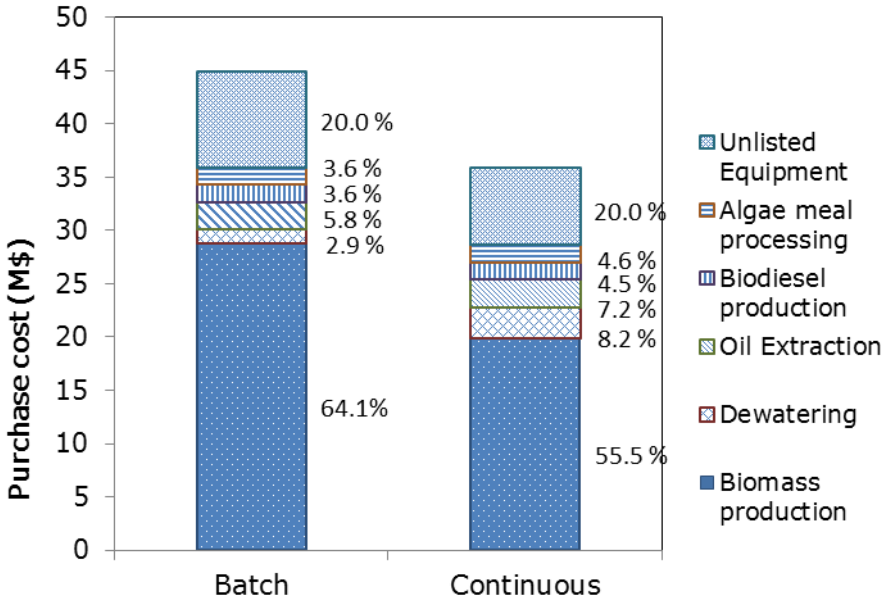

Figure 13. Equipment purchase costs for Batch and continuous mode

The fermenters alone are responsible for 95 % of the total purchase cost of fermentation section. The relatively low volumetric productivity of microalgae lipids is the most important factor for bioreactor sizing calculations and results in a high number (20, see Table 6) of large scale bioreactors.

Table 6. Equipment list of fed-batch and continuous microalgae plant

	BATCH		CONTINUOUS	
Equipment	Specification	Quantity	Specification	Quantity
FERMENTATION				
Seed Fermentor	1,000 L	5	1,000 L	1
Heat Sterilizer	2,010 L/h	5	2,010 L/h	1
Seed Fermentor	10,000L	5	10,000L	1
Heat Sterilizer	20,100 L/h	5	20,100 L/h	1
Seed Fermentor	100,000 L	5	100,000 L	1
Stirred Reactor	14.34 m3	1	14.34 m3	1
Rotary Vacuum Filter	57.13 m2	1	57.13 m2	1
Blending Tank	8033.03 L	1	257000 L	1
Blending Tank	14.55 m3	1	14.55 m3	1
Heat Sterilizer	14461.75 L/h	1	14461.75 L/h	1
Heat Sterilizer	13095.91 L/h	1	58,700 L/h	2
Fermentor	500.00 m3	20	500.00 m3	15
Centrifugal Compressor	1105.62 kW	2	2125 kW	3
DEWATERING				
Decanter Centrifuge	447.80 L/min	1	130727 l/min	1
OIL EXTRACTION				
Homogenizer	15743.40 L/h	1	15743.40 L/h	1
Blending Tank	255848.34 L	1	255848.34 L	1
Decanter Centrifuge	38839.56 L/h	1	38839.56 L/h	1
Evaporator	4.52 m2	1	4.52 m2	1
Condenser	28.42 m2	1	28.42 m2	1
Flat Bottom Tank	25756.30 L	1	25756.30 L	1
BIODIESEL				
Vertical-On-Legs Tank	839.50 L	1	839.50 L	1
Stirred Reactor	7423.95 L	1	7423.95 L	1
Decanter Centrifuge	3238.13 L/h	1	3238.13 L/h	1
Stirred Reactor	773.68 L	1	773.68 L	1
Decanter Centrifuge	2778.11 L/h	1	2778.11 L/h	1
Flash Drum	245.75 L	1	245.75 L	1
Stirred Reactor	100.77 L	1	100.77 L	1
Distillation Column	286.50 L	1	286.50 L	1
ALGAE MEAL				
Ultrafilter	67.58 m2	5	67.58 m2	5
Drum Dryer	19.34 m2	6	19.34 m2	6

In fed-batch case, the need for an inoculum cultivation structure with multiple staggered seed fermenters is a source of high capital costs, representing 15 % of the EPC. In continuous mode, the long times of uninterrupted operation result in lower demand of inoculation operations and, consequently, 15.8 % lower capital expense. However, the lower product concentration obtained in continuous cultivation has a negative impact over the medium preparation section. The purchase cost of medium preparation equipments (blending tanks V-101 and V-102 and heat sterilizers ST-103 and ST-104) is 2.4 times higher in continuous than fed-batch. For the same reason, biomass harvesting centrifuges (DC-102) have 4 times larger capacity with 2.3 times higher purchase cost. These cost differences, however, are not significant in relation to seed and production fermenter costs as demonstrated in Figure 12.

3.1.2 Direct Fixed Capital Estimation

The Direct Fixed Capital estimation from the EPC percentages is shown in Table 7. The DFC for the continuous process was estimated to be 79 % of the DFC for the fed-batch process. This can be explained by the lower number of fermenters needed for continuous cultivation, that drastically reduce the equipment purchase costs, as seen in the former section.

Table 7. Direct Fixed Capital factorial estimation (in thousands of US$).

Item	Batch	Continuous
EPC	44,950	35,870
a. Installation	22,480	17,940
b. Piping	26,970	21,520
c. Instrumentation	6,740	5,380
d. Electrical	4,500	3,590
e. Buildings	22,480	17,940
f. Yard improvement	2,250	900
DC	130,370	103,130
g. Design and Engineering	13,040	10,310
TPC	143,400	113,440
h. Contractor's fee	7,170	5,670
i. Contingency	14,340	11,340
DFC	164,910	130,450

The DFC was estimated to be 165 million for the batch and 130 million for the continuous process. In relation to the EPC, a Lang factor (multiplier of EPC for rough estimation of DFC) of approximately 3,6 was obtained. This is coherent with the Lang factors used for solid-liquid processing plants. [86].

These are large figures when compared with bioethanol refineries, whose capital investment are estimated to be around $ 250 million [47] for a capacity of 250 million litres of bioethanol per year. The main differences between the two processes are the productivity of the fermentation section and the purchase cost of the fermentation equipment. In comparison, ethanol productivity can reach up to 8 g L^{-1} h^{-1} against 0.35 g L^{-1} h^{-1} for microalgal lipids. In addition, bioethanol fermentation is performed in anaerobic fermenters, which have significantly lower purchase costs than aerobic fermenters, necessary for heterotrophic microalgae production.

