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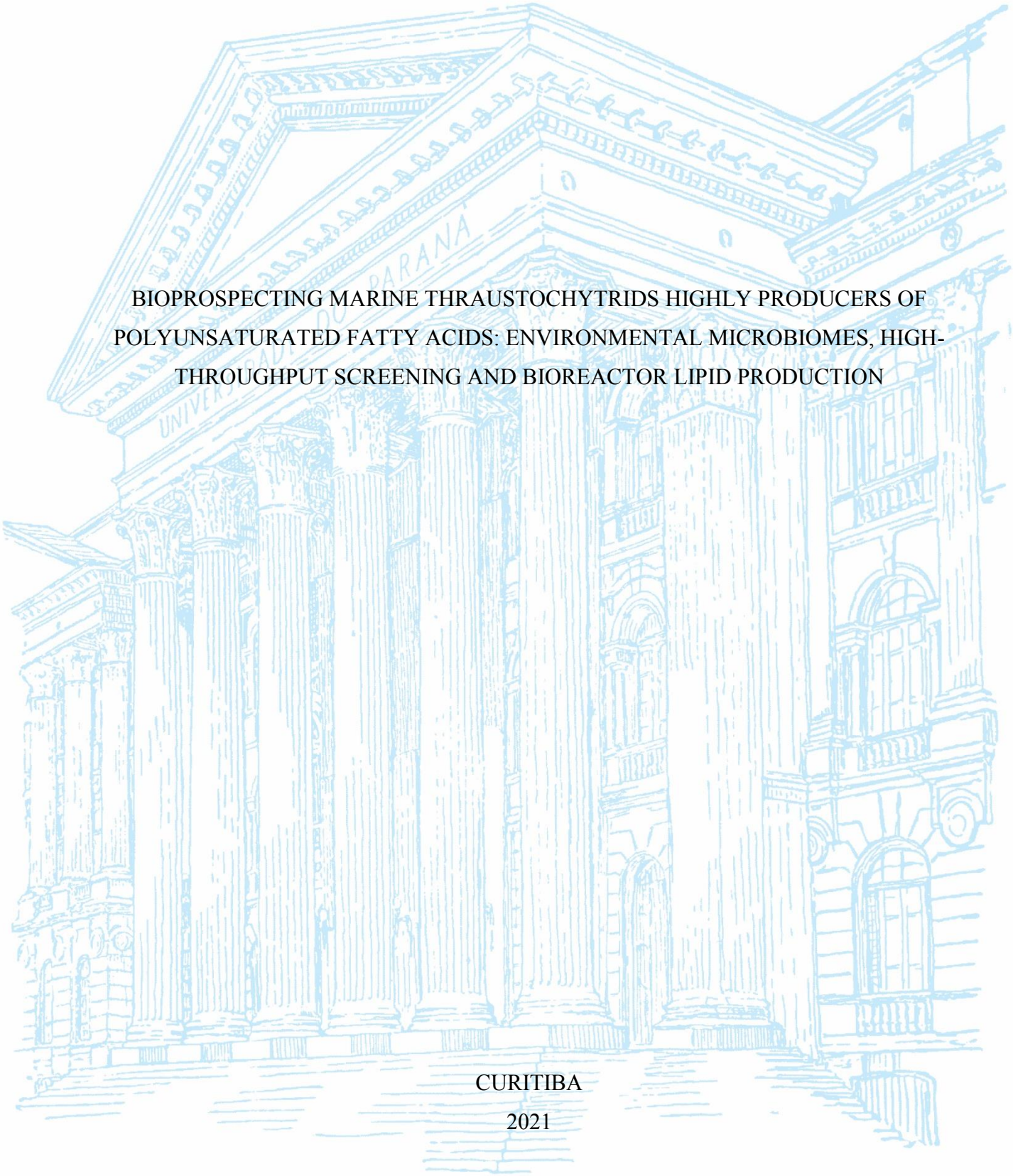

BIOPROSPECTING MARINE THRAUSTOCHYTRIDS HIGHLY PRODUCERS OF POLYUNSATURATED FATTY ACIDS: ENVIRONMENTAL MICROBIOMES, HIGH-THROUGHPUT SCREENING AND BIOREACTOR LIPID PRODUCTION

CURITIBA

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Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Carlos Ricardo Soccol

Coorientador: Prof. Dr. Gilberto Vinícius de Melo Pereira.

CURITIBA

2021

Catalogação na Fonte: Sistema de Bibliotecas, UFPR
Biblioteca de Ciência e Tecnologia

C719b Colonia, Brigitte Sthepani Orozco
Bioprospecting marine thraustochytrids highly producers of polyunsaturated fatty acids [recurso eletrônico] : environmental microbiomes, highthroughput screening and bioreactor lipid production / Brigitte Sthepani Orozco Colonia. – Curitiba, 2021.

Tese - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2021.

Orientador: Carlos Ricardo Soccol.
Coorientador: Gilberto Vinícius de Melo Pereira.

1. Ácidos graxos Ômega-3. 2. Óleos de peixe. I. Universidade Federal do Paraná. II. Soccol, Carlos Ricardo. III. Pereira, Gilberto Vinícius de Melo. IV. Título.

CDD: 612.01577

Bibliotecária: Vanusa Maciel CRB- 9/1928

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **BRIGITTE STHEPANI OROZCO COLONIA** intitulada: **BIOPROSPECTING MARINE THRAUSTOCHYTRIDS HIGHLY PRODUCERS OF POLYUNSATURATED FATTY ACIDS: ENVIRONMENTAL MICROBIOMES, HIGH-THROUGHPUT SCREENING AND BIOREACTOR LIPID PRODUCTION**, sob orientação do Prof. Dr. CARLOS RICARDO SOCCOL, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 16 de Junho de 2021.

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DEDICATÓRIA

Dedico a Tese ao meu marido, Rodrigo Malanski, aos meus pais, Judith Colonia Andrade e Jorge Arturo Orozco Ramirez, e a minha irmã Britney Orozco Colonia, que sempre me incentivaram e apoiaram incondicionalmente.

AGRADECIMENTOS

Agradeço em especial ao meu marido, Rodrigo Malanski, por todo o apoio, incentivo e carinho ao longo de todos esses anos juntos.

Aos meus pais, Judith Colonia Andrade e Jorge Arturo Orozco Ramirez pelo carinho e inspiração sempre e em todos os momentos da minha vida.

À minha irmã, Britney Orozco Colonia por ser minha amiga e motivadora sempre.

À minhas avós, Mery Andrade e Ana Virginia Ramirez (*in memoriam*) por sempre estarem dispostas a ajudar.

À minha família na Colômbia, tios, tias, primos e primas por toda a motivação e apoio desde longe durante estes 7 anos morando no Brasil.

À minha segunda família no Brasil, Milton Malanski, Rosalba Elisa dos Santos Malanski, Milena Malanski Fernandes e aos demais integrantes da família Santos e Malanski por terem me acolhido como uma filha, irmã, neta e sobrinha.

Agradeço em especial ao Prof. Dr. Carlos Ricardo Soccol pela orientação, confiança, motivação e por ter acreditado no meu potencial durante toda minha pós-graduação (mestrado e doutorado).

Ao Prof. Dr. Gilberto Vinícius de Melo Pereira por ter sido um coorientador sempre disposto a ajudar e guiar no desenvolvimento dos meus experimentos, redação de artigos e tese.

Ao Programa de Pós-Graduação de Engenharia de Bioprocessos e Biotecnologia da UFPR, aos professores, técnicos, e à secretária pelo conhecimento, apoio e disponibilidade durante o doutorado.

Aos meus amigos da pós, Amanda Oliveira, Elisângela Muynarsk, Felipe Mendoça, Raphael Boschero e Jair Junior pela parceria, risadas, momentos de nerd e pelas tardes de café.

Aos meus amigos, Eduardo Gochi, Sheila Nunes, Ricardo Morais, Mário Bier e demais pelos fins de semana de jogos, risadas e noites de vôlei.

Á Dr. Larissa Vuitika pela paciência e ensinamento no uso do fluorímetro de microplacas (Tecan Infinite M200) do Departamento de Biologia Celular da UFPR.

Á Dr. Jéssica Aparecida Viesser e ao Gabriel Favero pela ajuda no depósito de sequencias no GenBank da NCBI.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo financiamento do projeto e da bolsa de pesquisa.

A todos os colegas que cruzaram o meu caminho durante essa jornada.

"Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at. It matters that you don't just give up".

Stephen Hawking

RESUMO

O Brasil apresenta potencial biotecnológico para a desenvolvimento de novos produtos para atender a grande demanda da indústria de alimentos e farmacêutica, os quais aportem benefícios à saúde humana e animal. O consumo de alimentos ricos em lipídeos de alto valor como os ácidos graxos essenciais ômega-3 tem ganho grande destaque, tendo como principal fonte a extração a partir do óleo de pescados. Porém, alguns problemas relacionados à produção de ácidos graxos a partir de pescados incluem (i) a diminuição dos estoques de peixes devido à pesca predatória extrativista, (ii) a poluição dos mares tem causado graves problemas ambientais e (ii) a baixa disponibilidade de fontes alternativas para consumidores alérgicos ou com dieta vegana. Para tal se faz necessário a busca de alternativas biotecnológicas para produção desses biocompostos. Desta forma, os traustoquitrídeos aparecem como uma solução sustentável, eco-amigável e renovável de fontes microbianas de lipídeos de alto valor como o ômega-3, principalmente o DHA (ácido docosahexaenóico). Traustoquitrídeos são protistas ou microalgas heterotróficas que habitam o ecossistema de mangues e águas costeiras oceânicas. Nesta tese, um estudo sobre o uso de traustoquitrídeos e microalgas como fontes de lipídeos microbianos foi realizado. Diferentes abordagens sobre técnicas de isolamento, manutenção e cultivo celular foram discutidas. Uma análise do desenvolvimento destes protistas em biorreatores, estratégias para promover o acúmulo de lipídeos, técnicas de moleculares de melhoramento e inovações visando a produção em larga escala foram estudadas. Da mesma forma, uma revisão aprofundada sobre os traustoquitrídeos como fonte sustentável de ômega-3, tendencias, avanços tecnológicos e mapeamento de patentes foi realizado. Seguidamente, uma abordagem integrando metagenômica e cultivo em microescala junto com espectroscopia de fluorescência foi usada na bioprospecção de traustoquitrídeos acumuladores de lipídeos a partir de amostras de manguezal e água do mar costeira no sul do Brasil. Estas estratégias permitiram isolar eficientemente traustoquitrídeos, dos quais vinte isolados foram cultivados em fermentação submersa e seus perfis lipídicos foram caracterizados. O isolado B36 foi identificado como *Aurantiochytrium* sp. pelo sequenciamento do rRNA 18s e obteve uma alta produção de biomassa (25.60 g/l) e lipídeos (17.12 g/l) com 44.37% de AGPI-CL e 34.6% de DHA. Paralelamente, estudos de melhoramento do traustoquitrídeo *Aurantiochytrium limacinum* SR21 por técnicas de mutação UV e otimização de bioprocessos utilizando subprodutos agroindustriais de baixo custo foram realizados. Assim, modelos de regressão matemáticos e superfícies de resposta foram gerados a fim de obter as melhores condições de cultivo. O traustoquitrídeo otimizado foi escalonado em biorreator de tanque agitado de 10L utilizando melaço de cana, milhocina e creme de levedura. Estudos cinéticos fermentativos foram realizados nos cultivos em shaker e biorreator. A maior produção obtida no biorreator em batelada foi 39.29 g/l de biomassa e 14.98 g/l de lipídeos com um rendimento ($Y_{P/X}$) de 0.45 g de lipídeos/g de biomassa. Os resultados obtidos tanto dos isolados como da cepa SR21 otimizada foram promissórios visando ao aumento em escala de bancada e piloto. Portanto, a biomassa de traustoquitrídeos rica em lipídeos de alto valor poderia ser usada como uma alternativa sustentável na alimentação humana e animal.

Palavras-chave: Traustoquitrídeo. Ômega-3. Metagenômica. Bioprospecção. Biorreator.

ABSTRACT

Brazil has biotechnological potential for the development of new products to meet the great demand of the food and pharmaceutical industry, which bring benefits to human and animal health. The consumption of foods rich in high-value lipids such as omega-3 essential fatty acids has gained great prominence, where the main source is fish oil. However, some problems related to the production of fatty acids from fish include (i) the decrease in fish stocks due to overfishing, (ii) the pollution of the seas has caused serious environmental problems and (ii) the low availability of alternative sources for allergic or vegan consumers. Thus, the search for biotechnological alternatives for the production of these biocompounds is necessary. Thus, thraustochytrids appear as a sustainable, eco-friendly and renewable solution of high-value microbial sources of lipids such as omega-3, mainly DHA (docosahexaenoic acid). Thraustochytrids are protists or heterotrophic microalgae that inhabit the ecosystem of mangroves and oceanic coastal waters. In this thesis, a study on the use of thraustochytrids and microalgae as sources of microbial lipids was carried out. Different approaches to cell isolation, maintenance and culture techniques were discussed. An analysis of the development of these protists in bioreactors, strategies to promote lipid accumulation, molecular improvement techniques and innovations aimed at large-scale production were studied. Likewise, an in-depth review of thraustochytrid as a sustainable source of omega-3, trends, technological advances and patent mapping was performed. Next, an approach integrating metagenetics and microscale cultivation with fluorescence spectroscopy was used in the bioprospection of lipid accumulating thraustochytrid from mangrove and coastal seawater samples in southern Brazil. These strategies allowed the efficient isolation of thraustochytrids, where twenty isolates were cultivated in submerged fermentation and their lipid profiles were characterized. Isolate B36 was identified as *Aurantiochytrium* sp. by sequencing 18s rRNA and obtained a high production of biomass (25.60 g/l) and lipids (17.12 g/l) with 44.37% LC-PUFAs and 34.6% DHA. In parallel, studies on the improvement of the thraustochytrid *Aurantiochytrium limacinum* SR21 by UV mutation techniques and optimization of bioprocesses using low-cost agro-industrial by-products were carried out. Thus, mathematical regression models and response surfaces were generated in order to obtain the best growing conditions. The optimized thraustochytrid was scale-up in a 10L stirred tank bioreactor using sugarcane molasses, corn steep liquor and residual yeast cream. Fermentation kinetic studies were carried out in shaker and bioreactor cultures. The highest production obtained in the batch bioreactor was 39.29 g/l of biomass and 14.98 g/l of lipids with a yield ($Y_{P/X}$) of 0.45 g of lipids/g of biomass. The results obtained from the isolates and the optimized SR21 strain were promising, aiming at increasing bench and pilot scale. Therefore, high-value lipid-rich thraustochytrid biomass could be used as a sustainable alternative in human and animal feed.

Keywords: Thraustochytrid. Omega-3. Metagenetics. Bioprospecting. Bioreactor.

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LIST OF ABBREVIATIONS AND ACRONYMS

ALA – Alpha-linolenic acid

ADHD – Attention deficit hyperactivity disorder

CDW – Cell dry weight

CHD – Coronary heart disease

CIPO – Canadian Intellectual Property Office

CSL – Corn steep liquor

CRISPR – Clustered regularly interspaced short palindromic repeat

DHA – Docosahexaenoic acid

DO – Dissolved oxygen

DOC – Dissolved organic carbon

DPA – Docosapentaenoic acid

EFSA – European food safety authority

EPA – Eicosapentaenoic acid

EPO – European Patent Office

FAO – Food and Agriculture Organization

FAs – Fatty acids

FAMEs – Fatty acid methyl esters

FDA – Food and Drug Administration

FI – Fluorescence intensity

GOED – Global Organization for EPA and DHA Omega-3s

GRAS – Generally Recognized as Safe

HDL – High-density lipoprotein

HTS – High-throughput screening

IPC – International Patent Classification

ISSAL – International Society for the Study of Fatty Acids and Lipids

n-3 LC-PUFAs – Omega-3 long-chain polyunsaturated fatty acids

NGS – Next-generation sequencing

OD – Optical density

OTUs – Operational taxonomic units

PCT – Patent Cooperation Treaty

POC – Particulate organic carbon

PUFAs – Polyunsaturated fatty acids

RYC – Residual yeast cream

RFU – Relative fluorescence unit

SAR – Stramenopiles, Alveolata and Rhizaria

SCM – Sugarcane molasses

STBR – Stirred-tank bioreactor

SWOT – Strengths, weaknesses, opportunities and threats

TFA – Total fatty acids

USPTO – United States Patent and Trademark Office

VLC-PUFAs – Very-long chain polyunsaturated fatty acids

WIPO – World Intellectual Property Organization

SUMMARY

1. GENERAL INTRODUCTION

High-value lipids such as omega-3 have been highlighted in recent years as functional foods or nutraceuticals that provide benefits to human and animal health. Traditionally, fish has been the main source of these lipids, but some concerns regarding this source have been questioned such as the presence of contaminants and pollutants. This exclusive source has not been able to supply the world demand for omega-3 oils, which consequently has increased overfishing. Thus, there has been a need to search for alternative sources for the production of long-chain polyunsaturated fatty acids (LC-PUFAs). Therefore, microbial sources such as thraustochytrids appear as a sustainable and renewable omega-3 solution (OROZCO COLONIA; VINÍCIUS DE MELO PEREIRA; SOCCOL, 2020).