The estimation of equipment purchase costs can largely vary depending on the specifications, suppliers, materials and location factors. Brazil has a long experience with sugarcane processing engineering and there are many companies in the country specialized in fermentation plants construction. Note that the equipment costs for ethanol industry in Brazil are substantially lower than those estimated in this study. Location factors that adjust purchase prices are available; however they may also not be reliable due to fluctuations of construction material prices, inflation and other constraints. A brief review of similar studies and process design literature reveals a wide variability of fermenters purchase costs. The prices from some of these cases are summarized in Table 8.

Table 8. Fermenter prices estimates from literature

Purpose	Equipment description	Purchase Cost (thousands of US$)	Calculated price* V = 500 m^3 (thousands of US$)	Reference
Lipid production by oleaginous yeast	250 m^3 aerobic fermentor (STR)	$ 3,200	$ 4,850	[92]
Farnesene production by yeast	200 m^3 aerobic fermentor (STR)	$ 125	$ 217	[72]
Lipid production by heterotrophic algae	150 m^3 aerobic fermenter (STR)	$ 190	$ 391	[93]
	500 m^3 aerobic fermentor (STR)	$3,774	$3,774	Superpro Designer built-in database
This study	500 m^3 air-lift fermentor	$ 840 (reference year 2015)	$ 815	Superpro Designer built-in database

*For comparison purposes, the prices for 500 m^3 fermenters were calculated according to the scale up rule of thumb $C1/C2 = (V1/V2)^n$ with n = 0.6.

To determine the impact of the fermenter purchase cost and biomass productivities over the Direct Fixed Capital of the design, a sensitivity analysis is shown in Figure 14. Based on the data from Table 8, the purchase costs of fermenters was assessed from $500,000 to $3,000,000. Also the impact of biomass productivity over the DFC was assessed from 0.4 g L^{-1} h^{-1} to 1 g L^{-1} h^{-1} (base productivity: 0.68 g L^{-1} h^{-1}).

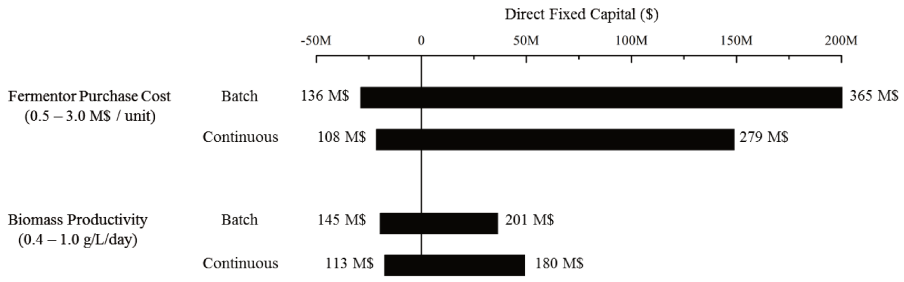

Figure 14. DFC sensitivity analysis of microalgae biodiesel plant

The fermenter cost had a strong impact over the direct fixed capital of the microalgae biodiesel plant. The highest value of DFC calculated for batch operation was, approximately, 3 times larger than the lower. Table 8 shows that the variability of equipment cost estimate found in literature (from $0.2 M to $ 5.0 M) is even wider than the range applied in the sensitivity analysis (from $0.5M to $ 3.0 M). The use of inaccurate estimations for equipment pricing can be misleading in process design studies and lead to very different conclusions.

3.2 Operating costs

3.2.1 Materials cost

In Table 9, the demand for raw materials is described. The cost of molasses is 56 % of the total raw materials cost. After molasses, the most important items for material cost estimation are glycine and KH_2PO_4. This is no surprise, since nitrogen and phosphorus are the two most important nutrients for microalgae growth after carbon [94]. Glycine, however, is not the most economical choice of nitrogen source, since *C. protothecoides* can utilize cheaper inorganic sources such as nitrate [95]. However, the highest biomass productivities to date were obtained when glycine was utilized as nitrogen source [17, 32, 96].

Table 9. Raw materials consumption and costs per year and per liter of biodiesel

Material Name	Unit Cost ($/kg)	Amount (Ton/year)	Annual Cost ($/year)	Cost / product ($/L biodiesel)	%
$CaCl_2$	0.7	24	16757	0.0008	0.07
Ethyl Alcohol	0.52	5,391	1,886,727	0.09	7.58
Glycine	1.5	3,990	5,984,541	0.30	24.05
HCl	0.1	148	14,803	0.0007	0.06
Hexane	0.4	233	93,199	0.005	0.37
KH_2PO_4	0.7	3,983	2,788,172	0.14	11.20
$MgSO_4$	0.35	2,627	919,289	0.05	3.69
Molasses	0.11	127,670	14,043,724	0.70	56.43
NaOH	0.4	160	63,835	0.003	0.26
Sulfuric Acid	0.07	402	28,106	0.001	0.11
TOTAL			31.9	1.70	100

No differences were observed in materials consumption for batch or continuous modes, with the only exception being water for culture medium. We disregarded water consumption as material cost, since we considered that most of it can be reused after waste treatment.

3.2.2 Utilities cost

The utilities demand and costs are described in Table 10 and Figure 15. Most of the electricity was consumed in fermentation section for aeration. The cooling water was consumed in highest amount in the fermentation section, for fermenter and compressors cooling. Chilled water was consumed mainly during oil extraction for the solvent recovery after evaporation. While the highest consumption of steam in the whole process was observed for the algae meal section, mainly during meal drum drying.

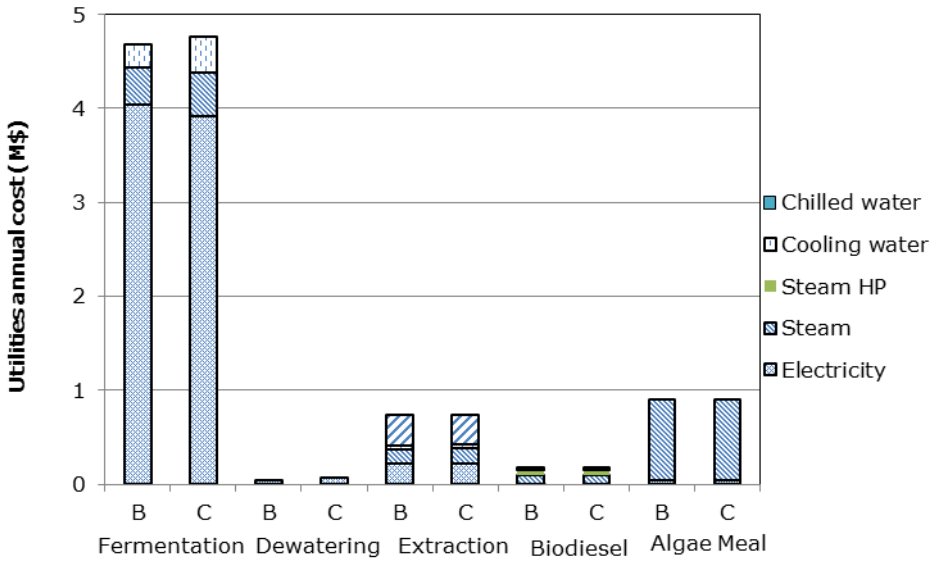

Figure 15. Utilities consumption per section of batch (B) and continuous (C) operation

The main energy sources consumed were electricity and steam (152 ºC). The process simulation indicated an overall consumption of electricity of, approximately, 4.2 kWh per litre of biodiesel. .

Table 10. Total utilities cost per year and per liter of biodiesel

	Batch			Continuous		
Energy Source	Annual demand	$ / year	$ / L biodiesel	Annual demand	$ / year	$ / L biodiesel
Electricity	84,600,000 kWh	4,400,000	0.22	82,870,000 kWh	4,309,000	0.22
Steam	167,800 MT	2,010,000	0.10	129,000 MT	1,547,000	0.08
Steam HP	2,500 MT	50,000	0.003	2,500 MT	50,300	0.003
Cooling water	9,910,000 MT	300,000	0.02	8,810,000 MT	441,000	0.02
Chilled water	807,000 MT	323,000	0.02	780,300 MT	312,000	0.02
Total		6,573,000	0.35		6,659,400	0.33

The continuous and batch processes had very similar overall energy demands (Figure 14). Although the higher product concentration reduced the energy consumption for biomass centrifuging operation (44 % lower than in continuous cultivation), the higher demand of energy for production and seed fermenters in batch cultivation resulted in a 2% higher overall consumption of electricity than in continuous operation. However, the overall costs for utilities for both process modes differed by only $0.04 per litre of biodiesel with $1.12 and $ 1.08 for batch and continuous cultivation, respectively.

3.2.3 Labour

The labour demand for both batch and continuous operation is shown in Table 11. The labour demand in continuous mode was 33 % lower than the obtained for batch mode. The explanation for this difference lies in the assumption that the sequential operations of inoculum preparation, fermentation, discharging, cleaning and sterilization in batch processing are more labour demanding than the long-term continuous cultivation.

Table 11 . Labor cost per year and per liter of biodiesel for batch and continuous operations

	Batch		Continuous	
Section	Annual demand	$ / L biodiesel	Annual demand	$ / L biodiesel
Fermentation	2,274,456.00	0.11	997,919.00	0.05
Extraction	325,040.00	0.02	324,330.00	0.02
Biodiesel	1,010,408.00	0.05	1,016,206.00	0.05
Algae meal	208,566.00	0.01	212,002.00	0.01
Total	3,818,470.00	0.19	2,550,457.00	0.13

3.2.4 Waste treatment

As mentioned before, the aqueous waste remaining from algae cultivation will be recovered from biomass centrifugation and algae meal utrafiltration. Both streams are composed of 98-99 % (m/m) water, cell debris (around 0.1 % m/m), remaining salts from culture medium and ashes (around 0.8-1.0 % m/m). From molasses clarification, a solid ash/gypsum-based residue with 28 % water content is separated in a decanter centrifuge.

Since 70 % dry weight of the solid residue is composed of minerals from sugarcane and gypsum formed during molasses clarification, this material could theoretically be used as fertilizer in sugarcane crops or as a supplement in animal feed. We considered that the solid residue will be disposed in landfills since there is not enough data to assure the direct use of it as fertilizer. However if this application is possible, it could represent a monetary gain since less fertilizer would be needed for the crops.

It was reported that the solid residue separated by centrifugation may contain 30 % of Calcium (in the form of gypsum), up to 50 % of K_2O, 3-4 % of MgO, 1-2 % of P_2O_5 and the remaining consisting of a variety of minerals (Al, Fe, Mn, Si, and others) [56]. K_2O, the largest fraction of the minerals found in the residue, is usually applied to crops in the ratio of 150-285 kg ha^{-1} in the form of potash [97], with a market price of around \$700 ton^{-1} K_2O (U.S. Geological Survey, Mineral Commodity Summaries, January 2015). If all the solid residue generated from molasses clarification was re-applied to the soil as fertilizer, around 4,250 ton yr^{-1} of K_2O could be replaced (50 % of 8,500 ton of residue, see Table 12), resulting in total savings of $ 2,975,000.

The high water content in continuous cultivation substantially increases the amount of aqueous waste and, consequently, waste treatment costs (Table 12).

Table 12. Waste treatment cost per year and per liter of biodiesel for batch and continuous processes

Cultivation mode	Unit cost ($/MT)	Amount (MT/year)	Cost ($/year)	Cost ($ /L biodiesel)
BATCH				
Solid Waste	50	8,568	428,413	0.02
Aqueous Waste	1.50	180,664	270,997	0.01
CONTINUOUS				
Solid Waste	50	8,568	428,413	0.02
Aqueous Waste	1.50	959,221	1,438,832	0.07

3.2.5 Facility Related costs

The facility related costs are summarized in Table 13. The depreciation costs are estimated in more than $ 15 million per year for the batch process and $ 12 million for the continuous process. This represents $0.79 per litre of biodiesel for the batch process and $ 0.62 for the continuous process. Maintenance and Insurance were estimated in 6.1 million for batch process and 4.9 million for the continuous process (table 13).

Table 13. Facility related costs for batch and continuous processes

	Batch			Continuous		
	Annual cost ($)	Cost per L biod ($)	%	Annual Cost ($)	Cost per L biod ($)	%
Maintenance	4,495,375.00	0.22	21	3,587,000.00	0.18	21
Depreciation	15,705,983.99	0.79	72	12,424,257.74	0.62	72
Insurance	1,649,128.32	0.08	7	1,304,547.06	0.07	8
Total	21,850,487.31	1.09		17,315,804.80	0.87	

The incorporation of depreciation increase the operational costs considerably. For low value added products, such as biodiesel, market prices are often based on the results of long-term operating plants, which are already 100 % depreciated [98].

3.3 Operating cost estimation

The composition the total operating costs are depicted in Figure 16. To determine the biodiesel operating costs, the revenues from the co-product algae meal were discounted from the total costs. The simulation indicated the possible annual production of 17.5 million kg of algae meal and the selling price of $ 0.45 per kg was considered for the revenue calculation.