Thraustochytrids are heterotrophic protists from the marine ecosystem. Brazil has large areas of rich microbial diversity that are still little explored. Thus, bioprospecting studies of these ecosystems would aim to explore new species with high biotechnological potential for the production of microbial lipids rich in omega-3. Metagenetics techniques could aid in the rapid study of desired microorganisms by reducing isolation time for samples that might not have an abundance of thraustochytrids (COLONIA et al., 2021). These microorganisms could be selected and evaluated as important omega-3 producers, including the possible discovery of new Brazilian species. Currently, there are only seven complete genomes in the world available in databases such as *Hondaea fermentalgiana* strain CCAP 4062/3, *Schizochytrium* sp. CCTCC M209059, *Aurantiochytrium* sp. T66, *Thraustochytrium* sp. ATCC 26185, *Aurantiochytrium limacinum* ATCC MYA-1381 and *Aurantiochytrium acetophilum* strain HS-399 (MORABITO et al., 2019).

This shows the great potential in discovering thraustochytrids and developing emerging technologies for their biotechnological study. Furthermore, it is still unclear how thraustochytrids are able to synthesize high levels of long-chain polyunsaturated fatty acids (LC-PUFAs), mainly DHA, and simultaneously produce secondary metabolites such as carotenoids, antioxidants and sterols. Also, there is still no well-established evidence as to why thraustochytrids are highly differentiated from photosynthetic microorganisms that also produce omega-3, but with high levels of EPA and low levels of DHA. Therefore, it is believed that advances and in-depth studies of functional genomics and metabolic engineering can elucidate issues related to taxonomy, cell life cycle and lipid biosynthesis through aerobic and anaerobic pathways.

1.1 GENERAL OBJECTIVE

The development of a sustainable bioprocess for the production of polyunsaturated fatty acids by marine thraustochytrids through bioprospecting, high-throughput screening, strain improvement, process optimization and bioreactor scale-up.

1.2 SPECIFIC OBJECTIVES

Study the production of microbial lipids from thraustochytrids and microalgae in order to discuss strategies for cultivation and accumulation of lipids, characterization of fatty acids, genetic improvements, technologies and trends aimed at large-scale production.

Analyze the sustainable production of lipids rich in omega-3 through the SWOT matrix (Strengths, Weaknesses, Opportunities, and Threats) as well as the patent landscape in order to identify trends, advances and innovations.

Explore the biotechnological potential of mangroves and coastal zones in southern Brazil through eukaryotic metagenetics.

Bioprospect thraustochytrids using integrated metagenetics and high-throughput screening techniques to obtain isolates producing long-chain polyunsaturated fatty acids.

Cultivate Brazilian thraustochytrids in submerged cultures and characterize the lipid profiles by chromatographic techniques.

Identify the Brazilian thraustochytrids producing long-chain polyunsaturated fatty acids through 18S sequencing.

Improve the production of biomass and lipids by thraustochytrids through ultraviolet mutagenesis, optimization of bioprocess variables and culture medium.

Recover agro-industrial by-products as substrates for the culture medium used in the production of lipid-rich biomass.

Build mathematical models and prediction profiles for the production of biomass and lipids by optimized thraustochytrid.

Scale-up bioprocess in 10L stirred tank bioreactor in batch mode by strain optimized using agro-industrial by-products.

Study the fermentative kinetic parameters of biomass generation and lipid production in shake flasks and bioreactor cultivation.

CHAPTER 1

LIPIDS PRODUCED BY MICROALGAE AND THRAUSTOCHYTRIDS

This chapter was submitted for publication in the book *Microbial Lipids – Processes, Products, and Innovations - Bioproduction for foods, nutraceuticals and biofuels* under Elsevier series on Biomass, Biofuels, Biochemicals.

ABSTRACT

Microalgae and thraustochytrids have appeared as an alternative renewable source of lipids in human nutrition, animal nutrition and biofuels. These microbial lipids seek to meet the demands for high-value oils and reduce dependence on petroleum-based fuels. This chapter aims to study and provides concepts, trends and innovations in terms of cultivation, growth, large-scale production, strategies for accumulating lipids, strains breeding and lipid profiles analysis. The findings show how further studies and advances are needed to develop more sustainable and cost-efficient bioprocesses. Important study sectors include improvement of the upstream (e.g., isolation, strain breeding, bioprospection, culture media and variables screening), midstream (e.g., bioprocess optimization, bioreactor scale-up, process control) and downstream (e.g., centrifugation, filtration, drying, lipid extraction and purification) stages of lipid production through new technologies. This whole approach would lead to obtaining bioproducts from microalgae and thraustochytrids that are more suitable for their application in human and animal nutrition, as well as biofuels.

Keywords: Microalgae; Thraustochytrids; Microbial Lipids; Single Cell Oils, Sustainable Bioprocess.

1. INTRODUCTION

Microalgae and thraustochytrids have recently stood out for being renewable and natural sources of microbial lipids. Most of them inhabit marine environments and watery bodies, such as neritic zone, intertidal zone, oceans, lakes, mangrove ecosystem, soils and plants. Microalgae are microscopic marine algae, which constitute phytoplankton blooms. They are usually photosynthetic microorganisms, which use carbon dioxide and light to grow. On

the other hand, thraustochytrids are non-photosynthetic stramenopiles (heterokonts), which are commonly called as microalgae or algae by industries and commercial brands. Thraustochytrids are heterotrophic protists from Labyrinthulomycetes class, and it was suggested that they lost their plastids through evolution. However, evidences have shown that it is more plausible that photosynthetic stramenopiles (Ochrophyta) have gained their plastids by endosymbiosis from a red algal plastid instead of non-photosynthetic stramenopiles (Labyrinthulomycetes) having lost their plastids in the past, which would justify the fact that thraustochytrids have a misattribution as algae (DORRELL; BOWLER, 2017; LEYLAND; LEU; BOUSSIBA, 2017).

Moreover, microalgae and thraustochytrids the primary source of omega 3 and other polysaturated fatty acids. They serve as food for zooplankton in the natural environment, which are subsequently consumed by herbivorous fish, and these become the food for predatory or carnivorous fish such as salmon (KIMURA; NAGANUMA, 2001; TAKAO et al., 2015). Thus, microalgae and thraustochytrids lipids are indirectly consumed by humans through carnivorous fish or as fish oil. However, overfishing, pollutants present in extracted oils, the decline of marine stocks, and the difficulty of consumption by people allergic to seafood has raised concerns. Therefore, these microorganisms appear as a sustainable, eco-friendly and animal-friendly alternative (OROZCO COLONIA; VINÍCIUS DE MELO PEREIRA; SOCCOL, 2020). Both microalgae and thraustochytrids have been developed to adapt their growth in controlled systems, which mostly seek to provide conditions similar to the natural. Thus, sustainable production directly from the microbial source producing lipid-rich biomass for human nutrition, animal feed, industrial oils and biofuels is known as "Cutting Out the Middle Fish" as shown in Figure 1.

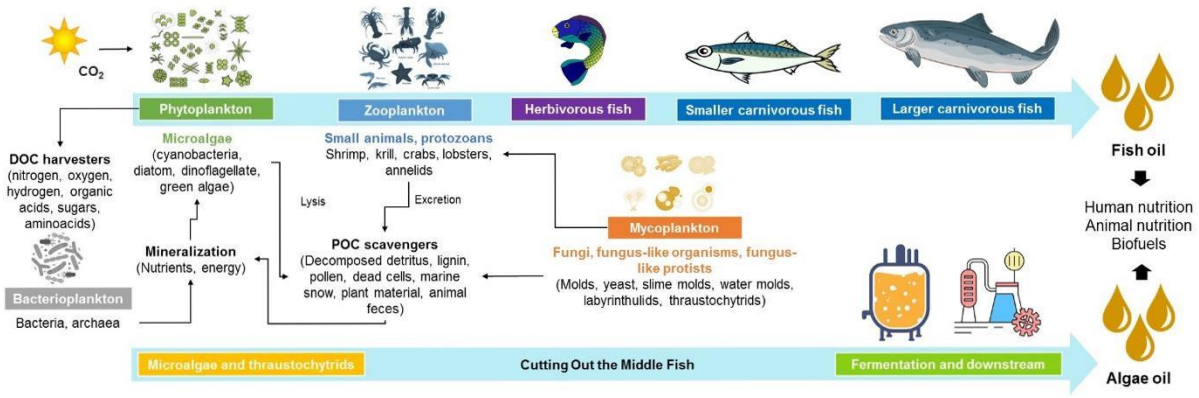

Figure 1. Cutting out the middle fish to produce sustainable lipids from microalgae and thraustochytrids adapted from (BASU; MACKEY, 2018; CELLANA, 2021; KIMURA; NAGANUMA, 2001; MOOMAW; BERZIN; TZACHOR, 2017; POLARIS, 2021; RAGHUKUMAR, 2002; TURNER, 2015).

In summary, this chapter provides valuable information on microalgae and thraustochytrids as sources of microbial lipids. Here, we review and contextualize definitions related to microalgae and thraustochytrids cultivation for large-scale production. Likewise, we cite different strategies to improve the accumulation of intracellular lipids and strain breeding techniques. We emphasize the importance and obtaining of fatty acids with a desired lipid profile according to its application. All of these concepts are finally described through relevant innovations and technologies, including important findings developed by Bioprocess Engineering and Biotechnology Department, at UFPR, Brazil.

2. MICROALGAE

Microalgae is an informal term for *oxygenic photosynthetic microorganisms* (CAVALIER-SMITH, 2007; MOLINA et al., 2019). The term has no rigorous taxonomic meaning – its polyphyletic grouping, which comprises unicellular to pluricellular, and eukaryotic to prokaryotic organisms. In common, microalgae have cell sizes generally below 50 μm and the ability to synthesize several compounds using CO_2 as carbon source and light as energy source (KIRNEV et al., 2018); however, its diversity is enormous, with about 50-100,000 species estimated (CONNELLY, 2014; HALDER; AZAD, 2019), and so is the substructure and chemical compositions of microalgal cells. Figure 2 presents a phylogenetic tree of common microalgae, many with industrial application.

Selected microalgal strains can grow fast, with growth rates from 0.013 h^{-1} to 0.11 h^{-1} for *H. pluvialis* and *C. vulgaris,* respectively (LEE, 2004). That may seem a moderate growth rate for fermentation, but can be translated to doubling times of 0.5 to 4.5 days (with a photoperiod of 12h). That is much higher than most land plants, and several microalgal species can accumulate large amounts of lipids, however with lower growth rates. For industrial purposes, microalgal strains should not only grow fast, but also be tolerant to conservation, contamination, be easy to grow and harvest, and easy to process.

2.1 MICROALGAE SOURCES, ISOLATION AND MAINTENANCE

Microalgae are ubiquitous and can be found in most biotopes – not only lakes and oceans, but also soils and plant surfaces. However, water bodies are more likely to have microalgae suitable for industrial cultivation, and these can be isolated by classical microbiological techniques such as streaking in agar plates or direct micromanipulation

(ANDERSEN; KAWACHI, 2005). Culture media play an important role in isolation, for selective conditions can be applied (minimal media, or specific residues) at the expense of isolates diversity. Antibiotics and antifungals can be used as aids for elimination of contaminations. Cultures can be conserved by recurrent transfer to fresh media, and many microalgae can be successfully conserved for long periods under liquid nitrogen with the additions of 10% DMSO or methanol. There are several microalgae culture collections worldwide, such as SAG (Germany), UTEX (USA) and MCC-NIES (Japan) (LOURENÇO, 2020).

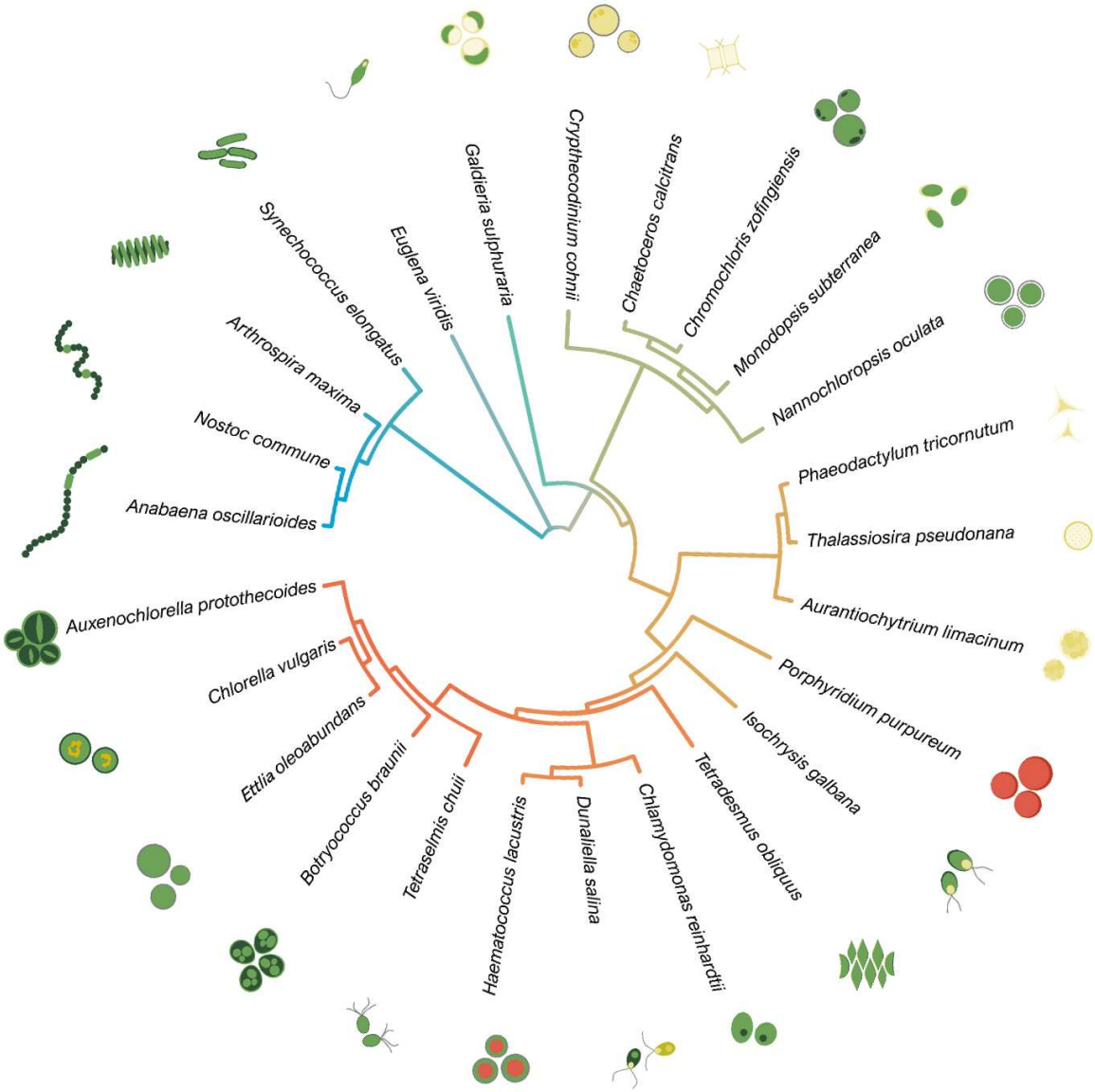

Figure 2. Phylogenetic tree of 25 industrially relevant microalgal species, grouped through 16S and 18S rRNA gene sequences retrieved from the GenBank database. Branch lengths represent the number of substitutions per site from (de Carvalho et al., 2020) with Elsevier permission.