Table 14. Operating costs composition of microalgae biodiesel plant

Cost source	Batch			Continuous		
	Annual cost ($)	$/L	%	Annual cost ($)	$/L	%
Waste	699,410	0.03	1.2	1,867,245.00	0.09	3.50
Labor	3,818,470	0.19	6.6	2,550,457.00	0.13	4.79
Utilities	6,813,372.78	0.34	11.7	6,658,733.80	0.33	12.50
Facility related	21,850,487.31	1.09	37.6	17,315,804.80	0.87	32.50
Materials	24,886,486.89	1.24	42.9	24,886,486.89	1.24	46.71
Total	58,068,226.97	2.90	100.0	53,278,727.49	2.66	100.00
Total - algae meal revenues $		2.51	Total - algae meal revenues $		2.27	

The total cost per liter of biodiesel was calculated as $ 2.51 for batch process and $ 2.27 for continuous process. Production costs are nearly two times higher than the world average 2014 diesel price of $ 1.16 / liter [49].

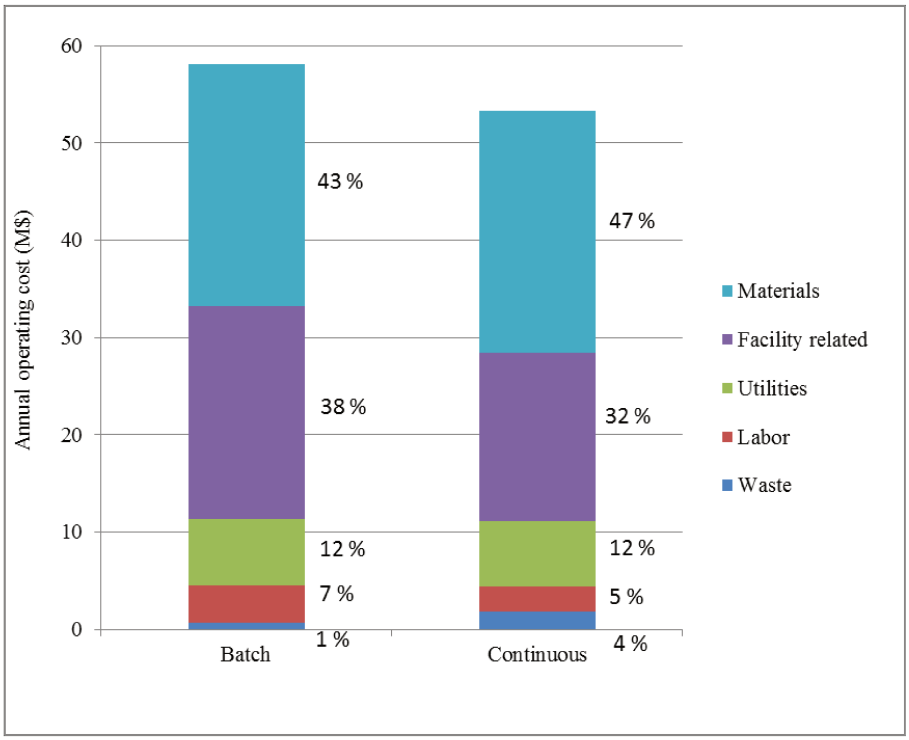

Figure 16. Annual operating costs composition for the microalgae biodiesel plant

The materials cost was the most important fraction of cost composition with 43 and 47 % of the total costs for batch and continuous operation, respectively. Facility related costs represent 38 and 32 % of the total production costs, which reflect the high capital costs of the process.

If depreciation is not considered, the production cost is reduced by 20 and 10 % for batch and continuous modes (Table 15).

Table 15. Operating costs composition without depreciation

Cost source	Batch			Continuous		
	Annual cost ($)	$/L	%	Annual cost ($)	$/L	%
Waste	699,410.00	0.03	1.7	1,867,245.00	0.09	4.6
Labor	3,818,470.00	0.19	9.0	2,550,457.00	0.13	6.2
Utilities	6,813,372.78	0.34	16.1	6,658,733.80	0.33	16.3
Facility related	6,144,503.32	0.31	14.5	4,891,547.06	0.24	12.0
Materials	24,886,486.89	1.24	58.7	24,886,486.89	1.24	60.9
Total	42,362,242.98	2.12	100.0	40,854,469.75	2.04	100.0

For bulk chemicals, the depreciation is sometimes not incorporated in the operating costs, but added to the capital charge, (profit plus depreciation). [99].

3.4 Working capital and Start-up cost

The working capital was calculated as the money necessary for 30 days of operating costs. Start-up cost was estimated as 5 % of the total DFC (Table 16).

Table 16. Working capital and start-up costs for batch and continuous processes

Item	Batch	Continuous
Working capital	5,300,900	5,226,600
Start-up cost	21,623,000	15,418,000

3.5 Financial Analysis

3.5.1 Microalgae plant as independent business

The feasibility of the microalgae process as an independent business was assessed through NPV analysis. The minimum biodiesel selling price that equals the NPV of the plant to 0 at a fixed IRR was calculated for different conditions. From the operating costs it is clear that the materials and capital expenses are the most important factors affecting the profitability of the business. Moreover, the co-product algae meal plays an important role, increasing significantly the total revenues. At the conditions of the base case and a fixed IRR of 7% and algae meal price of $ 0.45 per kg, the minimum selling price of biodiesel was 2.51 and 2.27 for the batch and continuous modes, respectively. The sensitivity analysis was performed for IRR values from 2 to 12%, molasses prices from $ 0 to $ 0.22, N-source prices from $ 0.5 to $ 2.5, fermentor purchase cost from $ 0.5 M to $ 3.0M, biomass productivity from 0.4 – 1.0 g L^{-1} d^{-1}, and algae meal selling price from $ 0.25 to $ 0.65 (Figure 17).

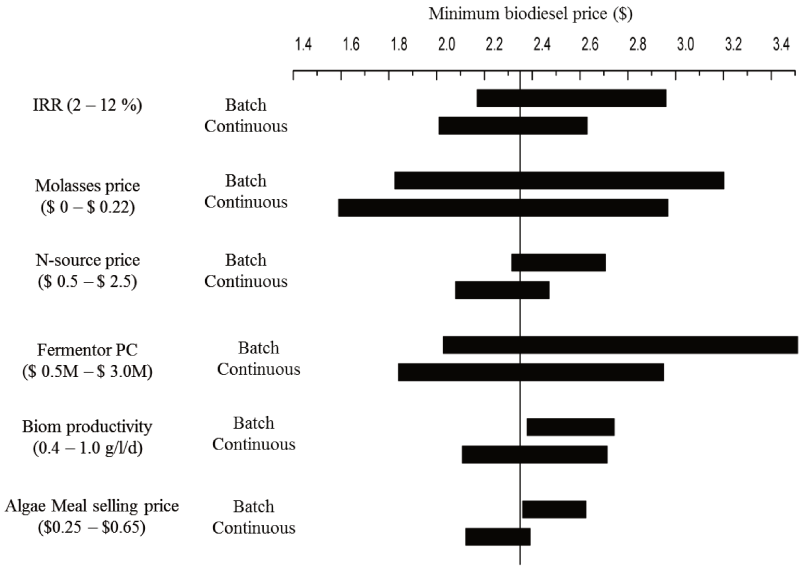

Figure 17. Sensitivity analysis of minimum selling price against most important cost factors

The sensitivity analysis (Figure 17) shows that different internal rates of return (IRR) caused the biodiesel minimum selling price to vary between $ 2.17 and $ 2.96 per liter for batch cultivation and $ 2.01 and $ 2.63 per liter for continuous mode. An IRR of 12 % is usually taken as the standard for attractive rate of return[100], so $ 2.96 and $ 2.63 would be the minimum required selling prices to assure the financial feasibility of the proposed design as an independent business. These prices are nearly 3 times larger than the diesel selling prices, which indicates that biodiesel and algae meal cannot generate income revenues sufficient for supporting the total costs of the plant.

Between the limits tested, molasses and fermentor purchase costs had strong impacts over the minimum biodiesel selling price and, consequently, over the economic feasibility of the plant. For molasses purchase costs between $ 0 and $0.22 per kg, the minimum biodiesel price had a variation of $ 1.4 per litre from the lower to the higher value. The variation of the fermentor purchase cost between $ 0.5 M and $3.0 M caused a variation of $ 1.5 in the minimum biodiesel price for batch mode and $1.1 for continuous mode. The stronger impact over the batch design was not surprising, as the batch cultivation requires a higher number of fermenters.

The N-source price did not show a strong impact over the biodiesel minimum price causing a variation of $ 0.4 of the biodiesel price for both cultivation modes, even though a wide range of N-source prices was tested ($ 0.5 – $ 2.5). The algae meal selling price between $0.25 and $0.65 had an effect of $ 0.3 over the biodiesel price for batch and continuous modes.

3.6.1 Integrated biorefinery

The main objective of the microalgae integrated system is the substitution of the diesel fuel consumed by the sugarcane biorefineries. In this proposal, utilities and molasses are supplied by the first generation biorefinery, which reduce biodiesel production costs. However, the production of microalgal based biodiesel and algae meal impact bioethanol and energy production, since part of the molasses previously used for bioethanol production are now consumed in the the microalgae process. The electricity sales revenue from cogeneration is also influenced since part of the power surplus is utilized in biodiesel production. Since it is not the goal of this study to present a detailed financial analysis of a sugarcane biorefinery, but to evaluate the effects of the integration to a microalgae biodiesel plant, a simplified analysis of gross profits was applied for feasibility assessment. The gross profit of the

base sugarcane biorefinery is calculated from literature data and compared with the estimate gross profit of the integrated microalgae-sugarcane plant (Table 17). In Figure 18 the inputs and outputs for both cases are depicted.

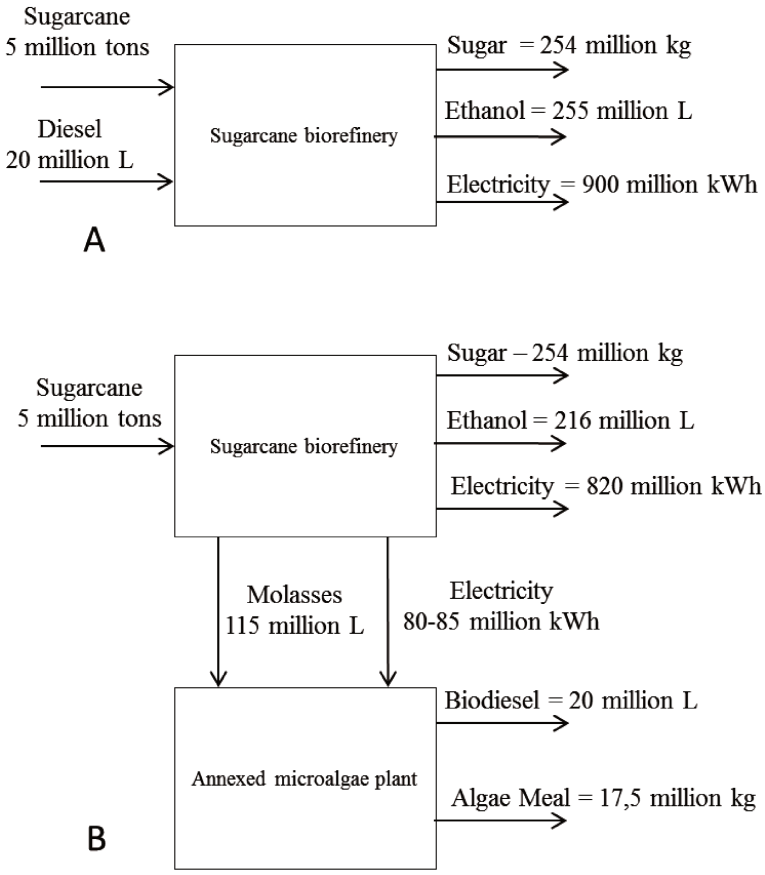

Figure 18. Diagram of integration of sugarcane biorefinery and microalgal biodiesel plant

The base sugarcane biorefinery (Figure 18 A) outputs were calculated from the data available in Cavalett *et al.* [46]. A sugar mill that processes 5 million tons of sugarcane is expected to produce 254,000 tons of sugar and 256 million litres of bioethanol. Assuming that the sugarcane biorefinery is equipped with modern high pressure cogeneration equipment, a surplus of 910 million kWh of electricity is produced from bagasse. These three outputs generate revenues of $ 316 million / year. From the estimate of diesel consumption it is calculated that around $ 20 million / year are spent with diesel purchase. The final result from revenues after discounting the diesel purchase expense and production costs is calculated as $ 90,350,000 million per year.

Table 17. Gross profit analysis of the base sugarcane biorefinery (A).

Product	Cost per unit $	Production costs $	Selling Price $	Revenues $
Sugar 254,500,000 kg	0.25/kg	63,625,000	0.43/kg	109,435,000
Ethanol 256,020,279 L	0.35/L	94,727,503	0.6/L	153,612,000
Electricity 910,000,000 kWh	0.052/kwh	47,320,000	0.058/kwh	52,980,000
Diesel 20,000,000 L	1 / L	20,000,000 (purchase)		
	Total Cost $	225,672,500	Total Revenue $	316,027,000
				Gross Profit ($) 90,350,000

For the integrated plant (Figure 18 B), the molasses and utilities demand were calculated as described in section Operating costs. As these resources are now consumed in biodiesel production, the ethanol and electricity revenues are reduced. The new electricity revenue is calculated as follows.