2.2 CULTIVATION AND GROWTH REQUIREMENTS

Similarly to other microorganisms, microalgae require carbon, nitrogen and nutrients for growth, and also light in the case of phototrophic growth. Carbon can be provided as gaseous CO_2, as dissolved carbonates, or even as organic carbon. Nitrogen can be of inorganic origin (NO_3^- or NH_4^+), or organic (urea or peptides). Phosphorus is usually provided as inorganic phosphates, and microelements such as Mg^{2+}, Fe^{2+} and Cu^{+2} and oligoelements such as Mo^{+6} or V^{+5}. Some microalgae can grow in minimal media, but many grow better with the addition of specific vitamins such as B_{12} (DE CARVALHO et al., 2019). There are many classical culture media using complex metal or vitamin solutions, and even using soil extract as a source of oligoelements and cofactors. However, these media were generally developed for isolation and maintenance of microalgal (ANDERSEN, 2005) and not for biomass production. For biomass production, both adaptation of the culture to the medium (e.g. high osmolality, specific carbon sources) and the medium to the culture (C:N:P ratio, inclusion of minor components) must be done (DE CARVALHO et al., 2019). In particular, culture media for large scale production can be prepared using agricultural fertilizers, (with lower costs compared to analytical grade reagents typically used in research and agroindustry wastewaters but also lower purity), and agroindustry wastewaters, which may aid in reducing the impact of culture media costs in biomass production (COLUSSE et al., 2019; DE CARVALHO et al., 2018; MARKOU; GEORGAKAKIS, 2011; MONTALVO et al., 2019). Although the growth using only CO_2 as carbon source and light as energy source (autotrophic) may seem more natural, most microalgae are capable of heterotrophic or mixotrophic growth – an ability that can be exploited for increasing lipid content.

2.2.1 AUTOTROPHIC GROWTH

Autotrophic growth of microalgae requires the addition of CO_2, and illumination. Sparging air (~0.042% CO_2), air enriched with CO_2 (usually at 1-5%), or using culture media that already carry a high concentration of carbonates, such as WC or *Spirulina* medium, supplies CO_2, while light can be supplied using sunlight or lamps – the latter requiring extra energy input. Illumination conditions depend on several factors, such as the algal physiology and the culture concentration, but generally, the growth is better with more light if below a photoinhibition intensity (GRIMA et al., 1996; NIKOLAOU et al., 2016).

2.2.2 HETEROTROPHIC GROWTH

Heterotrophic cultivation uses organic compounds as a source of carbon and energy. Many microalgae can use simple sugars such as glucose or acids (e.g., citric acid and acetic acid); some can produce hydrolytic enzymes (BRASIL et al., 2017) and therefore grow on complex carbon sources such as starch. Heterotrophic growth has the advantage of allowing cultivation in large reactors with a small footprint and without the need for distributed illumination. However, it makes more sense for production of nutritional lipids such as *Crypthecodinium cohnii* (SONG; KURYATOV; AXELSEN, 2020) and thraustochytrids (OROZCO COLONIA; VINÍCIUS DE MELO PEREIRA; SOCCOL, 2020), or the use of agro-industrial side streams reach in low-cost organic carbon; for biofuel production, the use of light as energy source is recommended such as oxygenic photosynthetic microorganisms.

2.2.3 MIXOTROPHIC GROWTH

Mixotrophic cultivation uses both light and organic carbon, the former as energy source and the latter as carbon and energy source. It can be seen as a way to boost autotrophic cultivation, or to induce the production of molecules otherwise not synthesized in heterotrophic culture. Also, some microalgae cannot grow solely in heterotrophy (PEREZ-GARCIA et al., 2011).

2.3 CULTURE PROGRESSION AND MONITORING

A typical microalgal culture progresses similarly to other microorganism cultivations, starting with a diluted culture that goes through adaptation and exponential growth, followed by a deceleration of the growth rate and eventual stabilization and decline. Because many of the microalgal commercial products are not associated to growth, it is common to have two cultivation phases – one with plenty of nutrients and light, for biomass production, and another with some sort of limitation of environmental stress – lack of nitrogen, phosphorus, iron, or excess salinity or light. Such conditions may favor the synthesis of protective substances such as astaxanthin, or carbon accumulation in starch or lipids (GONZALEZ et al., 2020). The stress phase usually has no increase in biomass – it may even have a decrease, but induces important changes in the biomass composition, as discussed in Section 4 (Chapter 1).

2.4 BIOREACTORS AND LARGE-SCALE PRODUCTION

Large-scale production of microalgae is commonly done in photobioreactors, i.e., vessels that are illuminated with natural or artificial light and that are agitated by sparging gas or mechanically, using paddle wheels, stirrers or turbines. There is an enormous variety of designs, with active research on the topic (KIRNEV et al., 2020). Briefly, bioreactors can be open or closed systems. Open systems, such as ponds and raceways, are cheaper and can be scaled-up to very large volumes, efficiently using sunlight but prone to biological contamination, i.e., the colonization by competing or predating organisms that reduce process efficiency. Open systems are also subject to evaporation, precipitation and temperature changes, affecting productivity, which is generally around 15 $g/l.m^2$ with final biomass concentrations about 1g/l. Closed systems such as tubular or bag photobioreactors generally cost more, but are much more controllable, and may have high productivities and final concentrations. In a nutshell, autotrophic production aimed at biofuel production should use open systems to allow for low-cost biomass production, while nutritional lipids can be produced using closed systems.

3. THRAUSTOCHYTRIDS

Thraustochytrids are a group of heterotrophic marine protists that require the presence of organic matter to grow. They can be found almost everywhere in the oceans across the world and in many depths and through different environmental conditions (RAGHUKUMAR; DAMARE, 2011; SINGH et al., 2014). They are commonly found in surficial sediment layers, mangroves, salt marshes and river effluents due to their saprobic function, since those environments presents a lot of organic matter, such as animal detritus and decaying vegetal matter (RAGHUKUMAR, 2017). These microorganisms are characterized by a non-cellulosic cell wall composed by overlapping layers of circular membranes, presence of an ectoplasmic net (EN), which is an extension of the plasma membrane and emerges from a subcellular organelle, and biflagellate zoospores with a short posterior and a long anterior flagellum (FOSSIER MARCHAN et al., 2018). Thraustochytrids have high biotechnological potential to produce omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and a wide variety of other biocompounds with high commercial value (GUPTA; BARROW; PURI, 2012a).

The first thraustochytrid description was in 1934, when (SPARROW, 1936) isolated the species *Thraustochytrium proliferum* from marine alga Bryopsis plumose in coastal waters (FOSSIER MARCHAN et al., 2018). The Thraustochytriaceae family was incorrectly classified over the years as belonging to the Oomycetes class until it was showed by 5S and 18S rDNA analysis that they do not belong to fungi class and instead they were classified as chromists heterokonts (CAVALIER-SMITH; ALLSOPP; CHAO, 1994). The Labyrinthulomycetes class was created to encompass the orders of Thraustochytrida, Labyrinthulida and Amphitremida, since thy present a series of common features between themselves (BENNETT et al., 2017; LEYLAND; LEU; BOUSSIBA, 2017; MORABITO et al., 2019). Therefore, the generally accepted thraustochytrids classification is presented in Figure 3. However, the number of genera present in Thraustochytrida family is still on debate since new analysis and different approaches have been made leading to significant taxonomic rearrangements (DELLERO et al., 2018; YOKOYAMA; HONDA, 2007; YOKOYAMA; SALLEH; HONDA, 2007). A series of rearrangements was achieved within the group of Thraustochytrids, by coupling genomic sequence information with morphological and physiological observation (FOSSIER MARCHAN et al., 2018).

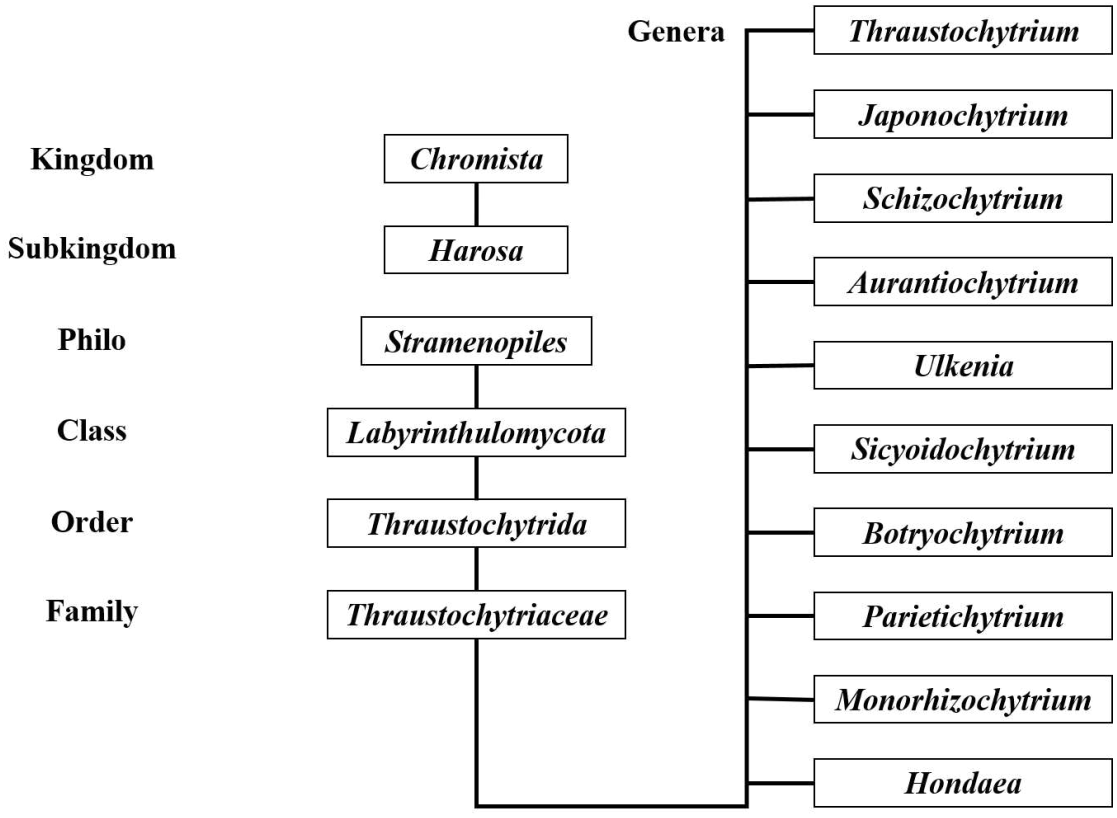

Figure 3. Taxonomic classification showing the position of thraustochytrids in the tree of life (ANDERSON; CAVALIER-SMITH, 2012; RUGGIERO et al., 2015).

3.1 THRAUSTOCHYTRIDS SOURCES, ISOLATION AND MAINTENANCE

The development of new isolation techniques for thraustochytrids have been received increasing attention, due to their biotechnological potential to accumulate lipids (Figure 4A), although there are some well-established methods that remain in use. One of the main techniques for the isolation of thraustochytrids is the use of sterile pine pollen grains as “bait” (GUPTA et al., 2013b). Pollen grains possess a highly nutritive interior and one of the most complex and resistant outer cell walls in higher plants. They are composed by sporopollenin, that only few microorganisms, like the thraustochytrids are capable to penetrate and colonizing (BONGIORNI et al., 2005; DOMÍNGUEZ et al., 1999) as showed in Figure 4B. The colonized pollen grains are transferred to agar plates or broth medium and incubated at room temperature for 3 to 5 days. Then, the pollen is examined for growth of thraustochytrids and followed by its subculturing (GUPTA et al., 2013b).

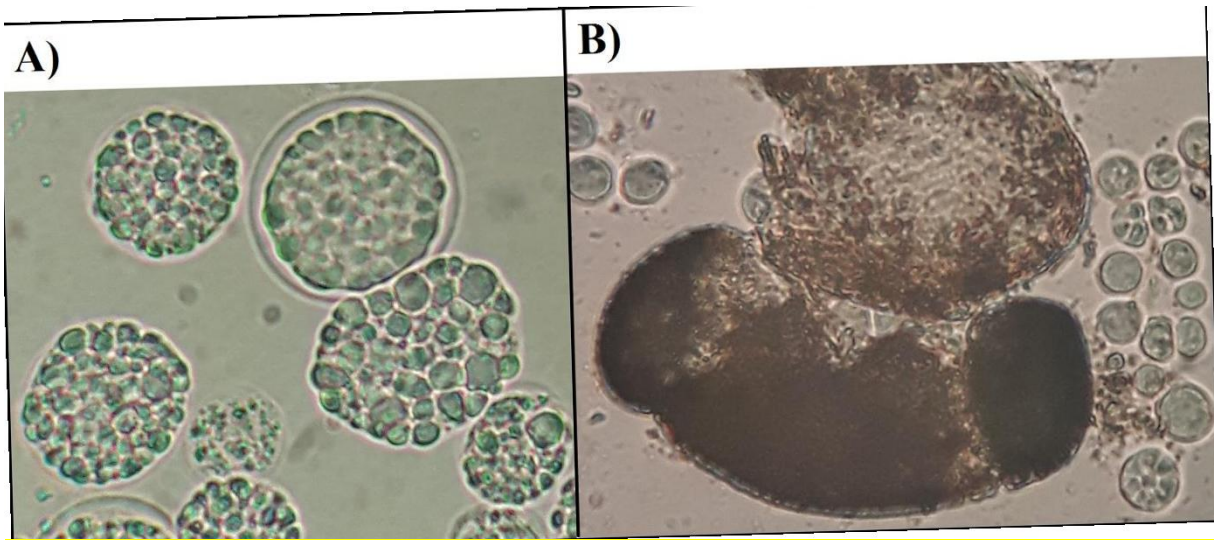

Figure 4. Microscopic view of thraustochytrids. A) Cells containing numerous lipid droplets. B) Cells showing adherence to pollen grains as bait.

Furthermore, another isolation technique is the direct plating from environmental samples on agar medium (ROSA; GALVAGNO; VÉLEZ, 2011). Several media can be used such as the Serum Seawater Agar, Honda medium, GYP (Glucose Yeast Extract Peptone) and the modified Vishniac's medium (KMV) presented great results for the culture of thraustochytrids (MARCHAN et al., 2017; UNAGUL et al., 2017; WANG et al., 2018b). These media are commonly supplemented with antibiotics and antifungal agents such as penicillin, streptomycin, rifampin and nystatin (GUPTA et al., 2013b; PANDEY; BHATHENA, 2014; WILKENS; MAAS, 2012). The pure cultures of thraustochytrids can be also preserved using

different cryoprotectants horse serum, glycerol, DMSO (dimethyl sulfoxide) (COX; HULSTON; MAAS, 2009).

3.2 CULTIVATION AND GROWTH REQUIREMENTS

One of the major concerns of the biotechnological exploitation of thraustochytrids is the optimization of the culture parameters to increase the production of biomass and high value products such as lipids for docosahexaenoic acid (DHA) and biodiesel production (CHEN et al., 2010; TROVÃO et al., 2020; WANG et al., 2018c). The cultivation temperature has great impact in the thraustochytrids growth, lipid profiles and fatty acids. The optimal temperature for biomass and DHA production ranges from 15 to 30º C. In addition, the oxygen dissolved in the medium is an important factor, where the excess of oxygen could result in oxidation of unsaturated fatty acids (FOSSIER MARCHAN et al., 2018; SINGH et al., 2014). The most commonly carbon sources used in cultivation are glucose, fructose and glycerol, follow by nitrogen sources such as yeast extract, peptone and monosodium glutamate (MSG) (HUANG; LU; CHU, 2012; NAZIR et al., 2018). A high carbon and nitrogen (C:N) ratio is desirable for lipid accumulation, which can be achieved by nitrogen limitation (PATEL et al., 2020c). They also can use organic residues such as lignocellulosic, agro-industrial and food residues for growth and lipid production (FAN; CHEN, 2007).

Moreover, thraustochytrids do not need a light source for growth, because they are heterotrophic. Therefore, they are more advantageous when compared to other lipid-producing phototrophic microorganisms, since their growth can be accelerated and controlled by the addition of nutrients increasing yields and productivity and do not require expensive photobioreactors to grow and accumulate lipids. However, thraustochytrids growth and lipid accumulation could be stimulated by light excitation in mixotrophic cultivation (FOSSIER MARCHAN et al., 2018; RAGHUKUMAR, 2017; ROMARI et al., 2013). In addition, they need sodium and phosphorus for growth. The sodium source can be natural seawater or artificial seawater to promote growth and lipid accumulation. The phosphorus can be supplement as KH_2PO_4 form for cell growth, nucleic acid and phospholipids layers formation. They also require trace elements such as vitamins B1 and B12. They have optimal pH for growth ranging from 6 to 8 (FAN; CHEN, 2007; KOTHRI et al., 2020).