Elect revenue (B) = Elect revenue (A) – Microalgae plant demand x Elec selling cost

The reduction of ethanol production is calculated based on the conversion of the sugar content of the molasses deviated to ethanol by using conversion factors of from literature data ($Y_{ethanol/molasses}$ = 0.45 g ethanol / g sugar from molasses [101]) .

Ethanol revenue (B) = Ethanol revenue (A) – Molasses to microalgae plant x $Y_{ethanol/molasses}$

In the integrated model, the diesel purchase is no longer accounted since it is substituted by microalgae biodiesel. The operation costs of biodiesel and algae meal production are added to the model as well as the algae meal revenues. Molasses costs and depreciation are not considered and electricity costs are accounted at the production cost of $ 0.052 / kWh.

Table 18. Gross profit analysis of the integrated microalgae-sugarcane biorefinery (A).

Product	Cost per unit $	Production costs $	Selling Price $	Revenues $
Sugar 254,500,000 kg	0.25/kg (Cavalett2012)	63,625,000	0.43/kg	109,435,000.00
Ethanol 216,000,507 L	0.35/L Cavalett (2012)	75,600,177	0.6/kg	129,600,304.18
Electricity 830,000,000 kWh	0.052/kwh Dias (2012)	47,320,000	0.058/kwh	48,322,600.00
Biodiesel 20,000,000 L		22,501,626		
Algae meal 17,450,000 kg			0.45/kg	7,852,500.00
	Total Costs $	209,050,000	**Total Revenues $**	295,210,404
				Gross Profit $
				86,163,000

The gross profit from the integrated sugarcane-microalgae biorefinery was $ 4.2 million lower than the base case biorefinery. To achieve feasibility, the integrated biorefinery depends on the selling prices of ethanol and biodiesel. If ethanol selling price is reduced and biodiesel price is increased, the integrated biorefinery becomes more attractive. To assess that assumption the difference of gross profit between the base and the integrated refinery were calculated for different prices of ethanol and biodiesel. The results are shown in Table 18.

Table 19 G*ross profits difference between base sugarcane biorefinery and integrated microalgae-sugarcane biorefinery*

		Biodiesel selling price ($/L)				
		0.6	0.8	1	1.2	1.4
Ethanol selling price ($/L)	0.9	-22,767,790	-18,767,790	-14,767,790	-10,767,790	-6,767,790
	0.8	-19,233,281	-15,233,281	-11,233,281	-7,233,281	-3,233,281
	0.7	-15,698,773	-11,698,773	-7,698,773	-3,698,773	301,227
	0.6	-12,164,264	-8,164,264	-4,164,264	-164,264	3,835,736
	0.5	-8,629,756	-4,629,756	-629,756	3,370,244	7,370,244
	0.4	-5,095,247	-1,095,247	2,904,753	6,904,753	10,904,753
	0.3	-1,560,738	2,439,262	6,439,262	10,439,262	21,508,279

Table 19 shows that the integrated refinery would be feasible only if a significant increase of biodiesel selling prices and a reduction of ethanol prices. At a selling price of $0.6 per liter of ethanol, the biodiesel price must be higher than $ 1.2 / L.

The yield of ethanol from sugars (~ 0.5 $g_{ethanol}/g_{sugar}$) is nearly two times the yield of lipid from sugars (~0.25-0.30 g_{lipids}/g_{sugar}). Moreover, the volumetric productivity of ethanol is many times higher than biodiesel and anaerobic processes have usually lower capital and operational costs [102]. These characteristics make difficult the feasibility of microalgae biodiesel against the opportunity cost of ethanol.

One possible way to increase profitability of the integrated plant is the exploration of high value added products from microalgae, in a similar way to what companies such as Martek S/A and Solazyme are doing for carotenoids or polyunsaturated fatty acids [103, 104]. Although some of these companies started their activities aiming at biofuels production, most of them are concentrating on higher value added products in order to finance their high production costs [105].

Despite the negative economic result, the substitution of fossil based diesel could improve the biorefinery overall renewability by eliminating the use of fossil fuels. In addition the plant would become independent of oil prices fluctuations or vegetable oils for biodiesel production. The gross profit difference values shown in Table 19 can be considered the cost of reducing the carbon emissions by replacing fossil base diesel. Considering a total reduction of 50,000 ton of CO_2-eq yr^{-1}, the cost per ton of CO_2 reduction can be calculated and compared with recent carbon price estimations. Carbon pricing can be considered as a measure of the economic impact due to domestic and global benefits from reduced air pollution and climate change and usually varies between $30 and $120 ton^{-1} CO_2 depending on the criteria [106]. Setting the goal carbon price of $100 ton^{-1} CO_2, Table 19 can be rewritten as cost of reduction per ton of CO_2 (Table 20). Considering the ethanol selling price of US$ 0.6 per liter, the selling price of biodiesel at US$1 is already advantageous compared to the base case, if considered the economic contribution of carbon reduction, since the cost of reduction of one ton of CO_2 is calculated as US$ 83.

Table 20. Cost of reduction of CO_2 emissions (US$ ton^{-1} CO_2)

		Biodiesel selling price ($/L)				
		0.6	0.8	1	1.2	1.4
Ethanol selling price ($/L)	0.9	-455	-375	-295	-215	-135
	0.8	-385	-305	-225	-145	-65
	0.7	-314	-234	-154	-74	6
	0.6	-243	-163	-83	-3	77
	0.5	-173	-93	-13	67	147
	0.4	-102	-22	58	138	218
	0.3	-31	49	129	209	430

Although, in theory, the diesel consuming machinery and trucks could be modified to use bioethanol instead of diesel, the technology is not well developed and the substitution costs are uncertain. Biodiesel, on the other hand, is a drop-in fuel that can be utilized in diesel consuming engines without any modification.

4. Conclusion

The process design for microalgal biodiesel resulted in high capital costs, mainly related to the fermentation section. A large number of fermenters were required due to the relative low volumetric productivity of microalgal oil. Sensitivity analysis showed that fermenter purchase cost and biomass productivity are critical for the profitability analysis of the process and must be carefully estimated. The main difference between batch and continuous modes was related to capital costs. The reduced downtime and lower need of inoculation equipment had a significant effect over direct capital cost. Materials and facility related costs were the most important components of operating costs for microalgae biodiesel. Sensitivity analysis showed that molasses is by far the most relevant operation cost.

At present average ethanol and biodiesel selling prices, the integrated plant (sugar/bioethanol/biodiesel) would be less profitable than a traditional first generation bio-refinery (sugar/bioethanol). However, the replacement of fossil based diesel fuel in sugarcane crop and transportation operations could increase the overall renewability and reduce carbon emissions.

It is clear that introducing renewable biodiesel to replace fossil diesel consumption in the sugar cane sector can not be cost-effective under all conditions, but in this study we showed that there are good opportunities to that require (1) scale-up and learning effects, (2) valorisation of by-products such as high-added value products as well as commodity products such as fertilisers and biogas, as well as (3) fiscal measures such as carbon tax. In terms of additional costs, the biodiesel-opportunity is comparable to other CO2-emission reduction measures on a carbon price basis. Hence innovation and political willingness are key for the success of this development.

5. Acknowledgements

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Chapter 7

Final Discussion and Future Outlook

Final Discussion and Future Outlook

The heterotrophic production of lipids from microalgae is an attractive alternative for liquid biofuels, while also providing a sustainable source of valuable chemicals and nutritional products. Heterotrophic growth allows the cultivation of microalgae biomass up to high cell concentrations, consequently reaching higher volumetric productivities (Chapter 2). This is a key factor for the economic feasibility of microalgae biodiesel production, as it impacts capital and operational costs of cultivation, by far the most expensive cost driver of the process (Chapter 5).

Biomass concentration and lipid content are also important factors in downstream operations, affecting biomass harvesting and lipid extraction equipment scale and energy consumption. However, high concentrations of biomass can result in a decrease of growth rates, leading to a trade-off between high biomass concentration and lipid productivity (Chapter 3). The choice of the optimal output depends on the economic analysis of process. For biodiesel, lipid volumetric productivities should be maximized, as the cultivation stage has a much higher weight than downstream operations (Chapter 5). There is an optimal point of operation, which depends on the biomass and lipid kinetic behavior of the strain (Chapter 3).

As a way of increasing overall productivity, continuous cultivation allows prolonged operation at optimal conditions, while at the same time reducing downtime operations. In addition, it also offers better control over biomass and fatty acid composition, by tuning growth rates and medium composition. We showed that the fatty acid composition may be tailored to a certain extent, by changing the unsaturation degree and chain length of fatty acids, both of which have significant impacts on biodiesel quality (Chapter 4).

Heterotrophic cultivation of microalgae is a possible solution for short-term production of biofuels from organic carbon sources. There is no need for the development of specific cultivation equipment, as conventional aerobic fermenters are perfectly suitable for it. In addition, the conversion of lipids into biofuels is a very well established technology in the case of biodiesel, and new technologies for the production of hydrocarbon based fuels from oils are presently being developed, e.g. aviation fuels through HEFA process [1].

The results presented in this thesis represent a step further toward the feasible production of biofuels from microalgae, expanding the understanding of heterotrophic cultivation and providing insights on the next steps for research and development. Still, a better understanding of the limiting factors in biomass production and lipid accumulation may increase the overall yields of the process and still need further research. The amount of information about heterotrophic production of lipids from microalgae is still small if compared to the literature about autotrophic production, and new approaches are yet to be explored.

The technology is currently being demonstrated as a large scale plant operation has started in Brazil, with the goal of producing biofuels, as well as high value added products [2]. However, the low oil prices have recently led the company in charge to give up on the biofuel production field and focus on ingredients for the food and personal care industries [3].

The volatility of oil prices creates short term uncertainty for the development of renewable technologies, which struggle to reach competitive prices against fossil based fuels and chemicals. If oil prices remain low, the efforts to decrease production costs of biofuels will be threatened, and subsidies may be necessary for the survival of the industry [4]. It is widely accepted that the availability and production costs of fossil fuels are no longer the limiting factors for its exploration, but rather the environmental effects caused by their increasing usage [5]. Therefore, governments must address the development of substitute technologies with urgency and create incentives and guarantees for companies and researchers willing to develop alternative renewable technologies.

In addition, there are still bottlenecks that need to be addressed for making heterotrophic microalgae biofuels feasible. Here presented are some suggestions of topics, which could have a significant impact over the yields and costs of the process. The first one is the cost and availability of feedstocks, which accounts for almost 50 % of operating production costs (Chapter 5). Lignocellulosic biomass is expected to be a cheap and widely available source of organic carbon; however hydrolysis technologies may still need years of research to decrease production costs down to competitive levels. Secondly, capital costs are still a major obstacle in scaling up new biofuel technologies, and heterotrophic microalgae process is no different. Higher volumetric lipid productivities and operation in non-aseptic conditions are key factors for enhancing feasibility, as upstream operations are responsible for most of the capital costs. In Chapter 5, the purchase cost of fermenters was shown to be more than 50 % of total capital costs. If non-aseptic reactors could be used, the capital and operational costs could be significantly reduced. A third point is the use of the non-lipid fraction of the biomass. At large scale production, this material should be converted into biofuels as well, increasing the global yield of the process. A few technologies are being currently developed with promising results, but there is still plenty of room for improvement. In Chapter 2, the global yield of conversion of glucose into biodiesel after cultivation, extraction and conversion was calculated as, approximately, 9 %.

In the next paragraphs, some of these points are discussed briefly, highlighting some of the possible solutions and current progress of research.

Substrate cost

Substrate costs represent a major fraction of total production costs of microalgae oils and biofuels. Considering that the maximum stoichiometric conversion yield of glucose into triacylglycerols is around 33% [6]; if pure sugar was used as substrate, just the cost of it ($0.90 / 3 kg sugar) would almost match the current diesel price ($1.02 / Liter - Brazil) (Chapters 2 and 5; Prices:[7]).

The rapid development of biomass hydrolysis technologies is likely to increase the availability of cheaper substrates. However, heterotrophic microalgae seem to have a relatively limited enzymatic repertoire for carbon metabolism. This is a major setback for the utilization of low cost substrates, such as those derived from biomass hydrolysis. While the hexose sugar present in lignocellulosic hydrolysates is efficiently metabolized, pentose sugars such as xylose and arabinose are not naturally utilized. The latter can account for up to 25 % of total feedstock composition [8].

Few studies have reported xylose utilization by microalgae in the presence of light [9, 10], and in those cases substrate consumption rates were much lower than the observed for glucose. Moreover, only one study has shown pure heterotrophic consumption of xylose by a microalgae strain in the absence of light [11].

The development of strains capable of metabolizing substrates other than the costly hexoses is an important step for the integration of lignocellulosic biomass hydrolysis and heterotrophic microalgae production.

CO_2 incorporation in heterotrophic growth

Mixotrophy is the combination of heterotrophic and autotrophic growth of microalgae. In this regime, respiration and photosynthesis occur simultaneously with assimilation of both organic carbon sources and CO_2 [12]. In comparison with heterotrophic systems, yields on substrate can be significantly higher as part of the carbon is assimilated as CO_2. Much is said about the high photosynthetic capacity of microalgae in comparison with terrestrial plants, and growing microalgae in pure heterotrophy, although productive, may be a waste of a greater potential. The combination of the two metabolisms could be an alternative for obtaining higher yields and increasing the feasibility of microalgae biofuels.