3.3 CULTURE PROGRESSION AND MONITORING

The most efficient operation mode for the bioreactor is the fed-batch, since it prevents inhibition of growth due to high substrate concentration and allows lipid accumulation (YE et al., 2020). The lipid production involves different stages of fermentation. A first stage with a nitrogen source for cell biomass growth and a second stage with limited nitrogen for lipid accumulation (SUN; XU; HUANG, 2020). A three-stage continuous fermentation was already described for *Schizochytrium* sp. fermentation, where lipid content, DHA content and DHA productivity were increased by 47.6%, 64.3% and 97.1%, respectively, in comparison with a two-stage process (GUO et al., 2018).

3.4 BIOREACTORS AND LARGE-SCALE PRODUCTION

The large-scale production is carried out under aerobic conditions. However, since many of those microorganisms presents thin-walled cell clusters, originated from binary division, excessive mechanical agitation in the presence of aeration can lead to disruption of the cells. Currently, the most used bioreactors are stirred tank bioreactors (STBRs) and airlift bioreactors. Airlift bioreactors appear to be the most suitable bioreactor configuration, due to their low shear stress and high biomass and lipids yields (CHEN; YANG, 2018a; PATEL et al., 2020c; SUN; XU; HUANG, 2020; WANG et al., 2018b).

4. LIPID ACCUMULATION STRATEGIES

The content of lipids in microalgae is between 20-50% of their dry weight when grown under normal conditions. However, this content can be increased with the exposure of cultures to stress conditions (HU et al., 2008). Thraustochytrids grown under normal cultivation conditions have a lipid content around 29% of cell dry weight (RAGHUKUMAR, 2008). Likewise, this lipid content can be increase to over 50% when different cultivation strategies are used such as nutrients limitation (GONZÁLEZ-BALLESTER et al., 2010), nitrogen limitation (JAKOBSEN et al., 2008; JU et al., 2020), temperature and luminosity variations (CORREDOR et al., 2021; SUN et al., 2018b), saline stress (CHEN et al., 2019), utilization of organic compounds as a carbon source (CHEN et al., 2020b; SINGH et al., 2016), carbon dioxide (DASAN et al., 2020); among others. These cultivation strategies lead to changes in the metabolic pathways of these microorganisms that cause cell reproduction to slow down, but reserve molecules like lipids are accumulated inside the cell (MILLER et al., 2010). Table 1

shows some studies with different strategies for stress conditions to increase lipid accumulation in microalgae and thraustochytrids.

Table 1. Different strategies or stress conditions for increase lipid accumulation in microalgae and thraustochytrids.

Microorganism	Strategies/Stress condition	Lipid content	Reference
Chlorella sorokiniana	Salinity (15mM KCl)	28.23%	(SRIVASTAVA; NISHCHAL; GOUD, 2017)
Desmodesmus	Salinity (5mM KCl)	30.17%	(SRIVASTAVA; NISHCHAL; GOUD, 2017)
Chlorella sorokiniana	Salinity (10 g/l NaCl)	0.36 g/l	(KIM et al., 2016)
Scenedesmus deserticola	Nitrogen limitation (5.8 mM/l)	54.4 %	(LI et al., 2013)
Scenedesmus obliquus	12% de CO^2	33.14%	(ABD EL BAKY et al., 2012)
Parachlorella. kessleri	CO^2 variation and luminosity	326 mg/l.d	(SERRANO-BERMÚDEZ; MONTENEGRO-RUÍZ; GODOY-SILVA, 2020)
Scenedesmus sp.	Photoperiod (6 h light/16h dark)	37.11%	(REN et al., 2020)
Chlorella sp.	Outdoor conditions	53.5%	(FENG et al., 2020)
Aurantiochytrium sp.	Glucose (30 g/l)	50.5%	(SINGH et al., 2016)
Aurantiochytrium sp.	Glycerol (30 g/l)	63.5%	(SINGH et al., 2016)
Schizochytrium sp.	Glycerol (30 g/l)	60.5%	(SINGH et al., 2016)
Chlamydomonas sp	Luminosity (600 µmol/m^2/s)	~30%	(TAN et al., 2016)
Antarctic thraustochytrids	C:N ratio 16:9	30%	(SHENE et al., 2020a)

4.1 NUTRIENT LIMITATION

Researchers showed that there was a significant difference ($p<0.05$) between the tests on the lipid accumulation by *Chlorella* sp., where there was an increase from 30.1% lipids under normal conditions to 36.28 and 48.65% lipids with phosphorus and nitrogen limitation, respectively (FENG et al., 2020). In a similar case, the regulation of lipid accumulation by *Schizochytrium* sp. was evaluated, where nitrogen limitation and corn steep liquor (CSL) as

alternative source showed high lipid concentration with 25.4 g/l and 37.2 g/l, respectively (JU et al., 2020). Moreover, the limitation of iron, phosphorus and nitrogen sources resulted in a high lipid content of 50.32% by *Chlorella pyrenoidosa* only with nitrogen limitation (FAN et al., 2014). Furthermore, the limitation of sulfur can also assist in the accumulation of fatty acids, it can cause oxidative stress in the cell followed by a higher lipid content in *Chlamydomonas* (GONZÁLEZ-BALLESTER et al., 2010).

4.2 LIGHT INTENSITY

Changes in light intensity is another way for stress induction. (SUN et al., 2018b) evaluated the variations in light intensity (90 to 360 μmol/m^2/s) during the cultivation of *Chlorella vulgaris*, where 33.54% lipids were obtained after 6 days under 360 μmol/m^2/s. Furthermore, photoperiod variations were evaluated in *Nannochloropsis* sp. growth with a high lipid production (31.3%) with 18h light and 6h dark, and luminosity of 100 μmol/m^2/s (WAHIDIN; IDRIS; SHALEH, 2013). Likewise, the microalgae *Scenedesmus* sp. had the highest lipid content (40.16%) under 18h dark and 6h light photoperiod (REN et al., 2020). In addition, the use of LED lamps with a luminous intensity of 560 μmol/m^2/s on *Chlorella vulgaris* cultivation resulted in 41.66% of lipids in cell dry weight (LIAO et al., 2017).

4.3 SALINITY STRESS

The stress generated by salinity variations on *Monoraphidium* sp. was reported by (Zhao et al., (2021), where 0.34 M NaCl was used to increase the lipid content from 35.96% (control) to 44.77% (treatment). Otherwise, the addition of 50 μM sodium azide and salinity 2.5 M in cultivation of *Dunaliella tertiolecta* increased the lipid yield and lipid content by 10% and 70.5%, respectively (CHEN et al., 2019). Then, increasing salt concentrations improves the intracellular lipid content which corresponds to triglycerides, saturated and monounsaturated fatty acids, however, high levels of salts could reduce the fraction of polyunsaturated fatty acids in microalgae and thraustochytrids (CHURCH et al., 2017; MIRIZADEH; NOSRATI; SHOJAOSADATI, 2020; PANDIT; FULEKAR; KARUNA, 2017; ZHU et al., 2007). Hence, salinity stress can be used strategically to obtain the desired fatty acids according to their future applications, whether food, pharmaceuticals, cosmetics or biofuels.

4.4 LOW-COST ALTERNATIVE NUTRIENTS

The use of glycerol as a low-cost carbon source used by *Thraustochytrium* sp. has been studied (CHEN et al., 2020b). In this study, the highest productivity of lipids (131 mg/l.h) was obtained with a pretreatment of pure glycerol, using acid and $CaCl_2$. Different concentrations of glycerol and glucose (0.5, 1, 2, 4, 6, 8 and 10%) were used by (GUPTA et al., 2013a) in Australian *Thraustochytrids* cultivations. The results obtained showed high concentrations of total fatty acids (61.6%) when 4% glucose was used, while with the use of 4% glycerol the concentration of saturated fatty acids was 48.8%. The use of 2% glucose and 2% hydrolyzed sugar were studied in thraustochytrid cultivation, where 71% and 59% of total fatty acids were reported, respectively (GUPTA et al., 2015). On the other hand, effects caused by temperature, nitrate content and pH were exemplified by (CORREDOR et al., 2021), where a higher concentration of lipids was obtained with a temperature of 30 ºC, 0.5 mM nitrate and without pH buffer.

5. FATTY ACIDS AND LIPID PROFILES

5.1 HIGH-VALUE FATTY ACIDS

Fatty acids (FAs) are important components of lipids and numerous studies have been carried out for the use of microalgae as a promising source of different FAs of commercial interest like on biorefinery, nutritional or medical applications and biomarkers in the determination of taxonomic groups. Like many microalgae, the thraustochytrids also naturally accumulate lipids and have been exploited as producers of very-long-chain polyunsaturated fatty acids (VLC-PUFAs). Many factors influence the quantity and the profile of the FAs, such as the strain, growth phase, medium and culture conditions but also the extraction methods used (DESHMUKH; KUMAR; BALA, 2019; LAGE; GENTILI, 2018; STAMENKOVIĆ et al., 2020).

Among the commercial value FAs produced by different microalgae, it can be mention polyunsaturated fatty acids (PUFAs), such as α-linolenic acid (ALA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), for their effects on human health and nutritional value (KUMAR et al., 2019). Other important FAs are oleic (18:1 n-9), palmitic (16:0), γ-linolenic (GLA, 18:3 n-6), stearidonic (SDA, 18:4 n-3), arachidonic (ARA, 20:4n-6), docosapentaenoic (DPA, 22:5 n-3 and 22:5 n-6) (DRAAISMA et al., 2013; DROUIN; RIOUX; LEGRAND, 2019). However, in biodiesel production only fatty

acid methyl esters (FAMEs) converted from neutral lipids can be used. Thus, an accurate analysis of the composition of FAs in lipids produced by microalgae is important in obtaining specific commercial products.

The content of lipids accumulated in the cells of thraustochytrids can be greater than 50% dry weight. LC-PUFAs are the main components, including the docosahexaenoic acid (DHA, 22:6 n-3), eicosapentaenoic acid (EPA, 20:5 n-3) and docosapentaenoic acid (DPA, 22:5 n-3), of which more than 25% is DHA (GUPTA; BARROW; PURI, 2012a). Although thraustochytrids can be considered a promising source of PUFA, only a few genera of *Thraustochytriaceae* family have been studied and explored biotechnologically, particularly species of *Schizochytrium*, *Thraustochytrium*, *Aurantiochytrium* and *Ulkenia*. Patents and products have been developed and commercialized mainly using *Schizochytrium* for microbial production of DHA (OROZCO COLONIA; VINÍCIUS DE MELO PEREIRA; SOCCOL, 2020). Thraustochytrids produce saturated FAs and LC-PUFAs. Among the PUFAs, DHA (22:6 n-3) and DPA (22:5 n-6) are the most abundant**,** followed by other commercially important compounds such as EPA (20:5 n-3), ARA (20:4 n-6). Oleic acid (18:1 n-9), γ-linoleic acid (18:2 n-6), and α-linolenic acid (18:3 n-3) are also reported as common 18 carbon unsaturated fatty acids (MORABITO et al., 2019). The FAs accumulation in *Aurantiochytrium* sp. cells has been reported to be greater than that achieved with *Thraustochytrium* species (QUILODRÁN et al., 2020).

The great demand for omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) stimulates the search of alternatives sources to conventional production from fish or plant oils. Due to high contents of DHA and EPA, biomass of microalgae and thraustochytrids are of important commercial interest. Moreover, the presence of other value-added compounds such as linoleic acid (LA), α-linolenic acid (ALA), arachidonic acid (ARA), and saturated FAs, being palmitic acid (C16:0) the most common, makes the cultivation of microalgae an innovative opportunity with applications either in the enrichment of healthy foods, in animal nutrition besides biofuel production.

5.2 FATTY ACIDS ANALYSIS

In this sense, different methods have been used for analysis of the lipids and FAs. The gravimetric method quantifies the crude extract which can also contains other substances such as proteins and carbohydrates, therefore, it is not a specific method for lipids (BLIGH; DYER, 1959). Otherwise, gas chromatography (GC) quantifies the individual FAs present and the total

concentration of the FAs but due to the nature of the crude extract matrix and the low volatility of the FAs, it is necessary to perform a derivatization of the FAs (transmethylation) before analysis by GC. Then, the later separation or purification techniques such as thin-layer chromatography (TLC) or solid-phase extraction (SPE) can be used to improve the separation between the peaks and consequently improve the GC analysis (LAGE; GENTILI, 2018).

The type of column, non-polar, polar or both and the type of detection coupled to the GC is important and also varies in the literature. GC-flame ionized detection (FID) and GC-mass spectroscopy (MS) systems have been performed being that MS is used when more precise analysis is needed since the FID is no-selective detector (NIEMI; LAGE; GENTILI, 2019; WOO et al., 2012). Co-elution cases are common in complex samples such as lipid extracts and alternatives have appeared as the use of two-dimensional GC-MS (GC/GC/TOFMS) with high separation efficiency (IZADMANESH et al., 2017).

In lipidomic studies, care with sample degradation is important. In addition to controlled extraction, and sample maintenance methods that prevent enzymatic activity, oxidation and hydrolytic degradation (JUROWSKI et al., 2017). Liquid chromatography (LC) is also an alternative, for example in the direct infusion of the sample was established by applying LC/MS. Moreover, hydrophilic interaction chromatography (HILIC) in combination with MS (DA COSTA et al., 2015) and MS/MS (OKAZAKI et al., 2013) just as ultra-performance liquid chromatography (UPLC) integrated with Q-TOF-MS or UHPLC/ESI/MS/MS has been reported as high resolution and sensitive methods in the study of microalgae lipidomics (CUTIGNANO et al., 2016; LU et al., 2013).

6. MOLECULAR BREEDING STRATEGIES FOR LIPID IMPROVEMENT

Despite all the benefits of using microorganisms to produce lipids, large-scale production is still not economically viable. The low lipid productivity (production rate) is one of the limiting factors in the scaling and industrial applications of microbial lipids. Cell resistance and specific characteristics required for industrial fermentation processes may not be found in nature. Genetic improvement techniques have been applied to increase lipid productivity of wild microbial strains, in addition to improving their adaptations in new substrates and modifying lipid profile for a more valuable content.

The techniques used for improving microalgae and Thraustochytrids are classified as conventional breeding (induced mutations and evolutionary engineering) and those based on molecular biology (Recombinant DNA Technology, CRISPR and Metagenomics). The

technique chosen is dependent mainly on the microbial group studied, in addition to available resources and the fermentation process in question. It is expected that a genetically improved strain will mainly present high lipid content, ability to grow in low cost media (non-cellulosic hydrolysates and wastewater), fast growth rate, genetic stability and easy of downstream processing and lipid extraction (ARORA; YEN; PHILIPPIDIS, 2020).

6.1 MUTATION THROUGH CONVENTIONAL BREEDING

Microbial improvement based on classical genetics, such induced mutations and evolutionary engineering, generate random improvements and are, therefore, less efficient. However, these techniques have the advantage of not requiring prior knowledge about the gene of interest (facilitated procedure) and do not generate genetically modified microorganisms by using genetic variability that occurs in nature. The mutation is the basis of microbial variability. These mutations can be accelerated in the lab for the generation of new microbial strains that accumulate functional lipids. The technique of induced mutation consists of the exposure of microbial cells to chemical (N-methyl-N'-nitro-N-nitrosoguanidine, ethidium bromide, proflavine, 5-bromodeoxyuridine, formaldehyde, hydroxylamine, and methoxamine) or physical (UV irradiation, X-ray, ion beams, and nuclear) mutagens until the death of 50-95% of the initial population. This step is followed by the selection of mutants with modified lipid accumulation in relation to the parental cell. However, a certain degree of targeting can be achieved by choosing the agent causing mutations. Ultraviolet (UV) radiation at 250–290 nm can be used to induce either formation of thymine dimers (transition of guanine (G) and cytosine (C) to adenine (A) and thymine (T)) or deletion of A-T base pairs in the DNA. Chemical mutagenesis by alkylating agents (methane sulphonate, methyl methane sulfonate, diethyl sulphate, and N-methyl-N-nitro-N-nitrosoguanidine) add an alkyl group to the hydrogen-bonded oxygen at N7 and N3 positions of G and A base pairs, respectively, resulting in depurination and pairing errors in DNA. It should be noted that physical agents has been extensively utilized for generating high lipid accumulating mutants of several oleaginous microalgae (ARORA; YEN; PHILIPPIDIS, 2020). For instance, UV mutagenesis resulted in an increase of up to 6% in total lipid yield of *Chlorella vulgaris* (SARAYLOO et al., 2017) and enabled the tolerance of *Scenedesmus* sp. to highly concentrated cellulosic ethanol wastewater (ZHANG et al., 2018a). Wang et al., (2020) obtained *Chlorella pyrenoidosa* and *Chlorella pacifica* mutants generated by three types of laser (He-Ne, Nd:YAG and SC) with higher lipid productivity, lipid content, and growth rate compared to wild strains. Through induced

mutations, hundreds of mutated colonies can be generated making the screening and selection of high lipid producing cells time consuming and challenging. Thus, the utilization of microtiter-based fluorescence procedures are used as mini-bioreactors for large-scale screening (DUETZ, 2007; KENSY; ENGELBRECHT; BÜCHS, 2009).

Evolutionary engineering explores the ability of microbial cells to adapt to adverse environmental conditions for the purpose of strain improvement. In brief, this technique consists of genetic modifications caused by the continuous growth of a microbial culture subjected to an appropriate selective pressure, such as a gradual increase in temperature, pH, and osmotic pressure (BENEDETTI et al., 2018; BONNEFOND et al., 2017; SUN et al., 2018a). This procedure results in the appearance of several mutations in the DNA, including single nucleotide polymorphisms (SNPs) and small-scale insertions and deletions, and activation of silenced genes (KUMAR et al., 2020). It takes up to 300 generations to cause an effect. The application of evolutionary engineering is mainly recommended when a given characteristic is coordinated by multiple genes; therefore, it is widely used to improve lipid-accumulating microorganisms.

6.2 GENETIC ENGINEERING

Recombinant DNA is DNA molecules that have fragments of DNA derived from two or more sources, usually from different species. Molecular cloning is the central technique of recombinant DNA methodology and consists of the transfer of a gene from one organism to another, that is, the recombination of DNA from different sources. The procedure involves isolating a gene with the desired potential (enzymes, proteins) and introducing it into a microbial cell. Thus, a population of recombinant cells (cloning) is established, where a single selected cell gives rise to a population of clones. At the end of the process, all resulting cells are expected to contain a copy of the vector carrying the gene of interest. Then the fermentation and multiplication step of the microorganisms and expression of the proteins of interest are carried out. Finally, after fermentation, a sequence of steps (downstream) must be performed to eliminate impurities and purify the product. Thraustochytrids are known to accumulate DHA and docosapentaenoic acid but not eicosapentaenoic acid or arachidonic acid. Using recombinant DNA technology, (KOBAYASHI et al., 2011) increased the production of eicosapentaenoic acid in thraustochytrids through thraustochytrid ubiquitin promoter-driven expression of a fatty acid {delta}5 desaturase gene. The gene was functionally expressed in *Aurantiochytrium limacinum* and the authors concluded that molecular breeding of

thraustochytrids is a promising strategy for generating beneficial lipid-accumulating microorganisms.

The clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system has also been widely studied as a tool for targeted gene modification (gene knockout and gene replacement) in numerous eukaryotic organisms. CRISPR elements are genomic structures that form a family of scattered repeats present in most archaebacteria and in half of the bacteria with sequenced genomes. Repeated sequences (DRs), usually specific to a given locus, are interspersed by variable sequences of constant and similar size, called spacers. These loci cause specific changes in the genomes and have been used as an efficient tool in microalgal genome editing (FAJARDO et al., 2020; JEON et al., 2017). (JIANG; WEEKS, 2017) transformed *Chlamydomonas reinhardtii* with Cas9/intron-sgRNA constructs and appropriately designed synthetic. As a practical application, a similar ssDNA oligonucleotide targeting the acetolactate synthase (ALS) gene and an appropriate Cas9/intron-sgRNA construct was used to create cells resistant to the herbicide, sulfometuron methyl.

7. INNOVATIONS AND TECHNOLOGIES

Different technologies have been developed in the last 20 years between 2000 and 2020 according to the PatentInspiration platform related to Boolean terms: (lipid* or oil* or "fatty acid*" or triglycerid* or pufa* or omega* or biodiesel or diesel or biofuel or dha or epa or ala or ara or oleic or linoleic or linolenic or arachidonic or eicosapentaenoic or docosapentaenoic or docosahexaenoic) and (thraustochyt* or labyrinthul* or protist* or traustoquit* or labirintul* or microalga* or "micro-alga" or "micro-algal"). The database search revealed a total of 4158 published patents, of which 1686 are granted and can be negotiated (Table 2).

The most technologies came from United States and China, mainly in 2015 and 2016. The most representative inventors were Scott Franklin from Solazyme Inc. and James G. Metz from Martek Biosciences Corporation. These patents were published mainly under the IPC codes C12P7/64 (Fats; fatty oils; ester-type waxes; higher fatty acids) and C12N1/12 (Unicellular algae; Culture media therefor), which refer to the main nouns as microalgae and oil. Consequently, the majority of companies, universities and inventors holding the technologies seek USPTO (United States Patent and Trademark Office) and CNIPA (China National Intellectual Property Administration) as main patent offices with commercial interest. They aim to protect their inventions in the two most potential countries in biotechnology area.

Table 2. Summary of results obtained in patent mapping for lipids produced by microalgae and thraustochytrids in the last 20 years (2000-2020) according to (PATENTINSPIRATION, 2021).

Patent search criteria	Results
Number of patents	• Published: 4158 • Granted: 1686
Top publication years	• 2015 • 2016
Top applicant countries	• United States • China
Top company applicants	• Martek Biosciences Corporation • Solazyme Inc.
Top inventors	• Scott Franklin • James G. Metz
Top nouns	• Microalgae • Oil
Top IPC codes	• C12P7/64 • C12N1/12
Top patent office requested	• USPTO • CNIPA

7.1 TECHNOLOGIES CLASSIFICATIONS AND APPLICATIONS

Patents published in all patent offices around the world receive one or more International Patent Classification (IPC) codes. In this case, most patents published in the last 20 years have been classified under the main codes C12P7/00 (Preparation of oxygen-containing organic compounds), C12N1/00 (Microorganisms, e.g., protozoa) and C12R1/00 (Microorganisms) (Table 3).

These tree main codes show the production of organic compounds such as lipids by microorganisms, where microalgae and thraustochytrids can be classified. It is also possible to observe the code C12N15/00 referring to mutation or genetic engineering, where molecular breeding strategies have been the object of study by companies and universities seeking to improve yields and productivities of the production of biomass, lipids and FAs by microalgae and thraustochytrids as discussed in Section 10.6. Likewise, the code C12N9/00 (Enzymes, e.g., ligases (6.)) list patents in the area of metabolic engineering, which seek to study, manipulate, improve or optimize the different pathways of the metabolism of these microorganisms to obtain desired bioproducts. In addition, the main applications shown by the IPC frequencies of these patents are as medicinal preparations, active ingredients, fuels, food fortification, foods, animal feed and cosmetics.

Table 3. Top 20 frequencies of International Patent Classification (IPC) maingroup codes for lipids produced by microalgae and thraustochytrids in the last 20 years (2000-2020).

IPC code	Description	Frequency
C12P7/00	Preparation of oxygen-containing organic compounds	1622
C12N1/00	Microorganisms, e.g., protozoa	1489
C12R1/00	Microorganisms	713
C12N15/00	Mutation or genetic engineering	599
C11B1/00	Production of fats or fatty oils from raw materials	524
C12N9/00	Enzymes, e.g., ligases (6.)	351
A61K31/00	Medicinal preparations containing organic active ingredients	308
C10L1/00	Liquid carbonaceous fuels	306
A23L33/00	Modifying nutritive qualities of foods	299
C12M1/00	Apparatus for enzymology or microbiology	271
A23L1/00	Foods or foodstuffs	240
A23D9/00	Other edible oils or fats, e.g., shortenings, cooking oils	228
A23K1/00	Animal feeding-stuffs	222
C11C3/00	Fats, oils or fatty acids obtained by chemical modification of fats, oils or fatty acids, e.g., by ozonolysis	180
A61K8/00	Cosmetics or similar toilet preparations	177
A61K36/00	Medicinal preparations of undetermined constitution containing material from algae, lichens, fungi or plants, or derivatives thereof, e.g., traditional herbal medicines	173
A23L17/00	Food-from-the-sea products	167
C12N5/00	Undifferentiated human, animal or plant cells, e.g., cell lines	160
C11B3/00	Refining fats or fatty oils	147
A23L29/00	Foods or foodstuffs containing additives	136

The IPC codes can be related to the highlighted nouns such as microalgae, microorganisms, protein, DHA, food, animal, fat and carotenoids (Figure 5). Microalgae noun shows the direct relationship with the cultivation, growth and generation of biomass rich in lipids, where it can later be extracted in oil for use as biodiesel. Otherwise, microorganisms noun highlights the production and synthesis of PUFAs mainly by thraustochytrids. Then, protein noun represents the manipulations or molecular breeding strategies through genes and enzymes important in the metabolism of lipids. In addition, DHA noun show the importance of this high-value lipid and FAs accumulation strategies such as saline stress to obtain desired lipid profiles. Consequently, food and animal nouns describe applications as ingredients, powders, flours and triglycerides for animal feed mainly aquafeed. Finally, carotenoids noun

shows secondary metabolites, which can be stimulated during autotrophic and mixotrophic growth such as β-carotene, astaxanthin, canthaxanthin and lutein. These microalgae pigments have recently gained importance due to their anti-oxidant, anti-inflammatory and anti-cancer properties (GONG; BASSI, 2016).

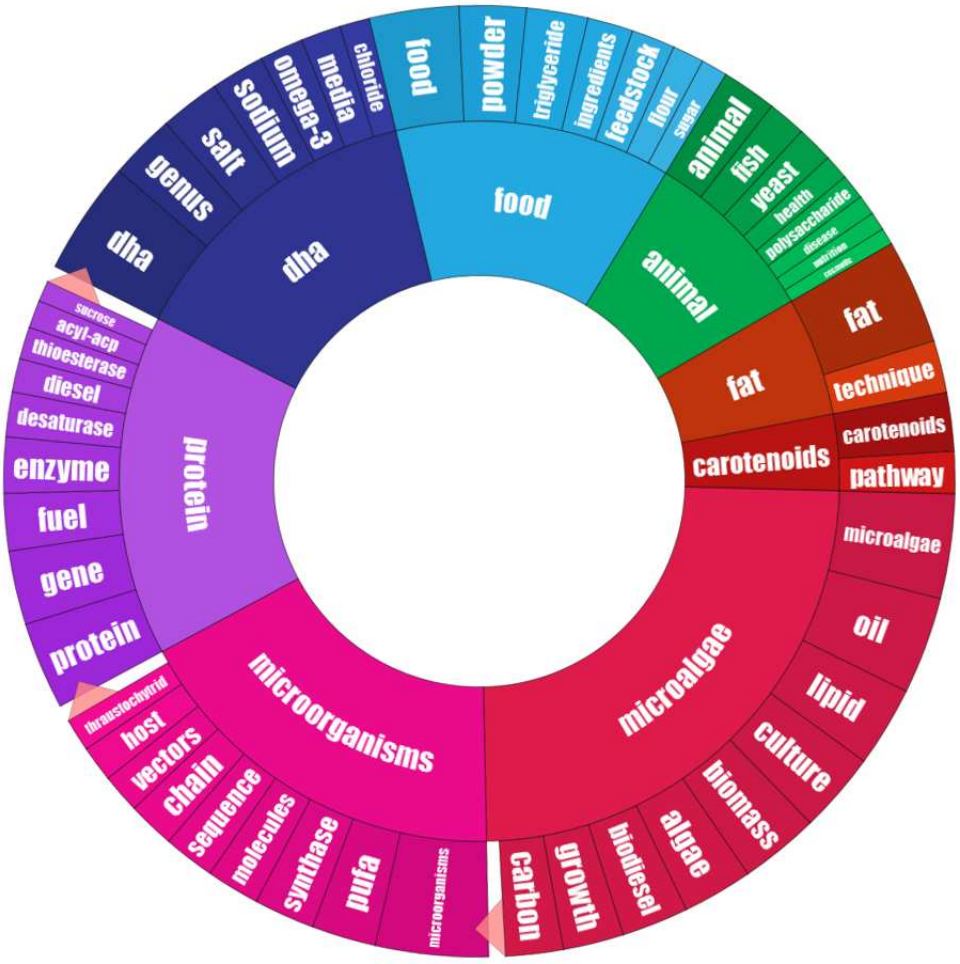

Figure 5. Nouns highlighted in the technologies and patents landscape for lipids produced by microalgae and thraustochytrids in the last 20 years (2000-2020).

7.2 INNOVATIVE COMPANIES AND TRADEMARKS

The lipids produced by microalgae and thraustochytrids began to stand out in 2008 with a major growth trend by the Top 5 companies: Martek Biosciences Corporation, Solazyme Inc., DSM IP Assets B.V., Roquette Frères and Corbion Biotech Inc (Figure 6). Martek Biosciences Corporation reached the maximum number of patents published with 88 patents in 2008 and 290 patents in the last 20 years. Martek produce fortified foods, beverages and dietary supplements, which are derived from microalgae. Martek was acquired by DSM IP Assets B.V.

in 2011, which operates in the field of health, nutrition and materials (JEANNET; SCHREUDER, 2015). This explains the decrease in patents published by Martek from the year 2012, while DSM increased the patents from that date. Life'sDHA® and Life's™OMEGA are trademarks of DSM, which are vegetarian nutritional lipids produced from microalgae for dietary supplements, foods and beverages, and infant nutrition. DHAgold™ is used for animal feed and pet food. Their products can be found in liquid and powder form. In addition, Life's®DHA+ARA is a combined liquid oil rich in omega-3 and omega-6 LC-PUFAs containing DHASCO-B® (Life'sDHA® from microalgae) and ARASCO® (Life's®ARA from fungi) specifically targeted for infant nutrition (DSM NUTRITIONAL PRODUCTS LTD, 2020). In 2018, DSM and Evonik launched Veramaris to produce omega-3 EPA and DHA for aquafeed. They are looking to meet the Sustainable Development Goals (SDGs): Zero hunger (Goal 2), good health and well-being (Goal 3), responsible consumption and production (Goal 12), life below water (Goal 14) and partnerships for the goals (Goal 17) (VERAMARIS, 2020).

Applicant	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	Total
MARTEK BIOSCIENCES CORP	0	5	6	12	7	20	24	33	88	15	32	32	10	3	1	0	0	0	1	0	1	290
SOLAZYME INC	0	0	0	0	0	0	0	4	3	13	33	19	23	36	27	24	30	6	3	1	0	222
DSM IP ASSETS BV	0	0	0	0	0	3	4	0	7	3	5	10	10	15	15	30	23	25	9	17	14	190
ROQUETTE FRERES	0	0	0	0	0	0	0	0	0	0	0	3	4	3	11	21	37	13	6	2	1	101
CORBION BIOTECH INC	0	0	0	0	0	0	0	0	0	0	1	1	1	5	1	5	13	6	17	17	10	77
FERMENTALG	0	0	0	0	0	0	0	0	0	0	0	0	5	9	8	11	4	11	6	4	1	59
FRANKLIN SCOTT	0	0	0	1	0	0	0	0	1	6	9	9	12	10	1	0	0	0	0	0	0	49
TERRAVIA HOLDINGS INC	0	0	0	0	0	0	0	0	0	0	1	0	0	3	4	4	16	11	9	1	0	49
CHINA PETROCHEMICAL CORP	0	0	0	0	0	0	0	0	0	0	0	0	1	8	10	9	9	6	0	3	0	46
DU PONT	0	0	0	0	0	3	0	8	13	4	7	1	2	2	0	1	0	0	0	0	0	41

Figure 6. Top 10 applicants, assignee names or companies for lipids produced by microalgae and thraustochytrids in the last 20 years (2000-2020).

Moreover, Solazyme Inc. is a biotechnology company initially created in 2003 to produce energy and biofuels from renewable microalgal source. In 2011, Solazyme communicated a joint venture with Bunge Limited in Brazil to produce renewable oils from algae using sugarcane as feedstock (BUNGE, 2012). Then, Solazyme changed its name to TerraVia Holdings Inc. in 2016 and announced AlgaPrime™ DHA as the first product from the join venture TerraVia and Bunge. Furthermore, Corbion Biotech Inc. acquired TerraVia in 2017 to bring solutions from microalgae with the trademarks AlgaPrime™ DHA for aquafeed, AlgaPūr™ for skin and hair care, AlgaVia® as a whole algae ingredient with protein and lipids, AlgaWise™ rich in omega-9 as a high stability oil for industrial cooking and frying (CORBION, 2017). On the other hand, Solazyme Industrials maintained its focus on biofuels and industrial oils from microalgae with some trademarks such as Encapso™ (industrial drilling lubricant), SoladieselBD® and SoladieselRD® both as biodiesel, Solajet™ as aeronautical

biofuel (SOLAZYME INDUSTRIALS, 2014). Therefore, Solazyme, Corbion and TerraVia had 222, 77 and 49 published patents, respectively, between 2000 and 2020 related to lipids produced by microalgae and thraustochytrids (Figure 6).