However, in traditional mixotrophic cultivations, cell densities are not as high, since mass and oxygen transfer in photo-bioreactors are not as efficient as in closed stirred reactors due to geometric differences. In addition, the need of light impose a significant complication in reactor design, and thus capital costs of photo-bioreactors can be orders of magnitude higher than conventional fermenters [13].

Another way of utilizing the autotrophic apparatus of microalgae is through the absorption of CO_2 during heterotrophic growth by activating the ribulose-1,5-bisphosphate carboxylase (Rubisco) enzyme, which is the key enzyme in the pentose phosphate pathway. Recently, researchers have shown that the expression of Calvin cycle enzymes and Rubisco increased in 10 % the yield of ethanol production in genetically modified yeast *Saccharomyces cerevisiae*, with CO_2 incorporated as co-substrate [14]. Another study reported the re-incorporation of CO_2 in heterotrophic conditions by the microalgae *Chlorella protothecoides*, resulting in 69 % higher yield when compared to regular heterotrophic growth. According to the authors, this result was obtained by growing the algae autotrophically, harvesting and re-suspending the cells in heterotrophic conditions, which was shown to activate the Rubisco system in the cells. [15]. While more studies are needed in order to clarify the interaction of the CO_2 incorporation apparatus with heterotrophic growth, there is evidence that this could be a way of using the full potential of microalgae for carbon assimilation.

Contamination and open cultures

As discussed in Chapters 3, 4 and 5, heterotrophic continuous cultivation of microalgae has many advantages over batch process, due to the reduced downtime operations and higher control over product quality. However, the maintenance of a heterotrophic continuous process over long periods of time demands a rigorous contamination control system. Most bacteria and yeast can grow faster than microalgae and, consequently, are able to quickly dominate the culture. For this reason, sterilization and aseptic operation are often required to maintain a productive culture [16], increasing energy and capital costs significantly and possibly preventing the economic feasibility of the process [17]. Contamination of heterotrophic cultures becomes even more important if waste products are used as substrates, since the presence of excessive bacterial populations in these streams may result in competition with microalgae and, consequently, in low yields [18].

Few solutions have been proposed to avoid contamination without the need of sterilization and aseptic operation, and one of the most promising is growing extremophile algae in selective environments, e.g. low or high pH, high salinity or high temperature [19]. Some microalgae, isolated from extreme environments, can perform well at inhospitable conditions for most organisms, potentially reducing the risk of proliferation of invading species [20]. This strategy has been successfully applied in open pond autotrophic cultivations, which also suffer from contamination issues, although in much lower extent than in heterotrophic cultivation [21]. An example of promising extremophile algae is *Galdieria sulphuraria,* a red algae that can live at pH as low as 0, temperatures as high as 57 ºC and salinity of up to 10 % (w/v) [22]. In addition, it is capable of heterotrophic growth, reaching high cell densities (80-110 g L^{-1}) and lipid content around 19 % (w/w) [23, 24]. Cultivation at such extreme conditions could inhibit most of the competing microorganisms, reducing the necessity for sterilization and aseptic processes and drastically reducing capital and operating costs of industrial scale cultivation. Therefore, more studies about the competition dynamics between extremophile algae and bacteria in heterotrophic conditions are necessary for the development of robust non-aseptic algae cultivation.

The photosynthetic apparatus of the algae can also be used as base for selective pressure, as recently demonstrated for autotrophic mixed cultures. By providing CO_2 and light during the day and nitrogen only during the night, only microalgae which stored energy and carbon were able to assimilate nitrogen and grow. This way, the microalgae with high capacity for carbon storage became the dominant species in the culture [25]. It is possible

that the same concept can be applied to mixotrophic cultures, which could increase even more the yields of this inventive strategy.

Conversion of non-lipid material

The production, extraction and conversion of lipids is the most common approach for biofuel production from heterotrophic algae. However, the total lipid fraction accounts for only 20-60 % of the microalgae biomass. The non-lipid fraction of microalgae biomass is roughly composed by carbohydrates and proteins and usually considered a by-product to be sold as animal feed or as high value added products, such as fine chemicals, pharmaceuticals and cosmetics [26]. In the microalgae bio-refinery concept, the revenues of these higher value products could help offset the production costs of lipids and biofuels and improve the feasibility of the process [27]. However, there are large disparities between the market sizes of biofuels and these potential co-products, which make this idea less plausible [28]. For example, if all biodiesel consumed in Europe was produced from microalgae, the amount of protein generated would be 40 fold higher than the current soy protein imports [29].

For large scale production, a more plausible option may be the conversion of the non-lipid material into energy and biofuels as well, increasing the total yield of biofuels. Energy can be generated from anaerobic digestion or direct burning of the biomass and liquid biofuels can be produced from thermochemical conversion technologies, such as hydrothermal liquefaction (HTL) and pyrolysis [30]. Hydrothermal liquefaction seems to be an attractive option, as it can be applied to wet biomass and generate high yields of bio-crude-oil, which can be upgraded to liquid fuels through hydrotreating and hydrocracking [31]. Yields of up to 45 % (w/w) of bio-oil have been achieved from lipid extracted algae biomass through the HTL process, and the resulting product had energy content (35–37 MJ/kg) similar to shale oil (41 MJ/kg) [30]. The further development and the optimization of this relatively new technology could enhance even more the yield of fuels from feedstock. It is not clear yet if it is preferable to apply HTL technology over lipid-extracted microalgae or over the whole cells, and different studies are trying to address this question [31, 32]. Some authors also argue that lipid accumulation would not be necessary, as HTL can convert proteins and carbohydrates into bio-crude oil, and therefore cultivation productivities could be enhanced by avoiding slow lipid accumulation [33]. However, the performance of HTL process seems to be directly affected by the lipid content of the microalgae, as the conversion of carbohydrates and proteins result in lower yields of bio-oil and in higher contents of oxygen and nitrogen in the final product [34, 35].

The following table summarizes the points discussed above and serves as a suggestive guide of bottlenecks that need to be addressed for the feasible production of biofuels.

Bottlenecks	Effects	Solutions
Short term instability caused by oil price fluctuation	High risks scare away investors and threaten the start and survival of biofuel projects	Government and society support of the development of renewable technologies
Low versatility for substrate utilization	High substrate costs	Development of strains with capacity for absorption and metabolism of alternative substrates
Contamination of cultures	High capital and operational costs for sterilization and aseptic operation	Use of extremophile algae may eliminate the need of aseptic operation
Non-lipid material is not used for biofuel production, but sold as co-products	Low yields and limited market size for co-products	Optimization of technologies for conversion of non-lipid material into fuels
Idleness of CO_2 incorporation machinery of microalgae	Low yields in comparison with full potential	Find ways of stimulating Rubisco enzyme system during heterotrophic growth

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