8. CONCLUSIONS AND PERSPECTIVES

The use of microalgae and thraustochytrids as renewable sources of microbial lipids has been promising in recent years. Companies have shown how it is possible to reach different market niches through innovation, technology and products. However, there are still technologies that could be improved either by making the process more cost efficient or with the aid of omics tools such as metagenomics, transcriptomics, proteomics, metabolomics, lipidomics or nutrigenomics. This approach of molecular techniques has shown great findings and are aiming to be used in the study of natural marine environments for bioprospecting of new potentially lipid-producing organisms. It is also important to note that ecological studies of interactions of these microorganisms in marine food web have become key to understanding and exploring many marine species normally grown or that have not yet been grown on a laboratory scale.

The sustainable production of lipids by microalgae and thraustochytrids still needs improvements in its stages of upstream, midstream and downstream. Strain breeding by molecular techniques (mutation, genetic engineering) or through adaptation to the culture medium and conditions such as temperature, pH, agitation, aeration has still been a major bet of recent research. Likewise, the use of stress strategies such as salinity, nutrient limitation, light intensity is still widely studied to stimulate the accumulation of lipids, achieve desired fatty acid profiles, increase yields and productivity. However, it is important to highlight the extent to which a strain can be improved without making it GMO (Genetically modified organism), which could be rejected by consumers. So, the bioprospecting of wild species has come with a wave of bioproducts of natural origin and minimally manipulated and processed. Thus, companies, research centers and industries could target GMO strains for non-food applications such as use in biofuels, industrial oils and lubricants.

On the other hand, the processes of separation and recovery of microbial oils in downstream stage tend to make the final product more expensive, especially if the purification of some high-value fatty acid such as polyunsaturated fatty acids (PUFAs) is sought. Steps that require less separation processes such as the use of whole algae biomass in powder form for nutrition have been a recent trend, which serves as a source of proteins and lipids. Additionally,

the search for green extraction technologies with less aggressive solvents has been approached as an alternative for the production of oils in liquid form used in formulations. Currently, new technologies for obtaining oils from microalgae and thraustochytrids through mild and more sustainable processes are gaining attention. Finally, further studies and tests on the application of these oils in human and animal nutrition, evaluating their bioavailability, digestibility, nutrient adsorption, nutritional profile and health effects are needed. Likewise, the development of oils rich in saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) more suitable for industrial use, such as biofuels and biodiesel, are an alternative to reduce our dependence on petroleum-derived fuels.

CHAPTER 2

OMEGA-3 MICROBIAL OILS FROM MARINE THRAUSTOCHYTRIDS AS A SUSTAINABLE AND TECHNOLOGICAL SOLUTION: A REVIEW AND PATENT LANDSCAPE

This chapter was published in the journal *Trends in Food Science & Technology* (2020). Impact factor: 11.077 (JCR 2019) and doi: 10.1016/j.tifs.2020.03.007 (Attachment 1).

ABSTRACT

Oils rich in omega-3 have been of industrial and economic importance, mainly for its benefits in human and animal health. Fish is considered the primary source for obtaining omega-3. The resurgence of interest in the use of omega-3 as a functional food has led to the need of alternative sources in this growing market. Omega-3 produced by marine thraustochytrids have appeared as an alternative to fish oil and an eco-friendly solution to overfishing. Here, we review key mechanisms that enable sustainable production and technological prospection of omega-3 from thraustochytrids. Sustainability analysis identified strengths, weaknesses, opportunities and threats for omega-3 oils from thraustochytrids. Thus, this study shows the technological prospection based on the worldwide patent landscape. Thraustochytrids have also been known as microalgae on the international market, which has been well received by consumers as a natural or vegan source of omega-3 supplementation. Thus, omega-3 oils can be developed in controlled bioprocess systems preventing the production of toxins and pollutants. Hence, the technological prospection showed a growing trend with 731 patents published between 1999 and 2018. Most patents were classified within the chemical and human areas, which highlighted omega-3 oils involving biosynthesis by microorganisms such as *Aurantiochytrium* in food applications. These technologies have been mainly developed by countries, such as the United States, China, Netherlands and Japan, which refer to bioreactor cultivation, strains improvement and food formulations. Thus, global patent search allowed to analyze technological advances, trends and market gaps of omega-3 oils from thraustochytrids as a renewable technological solution.

Keywords: Omega-3; Docosahexaenoic acid; Thraustochytrid; Microalgae oil; Sustainable bioprocess; Functional foods.

1. INTRODUCTION

The consumption of omega-3 fatty acids has gained relevance in the world. There are different sources of omega-3 fatty acids found in nature. Both short-chain and long-chain omega-3 fatty acids are essential and confer health benefits, having different functions in the body (ZHANG; DOWNIE, 2019). Alpha-linolenic acid (ALA, C18:3 n-3) is a short chain polyunsaturated fatty acid from the omega-3 family found mainly in plants, such as nuts, flaxseed, chia and others. For example, flaxseed oil can contain more than 39.90% ALA and has been used in some medical formulations for the treatment of different diseases, such as eczema, muscle contusions and atherosclerosis (GOYAL et al., 2014). In addition, an important function of ALA is to serve as a precursor for the biosynthesis of omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs) (BRENNA et al., 2009; SOKOŁA-WYSOCZAŃSKA et al., 2018; TURCHINI et al., 2012). However, terrestrial plants do not produce n-3 LC-PUFAs when compared to marine sources (TOCHER et al., 2019).

The n-3 LC-PUFAs are mostly found in fish and, more recently, from marine microbes. They mainly comprise eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). These fatty acids are classified as n-3 because the first double bond occurs at carbon 3 in the chain. The eicosapentaenoic acid (EPA, C20:5 n-3) is composed of a structure of 20 carbons and 5 double bonds, the docosapentaenoic acid (DPA, C22:5 n-3) of 22 carbons and 5 double bonds and the docosahexaenoic acid (DHA, C22:6) of 22 carbons and 6 double bonds (DAVE; ROUTRAY, 2018; MOZAFFARIAN; WU, 2011). The n-3 PUFAs biosynthesis is performed by a set of elongases and desaturases enzymes (Figure 7). The desaturases add double bonds and the elongases catalyze the extent of the carbon chain, starting from alpha-linolenic acid (ALA) which undergoes desaturation and elongation to produce EPA. Thus, the EPA uses several enzymes to be transformed into DPA and, ultimately, into DHA. Humans cannot efficiently synthesize the conversion of ALA to DHA due to the lack of these enzymes. Therefore, EPA, DPA and DHA are considered essential fatty acids because they must be obtained in food or supplementation (BRADBURY, 2011; LAYÉ et al., 2018; PETRIE et al., 2018; SAINI; KEUM, 2018).

Some reported physiological and cognitive functions of n-3 LC-PUFAs include fetal development and vision in infants, anti-inflammatory properties, maintenance of brain functions, ocular tissues, inhibitors of macular degeneration in old people as well as ability to raise levels of HDL cholesterol in the blood decreasing the risk of heart disease (AHMAD et al., 2020; AHMMED et al., 2020; MALLICK; BASAK; DUTTAROY, 2019).

Docosahexaenoic acid (DHA) is found in the human body (15–20% in the cerebral cortex and 30–60% in the retina), breast milk and sperm (APT et al., 2018; MATHUR et al., 2018; SWANSON; BLOCK; MOUSA, 2012).

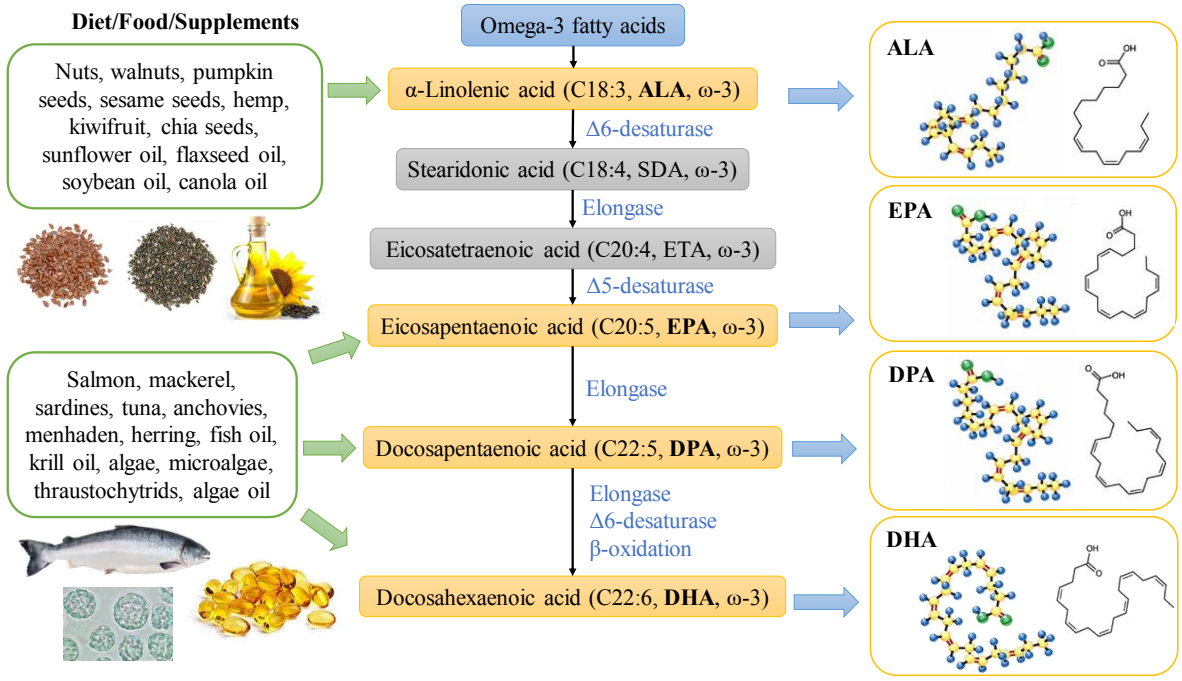

Figure 7. Sources, biosynthesis and structure of omega-3 polyunsaturated fatty acids (PUFAs) adapted from (MOZAFFARIAN; WU, 2011).

The market value of omega-3s is estimated to be about USD 57.07 billion by 2025, with a compound annual growth rate (CAGR) of 6% during the forecast period 2018–2025 (GRAND VIEW RESEARCH, 2017). The Global Organization for EPA and DHA Omega-3s (GOED) has established the following DHA+EPA intake recommendations, according to scientific evidence: 500 mg/day for healthy adults, 700–1000 mg/day for pregnant and lactating women, 1 g/day for patients with coronary heart disease (CHD), > 1 g/day for people with health conditions such as blood pressure and triglycerides (CUNNANE et al., 2004; ESLICK et al., 2009; EUROPEAN FOOD SAFETY AUTHORITY (EFSA), 2009; KOLETZKO et al., 2014; KRIS-ETHERTON; HARRIS; APPEL, 2003). Studies on DHA intake alone or combined with EPA have encouraged researchers and medical staff. A study conducted on the effects of DHA and DHA/EPA intake on specific domains of memory in healthy adults, showed improved memory in older adults with mild memory complaints with at least 1 g per day of DHA/EPA supplementation (YURKO-MAURO; ALEXANDER; VAN ELSWYK, 2015). Likewise, researchers have shown that a high intake of EPA (1.2 g/day) for 12 weeks improved

cognitive symptoms and impulsivity in children and adolescents with attention deficit hyperactivity disorder (ADHD) (CHANG et al., 2019).

Furthermore, omega-3-rich microbial lipids from thraustochytrids have appeared as an emerging alternative to fill the gap between the demand and supply of n-3 LC-PUFAs. Thraustochytrids are known as heterotrophic microalgae, which can grow in controlled systems with the ability to accumulate intracellular lipids rich in EPA and DHA (CHEN et al., 2020a; SOHEDEIN et al., 2020b). They can be used for both human and animal nutrition, including as fortified foods and beverages, dietary supplements, infant formula, clinical nutrition and medical foods, pharmaceuticals and pet foods (ROBERTSON et al., 2013). Moreover, thraustochytrids source is an option even for vegans and people with allergies to fish and crustaceans with the advantage that they simultaneously produce antioxidants, carotenoids, astaxanthin and squalene (FANG et al., 2019; PARK et al., 2018a; WANG et al., 2018d; WATANABE et al., 2018). Likewise, researches on the supplementation of omega-3 oils in fish, poultry and cattle animal feed aims to increase levels of these fatty acids and their nutritional value (AO et al., 2015; PARK; UPADHAYA; KIM, 2015; SPRAGUE; BETANCOR; TOCHER, 2017).

This article reviews and discusses the importance, sustainability and technological prospection of marine thraustochytrids as a green and eco-friendly alternative of omega-3 for human and animal nutrition. A study of microbial family concepts was performed as well as a SWOT analysis to evaluate the sustainability of the system. In addition, a technological prospection of the last 20 years (1999–2018) was performed to understand how technologies have evolved over the years looking for renewable solutions as well as the trends reflected in the global patent landscape.

2. RESEARCH METHODOLOGY

This review was divided into two parts: 1) a review of omega-3 sources with an analysis of sustainable omega-3 production from thraustochytrids as an alternative source, and 2) a technological prospection based on the mapping of patents related to the production of omega-3 fatty acids from thraustochytrids. The data presented were the result of in-depth and detailed research on articles, reviews, books, patents, case studies, and business reports. Different databases were used such as Google Scholar, Scopus, FishStatJ, Patent Inspiration, Patent Scope, Latipat-Espacenet.

2.1 OMEGA-3 MARINE SOURCES AND SUSTAINABLE SYSTEM ANALYSIS

Different sources of omega-3 have been studied and related to omega-3 fatty acid biosynthesis (BISHOP et al., 2015; USHER et al., 2017). In the present review, fish was studied as a traditional source of omega-3 fatty acids. Thus, data on fish production were obtained and analyzed using the FishStatJ software of the Food and Agriculture Organization (FAO). This tool showed historical data from capture fisheries and aquaculture. Similarly, commodity production was also analyzed by filtering mainly oil and meal production. Concerns and consequences of obtaining omega-3 oil from fish were highlighted.

Additionally, thraustochytrids were analyzed as a marine source for the production of omega-3 rich oils. Some taxonomic descriptions of the thraustochytrid family have been addressed. In addition, metabolic characteristics of lipid accumulation have been described. Hence, a sustainability analysis using thraustochytrids as an alternative source to fish was presented. Similarly, the sustainability cycle has been studied by highlighting thraustochytrids as a renewable source in an integrated bioprocess study. This study analyzed the strengths, weaknesses, opportunities and threats (SWOT) of omega-3 production from thraustochytrids.

2.2 PATENT MAPPING FOR OMEGA-3 FROM THRAUSTOCHYTRIDS

The technological prospection of patents was carried out using Patent Inspiration database. Patent Inspiration is a tool that allowed the searching of patents around the world. This application generates data mining graphs and customized tables. The World Intellectual Property Organization (WIPO) was also consulted by the Patent Scope tool, which provides access to international Patent Cooperation Treaty (PCT). Previously, an analysis of the filters for Patent Inspiration and query languages were performed to adapt the topics to the search system. The terminology was used for researching the title and abstract of patent. The patent searches were also carried out with all genera belonging to the thraustochytrids family, where those that do not show results were not considered in the patent analysis. The terms and synonyms related to omega-3 fatty acids were placed in the research due to the possible existence of other patents derived from thraustochytrids metabolites for other purposes. The data were filtered for the last 20 years (1999-2018).

The query languages were English, Spanish and Portuguese. The terminology used with Boolean operators was: ("omega-3" or "ômega-3" or "ômega 3" or dha or epa or dpa or "docosahexaenoic acid" or "eicosapentaenoic acid" or "docosapentaenoic acid" or "ácido docosahexaenoico" or "ácido eicosapentaenóico" or "ácido docosa-hexaenoico" or lipid* or

lipíd* or fat* or oil* or grasa or triglyceride* or triglicérid* or pufa or agpi or "poli-insaturado" or "microbial oil" or óleo or azeite or "óleo microbiano" or aceite or "aceite microbiano" or "ácido graxo" or "ácido graso" or "delta-6 desaturase") and (thraustochyt* or labyrinthul* or protist* or traustoquit* or labirintul* or thraustochytrium or schizochytrium or aurantiochytrium or ulkenia or parietichytrium or japonochytrium or sicyoidochytrium or botryochytrium or monorhizochytrium or oblongichytrium or althornia or hondaea or aplanochytrium).

The results were analyzed by timeline publication, International Patent Classification (IPC), origin of technologies, patent offices and applicants. The results obtained in Spanish and Portuguese in the Patent Inspiration were verified with the Latipat-Espacenet database. The results generated with WO number (WIPO) were separated and regrouped in the analysis of the publications in the patent offices and origin of the technology. Moreover, the Scopus database was also consulted using the same terminology as Patent Inspiration to analyze the timeline of published patents found by Patent Inspiration with non-patent documents in the world such as articles, reviews, book chapters, conference papers, etc. The query language used in Scopus search was English.

3. MARINE SOURCES OF OMEGA-3 FATTY ACIDS

3.1 FISH

Nowadays, the main source of polyunsaturated fatty (PUFAs) is fish oil (e.g., salmon, tuna, sardines) (VELEA; OANCEA; FISCHER, 2017); however, this source has been questioned due to the risk of ocean-borne contaminants, pesticide residues and depleted stock of fish (JACOBS et al., 2004; REN et al., 2018). Pollutants in the ocean, such as mercury, polychlorinated biphenyls (PCBs) and dioxins, have been reported in fish oil. Other complications with fish oil include taste, odor and low stability (JI; REN; HUANG, 2015). In addition, its quality variation will also depend on the fishing season, location and climatic variation (MARTINS et al., 2013; PARK et al., 2018b; RYCKEBOSCH et al., 2014). Some authors have proposed alternatives for decontamination of fish oil and fishmeal, such as adsorption with activated carbon, steam deodorization, short path distillation, solvent extraction with hexane, isopropanol, and ethanol. However, these processes are still in the research phase, and it is necessary to minimize operating costs, optimize and increase the efficiency of the process, and avoid the possible depletion of some beneficial constituents (FERNÁNDEZ-

GONZÁLEZ et al., 2014; KNUTSEN et al., 2017, 2018; LALL, 2010; OTERHALS et al., 2007; OTERHALS; KVAMME; BERNTSSEN, 2010; OTERHALS; NYGÅRD, 2008). Therefore, these concerns have increased interest in new sustainable omega-3 alternatives from microalgae, protists, yeast, fungi, and some plants.

In terms of omega-3 sources, fish oil has been the largest traditional source of omega-3, especially by its high DHA content (GAMSIZ; KORKUT; KOP, 2019; SOLOMANDO; ANTEQUERA; PÉREZ-PALACIOS, 2020). However, studies indicate that the wild fish capture has generated environmental, ethical and economic problems, which may not support the growing demand for omega-3 oils (STEINRÜCKEN et al., 2017). The fight against overfishing belongs to goal 14 (Life below water) from the United Nations Sustainable Development Goals (SDGs). Goal 14 includes different targets, such as sustainable fishing, conserve coastal and marine areas, end subsiding contributing to overfishing and increasing economic benefits from sustainable use of marine resources, where aquaculture has appeared as an alternative to wild capture (UNITED NATIONS DEVELOPMENT PROGRAMME, 2019). Overfishing occurs for a rapid rate, and ocean cannot replace species in time leading to their extinction and scarcity (KAR; MATSUDA, 2006). Figure 8 shows the reality of the marine world, where wild capture has been the most prevalent source of seafood through the years. On the other hand, aquaculture (marine/freshwater/brackishwater) started to grow from the year 2000, when it reached almost half of production with 43.01 million tonnes compared to wild capture with 94.79 million tonnes. Thus, aquaculture was able to surpass production with a total of 94.98 million tonnes compared to wild capture with 90.93 million tonnes in 2013, due to the efforts the planet has been making to conserve marine species and increase sustainable fish production. However, even with aquaculture's steady growth, wild capture has been kept constant for the past 20 years without significant decreases, unfortunately contributing to overfishing. Similarly, processed products are obtained from capture and aquaculture such as oils and meals, as illustrated in Figure 8. Fish oil production has been kept practically constant from 1976 to 2017, with 1.06 million tonnes in 2017. However, meals have declined considerably in recent years from a peak of production in 1994 with 7.51 million tonnes to 4.84 million tonnes in 2017. This diminution may be due to the recent search for alternative sources of meals, including microorganisms, plants and agro-industrial residues with relevant nutritional properties. Likewise, fish oil production is low compared to fish meals due to the low yields and oil content after the production process (FAO, 2019a, 2019b).

Aquaculture has provided the planet with great benefits, where fish can be farmed to complete their life and reproductive cycle, reducing the environmental impacts of overfishing.

Thus, aquaculture has opened great opportunities and markets by seeking alternative sources for their maintenance and feeding beyond fish. The main target of fish oil is aquaculture about 70% for feeding salmonids and a small portion for the development of functional foods for human consumption (ACIÉN et al., 2017; FINCO et al., 2017). Humans and animals lack or have low-capacity elongating n-3 LC-PUFAs from natural sources. Thus, n-3 LC-PUFAs need to be supplemented in the diet and they act as precursors for an active metabolism (UDAYAN; ARUMUGAM; PANDEY, 2017). The demand for omega-3 fatty acids has increased in recent years, which make mass fishing less sustainable. The search for sustainable sources of PUFAs with applications in pharmaceuticals, functional foods, dietary supplements and aquafeed industries shows the need for safe alternative sources (DE MEESTER; WATSON; ZIBADI, 2013; JI; REN; HUANG, 2015; STEINRÜCKEN et al., 2017).

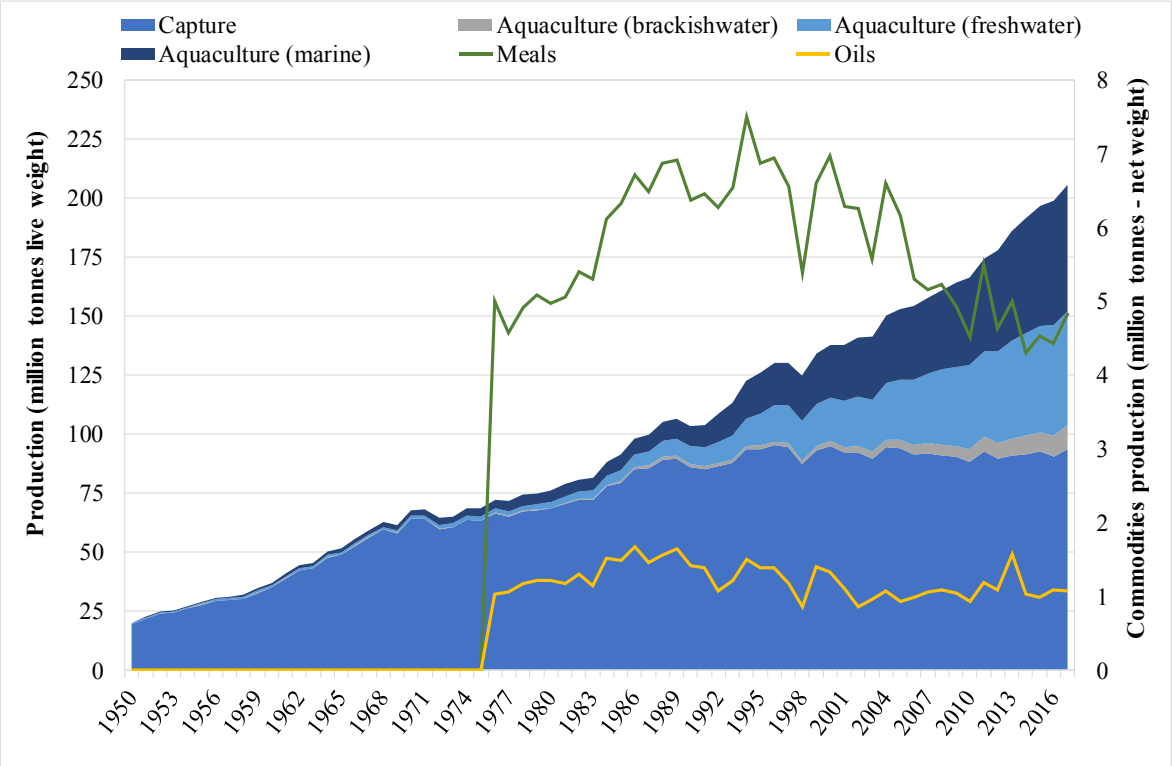

Figure 8. Global fish production from capture and aquaculture compared to fish oils and meals production reported by FAO Fisheries and Aquaculture Department (FAO, 2019a, 2019b).

3.2 THRAUSTOCHYTRIDS

Thraustochytrids are osmo-heterotrophic marine protists commonly found in estuarine, coastal and mangrove environments (LEE CHANG et al., 2012). Previously, thraustochytrids were classified as microalgae; however, this classification became less used

by taxonomists because they are not phototrophic like most microalgae (LEYLAND; LEU; BOUSSIBA, 2017). Notwithstanding, most products in the omega-3 market are namely using microalgae-related domination, such as Algal Oil Omega-3, Algae Omega-3, Vegan Omega-3, DHA Algae Omega-3, Veggy DHA, and Non-fish Omega-3s, even its origin being marine thraustochytrids.

Thraustochytrids are also known as fungal-like organisms with an ectoplasmic net, monocentric vegetative thalli and biflagellate zoospores (JARITKHUAN; SUANJIT, 2018a; JASEERA et al., 2019). Thraustochytrids belong to the class Labyrinthulomycetes. The Labyrinthulomycetes have been reported to have mostly microbial species producing omega-3 fatty acids. The orders Labyrinthulida (Labyrinthulales), Oblongichytriida (Oblongichytridiales) and Thraustochytrida (Thraustochytridiales) have been the most prominent in this case. These orders include microbial genera such as *Aplanochytrium, Labyrinthuloides, Stellarchytrium, Labyrinthula, Oblongichytrium, Thraustochytrium, Japonochytrium, Schizochytrium, Aurantiochytrium, Ulkenia, Sicyoidochytrium, Parietichytrium, Botryochytrium, Monorhizochytrium, Labyrinthulochytrium* (BENNETT et al., 2017; FOSSIER MARCHAN et al., 2018; NAKAI; NAGANUMA, 2015a). Recently, a new genus from the family Thraustochytriaceae has been discovered and identified as *Hondaea* (DELLERO et al., 2018).

Thraustochytrids have called attention mainly by the discovery of new metabolites produced by them, including enzymes, carotenoids (e.g., β-carotene, astaxanthin, canthaxanthin), antioxidants, sterols (e.g., squalene) and n-3 LC-PUFAs (e.g., EPA, DHA) (MORABITO et al., 2019). They accumulate lipids as a defense mechanism to stress factors of nitrogen limitation, starvation, low temperatures, among others. Thus, these protists activate their energy reserves by transforming them into high concentrations of fatty acids (ALLEMANN; ALLEN, 2018; SAJJADI et al., 2018). They can accumulate more than 50% of their biomass or dry weight as lipids. Lipid droplets are accumulated inside the cell, where the polyunsaturated fatty acid DHA typically accounts for more than 25% of the total fatty acids (TFA) (GUPTA; BARROW; PURI, 2012b). Some strains can use diverse sources of carbon and energy for their biomass production and accumulation of lipids, including the use of some industrial waste (FERREIRA et al., 2019). The production of biolipids rich in n-3 LC-PUFAs such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) from these marine protists has appeared as an alternative to fish oil. The process of production of single cell oil begins with the preparation of the culture medium and pre-inoculum. After this, the pre-inoculum is added to the bioreactor. Fermentation occurs for the

formation of cellular biomass and subsequent accumulation of lipids within the cell. The biomass undergoes cell disruption followed by solid-liquid separation and filtration. The recovered biomass can follow two pathways: (1) drying and milling for animal consumption or (2) lipid extraction, centrifugation and evaporation for human consumption (FINCO et al., 2017; LOPES DA SILVA et al., 2019).

4. SUSTAINABLE PRODUCTION OF OMEGA-3 RICH OILS FROM THRAUSTOCHYTRIDS

Sustainability (sustainability and ability) is defined as the ability of a system to remain diversified and productive without compromising ecological balance. In a nutshell, it is the resistance/endurance of systems and processes (JAMES et al., 2015). The term sustainability can have several definitions starting from each stage of its productive chain and finally based on a sustainable system. Thus, several aspects that support sustainable production of omega-3 rich oils should be analyzed. Winkler (2013) defined omega-3 sustainability as "securing regular supplies of LC-Omega-3 sufficient to meet the nutritional needs of the global population". In an ideal scenario, an omega-3 biorefinery should be sustainable from an economic, nutritional and environmental perspective, where the omega-3-producing organism, raw material, renewable source, extraction, purification and packaging of lipids would be inexpensive and easily accessible (JI; REN; HUANG, 2015).

The sustainability cycle starts from the ecosystem where thraustochytrids live, their isolation and improvement in the laboratory, through the bioreactor bioprocess for biolipid production, separation and obtaining high added value products. These products can be used for both human and animal feed, which allows thraustochytrids to reach the marine ecosystem again (Figure 9). The main marine carnivorous species (e.g., salmon and cod) lack or have low n-3 LC-PUFAs production capacity, which is why it is needed in diets. Thus, microalgae are the primary producers of n-3 LC-PUFAs in the marine environment, and fish contains n-3 LC-PUFA because they largely consume other fish or zooplankton that have themselves consumed microalgae, thraustochytrids or marine microbes (VENTURA et al., 2017). Microalgae omega-3 has been the name commonly used in industry and in the market to refer to omega-3 from thraustochytrids despite its taxonomy. Then, thraustochytrids produce n-3 LC-PUFAs and they are the main producers of eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) (FOSSIER MARCHAN et al., 2018; JI; REN; HUANG, 2015). Moreover, advantages of marine microbes compared to fish oil were reported, including (i) ease of

cultivation under controlled conditions, (ii) they are a vegan source, (iii) exacerbated fishing can be avoided by cutting out the middle fish, (iv) oxidative stability due to the presence of carotenoids synthesized simultaneously by these microbes and (v) they are less susceptible to environmental contamination by chemicals (ADARME-VEGA et al., 2012; MOOMAW; BERZIN; TZACHOR, 2017; UDAYAN; ARUMUGAM; PANDEY, 2017). In addition, microalgae or marine thraustochytrid biomass has high productivity compared to fish and plant sources (CHEN; YANG, 2018b). Omega-3 yields relative to its source have been presented, such as 30 kg per ton of fish, 49 kg per ton of vegetable and 300 kg per ton of microalgae biomass (FINCO et al., 2017). The accumulation of omega-3 fatty acids to obtain microbial oils is known as "Omega-3 Biotechnology" (GUPTA; BARROW; PURI, 2012b). Thus, green and sustainable biorefineries will benefit different market niches.

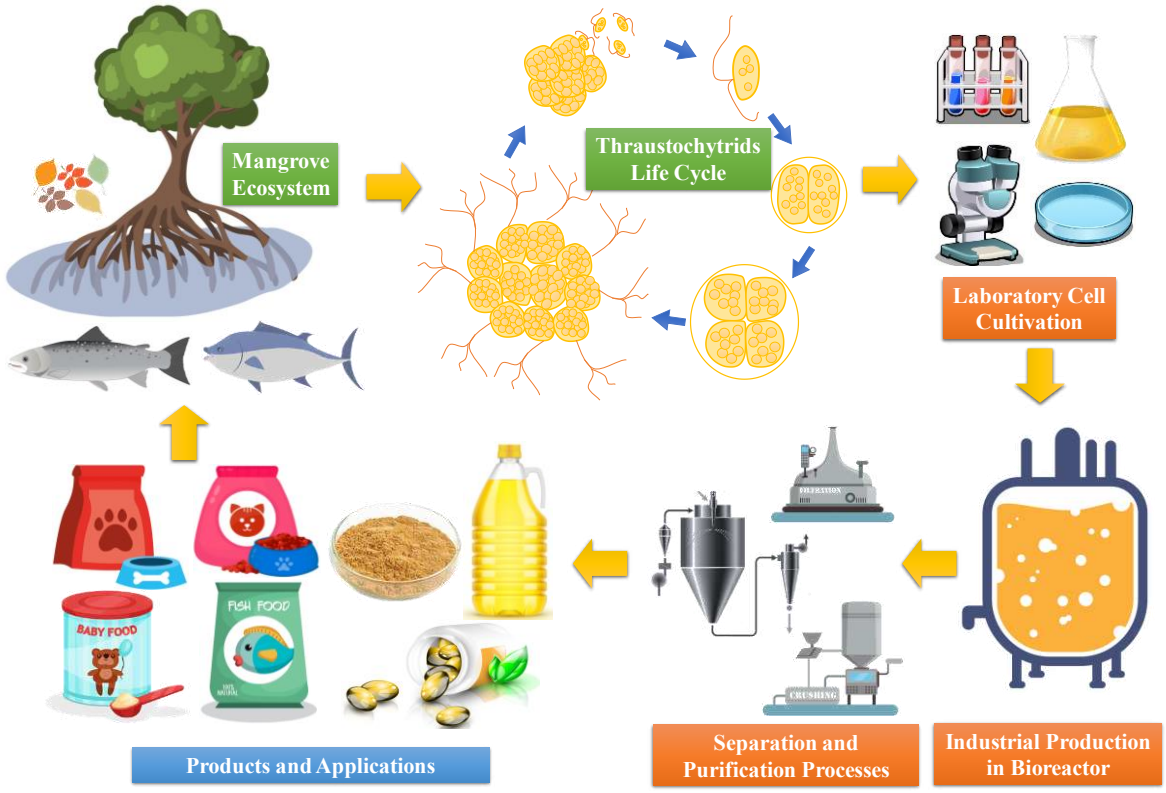

Figure 9. Omega-3 sustainable cycle from marine thraustochytrids.

Omega-3 fatty acids from thraustochytrids appear as a sustainable and productive alternative. They can grow phototrophically, heterotrophically and/or mixotrophically depending on the species (RYCKEBOSCH et al., 2012). Microalgae are prominent producers of bioactive compounds such as omega-3 fatty acids (DHA and EPA), vitamins, pigments (e.g., astaxanthin, lutein) and proteins (RIZWAN et al., 2018). Strains of *Schizochytrium* spp.,

Aurantiochytrium spp., *Thraustochytrium* spp., *Crypthecodinium* spp., *Nitzschia* spp., *Chlorella* spp., and *Haematococcus* spp. have been studied mainly by their high lipid content. Currently, heterotrophic microalgae are the most promising sources, where the families Thraustochytriaceae and Crypthecodiniaceae are highlighted by their high DHA intracellular production (DENIZ; GARCÍA-VAQUERO; IMAMOGLU, 2017; HAYES et al., 2017; JI; REN; HUANG, 2015; UDAYAN; ARUMUGAM; PANDEY, 2017).

Thraustochytrids use carbon source for cell growth and metabolism in the heterotrophic fermentation. This type of fermentation can be conducted in a well-controlled system, they can grow in dark, less contamination than open system, several carbon sources can be tested to reduce costs (CHEN; YANG, 2018b). In contrast, photoautotrophic microalgae are highlighted by the high accumulation of intracellular EPA instead of DHA. Consequently, some commercial products (biomass or oil) can lack high amounts of DHA or EPA, which depends on whether the source is heterotrophic microalgae (e.g., thraustochytrids) or photoautotrophic microalgae (e.g., *Nannochloropsis* sp.) (JIMÉNEZ CALLEJÓN et al., 2020; SÁ et al., 2020).

Furthermore, some authors claim that DHA produced by microalgae or thraustochytrids is readily absorbed by humans, where the separation of fatty acids is easier and tends to be more abundant in polar fraction (phospholipids and free fatty acids) when compared to fish DHA (PARK et al., 2018b; RYCKEBOSCH et al., 2012). Therefore, alternative sources to find omega-3 producers are necessary, where microbe isolates have high biomass and a high capacity to accumulate lipids within the cell. Finally, omics tools (e.g., metagenomics, genomics, proteomics, metabolomics, nutrigenomics) can help in the research of new strains (JI; REN; HUANG, 2015; LENIHAN-GEELS; BISHOP; FERGUSON, 2013). Metagenomics have allowed the study of the biodiversity, abundance and phylogeny of labyrinthulomycetes and thraustochytrids in coastal marine environments, which allows the analysis of ecological interactions between different communities, prospecting for new species and uncultured organisms (BOCHDANSKY; CLOUSE; HERNDL, 2017; LIU et al., 2017; PAN; DEL CAMPO; KEELING, 2017; WANG et al., 2019; XIE et al., 2018). Likewise, lipidomics and transcriptomics have allowed to study metabolic pathways of fatty acids, enzymes involved as PUFA synthases and important genes for the overexpression of n-3 LC-PUFAs in thraustochytrids (DIAO et al., 2019; HEGGESET et al., 2019; JIANG et al., 2019; MORABITO et al., 2019; YANG et al., 2020; ZHANG et al., 2018b).

A SWOT analysis is shown in Figure 10. This analysis assists to recognize internal (strengths and weaknesses) and external (opportunities and threats) factors in a system. In this case, the production of omega-3 rich microbial oil from thraustochytrids will be used as a

model. After SWOT analysis, weaknesses and threats can be seen as opportunities to find new strategies, e.g., find energy alternatives to reduce costs, optimize the process to find better large-scale yields and study new solvent-free extraction processes. Likewise, balance the omega-3 microbial cost with its quality and functionality and evaluate alternative feedstocks as low-cost nutrients and substrates. In addition, provide different packaging alternatives for the final product to balance oil purification steps, such as softgel capsules, microencapsulation by spray dried, lyophilization and others.

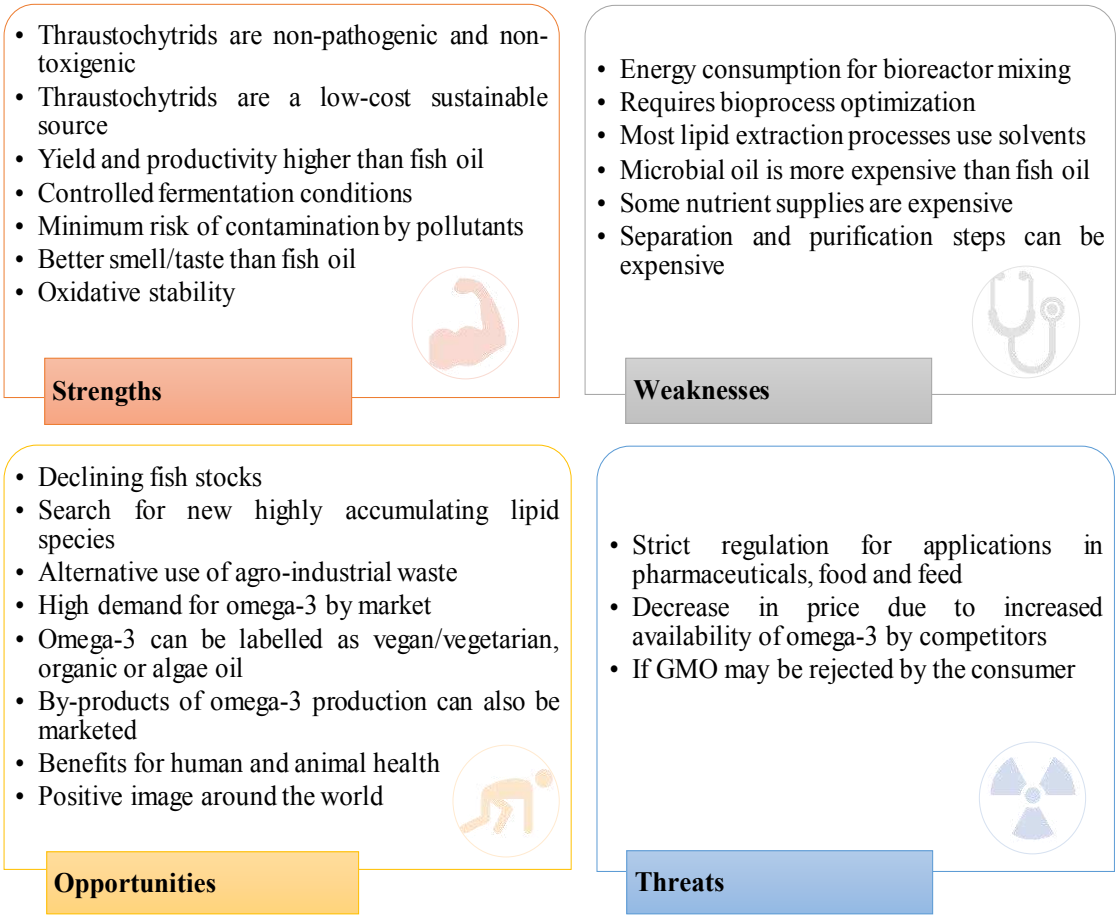

Figure 10. SWOT analysis of sustainability for omega-3 fatty acids from thraustochytrids related to micro-, macro- and socio-economic factors (JEGATHESE; FARID, 2014; POSSER et al., 2018; STEINRÜCKEN et al., 2017; VAN DER VOORT et al., 2017).

In addition, isolate novel wild and non-GMO strains producing n-3 LC-PUFAs and ensure the production of omega-3 within the regulation. These GMO strains refer to organisms that have been genetically improved by the introduction of specific genes to improve or highlight a desired characteristic (transgenesis). However, it is important to consider that each country has different regulations for GMOs. Some may not consider GMO strains those who

have undergone mutagenesis or protoplast fusion, even if these techniques lead to genetic modifications (ECKERSTORFER et al., 2019).

Furthermore, steps of lipid extraction and purification can be avoided when microbial biomass is used directly in animal feed, but it would need to be adapted or designed for this purpose. Likewise, it is important to consider that feed formulations in aquaculture generally work with oils and meals separately, often microencapsulated oils and spray dried meals to avoid possible obstructions in the extruders due to high levels of inclusion of lipid-rich biomass in the production of pellets (BAKRY et al., 2016; CAMACHO-RODRÍGUEZ et al., 2018; SHAH et al., 2018).

Finally, the use SWOT tool allows a global view of the sustainability of the process and how the negative aspects can be transformed into positive improvement options. In conclusion, the development of a sustainable, economical and efficient system is the biggest challenge of microbial omega-3 production, where many companies, universities and research centers are unleashing new patents and advances on these green technologies.

5. TECHNOLOGICAL PROSPECTION OF OMEGA-3 FROM THRAUSTOCHYTRIDS

5.1 ADVANCEMENT OF PATENTS IN THE WORLD

The published patent timeline has grown in recent years (Figure 11). The research performed in Patent Inspiration showed different but complementary results compared to the results found in Scopus database. Patent Inspiration showed 731 total records, where 64 of them belonged to publications of worldwide interest carried out by WIPO. This database allowed the visualization of patents published in English, Spanish and Portuguese. Only 296 results match with patents granted in the world. A granted patent grants rights to the inventor by protecting it from competitors and imitation for a period of time (ERNST, 2003). In contrast, the 1068 results showed by Scopus indicate that most research is found in articles, reviews and books; however, few are developed in patents that can generate products available on the market. The results over the years range from isolation processes, biochemical characterization, genetic improvement of strains, fermentative processes, optimization of cultures and bioreactors, methods of cell lysis, recovery and extraction of lipids. Some results have also shown how these innovations have been made available in the market such as biomass, flour, powder, softgel

capsules, bulk oil, food supplements, nutraceutical ingredient and enrichment of food formulations like infant milk and animal feed.

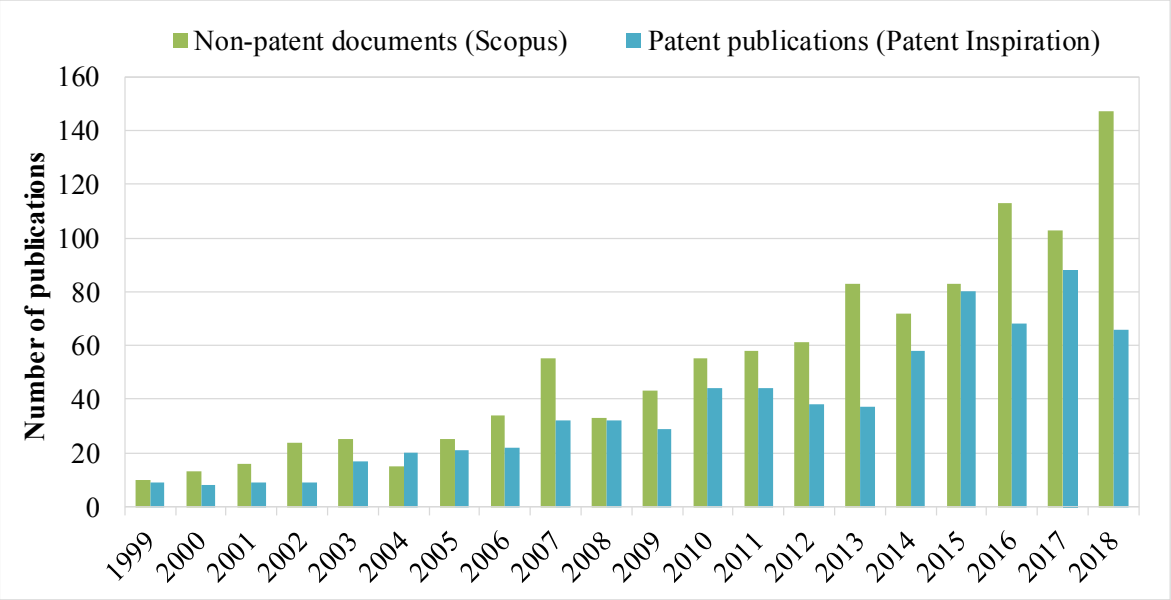

Figure 11. Timeline of patents published around the world on omega-3 rich microbial oils from marine thraustochytrids according to Patent Inspiration compared to non-patent documents found by Scopus database in the last 20 years (1999–2018).

The first company to publish patents in this subject was the American company PhycoTech Inc. in 1991, where it was published by the CIPO (Canadian Intellectual Property Office) followed by the international publication WO at WIPO. This first patent was entitled: Process for the heterotrophic production of products with high concentrations of omega-3 highly unsaturated fatty acids (BARCLAY, 1991). They have claimed the use of thraustochytriales on growing conditions that include: salinity level yielding conductivities up to 40 mmho/cm, a temperature near 15 ºC and the use of biomass as animal and human food. In this case antioxidants were added to the fermentation medium during harvesting of the biomass such as butylated hydroxytoluene (BHT) among others. They also included biomass extrusion to assess their bioavailability. Primarily the strains can be *Schizochytrium, Thraustochytrium* or a mixture of both. This patent included a total of 73 claims and was classified into 26 IPC codes. Thus, in 1992 OmegaTech Inc. entered into partnership with PhycoTech and the patent was published in the European patent office (EPO) and the United States Patent and Trademark Office (USPTO). OmegaTech Inc. started to publish this patent without PhycoTech from 1994. Finally, Martek Biosciences Corporation began to make

publications of this patent in the year 2003 after the acquisition of OmegaTech Inc. in 2002 (ISMAIL, 2002).

However, only after 10 years in 2001 that omega-3-rich lipids from thraustochytrids began to grow significantly (Figure 11). Subsequently, the peak of publications was in the year 2017, where 88 patents were published, stabilizing the growth of these technologies. In addition, the Scopus database showed 147 publications in 2018, which indicates that the topic is at the highest research peak as the trend shows. Nevertheless, the study of this omega-3-producing microbial family has generated many positive expectations by calling the attention of large companies.

5.2 CLASSIFICATION OF TECHNOLOGIES

The International Patent Classification (IPC) allowed visualizing the hierarchical classification of the content of patents in the world (Figure 12). Most of the codes were classified in section C (Chemistry; metallurgy) and A (Human necessities), which indicates a relation in the use of omega-3 fatty acids for human consumption, including those used in the enrichment of animal rations, which will be transformed into products with high added value in the diet. Therefore, the main sub-group code found in section C was C12P7/64, where 474 from 731 published patents had this classification. This code has the following description according to the IPC publication found in WIPO: Section C (Chemistry; metallurgy), class C12 (Biochemistry; beer; spirits; wine; vinegar; microbiology; enzymology; mutation or genetic engineering), sub-class C12P (Fermentation or enzyme-using processes to synthesize a desired chemical compound or composition or to separate optical isomers from a racemic mixture), group C12P7 (Preparation of oxygen-containing organic compounds) and sub-group C12P7/64 (Fats; fatty oils; ester-type waxes; higher fatty acids, i.e. having at least seven carbon atoms in an unbroken chain bound to a carboxyl group; oxidised oils or fats). Likewise, the main sub-group code found in section A was A23K10/16 with 102 counts from 731 published patents. The description of this code is described as follows: Section A (Human necessities), class A23 (Foods or foodstuffs; their treatment, not covered by other classes), sub-class A23K (Feeding-stuffs specially adapted for animals; methods specially adapted for production thereof), group A23K10 (Animal feeding-stuffs) and sub-group A23K10/16 (Addition of microorganisms or extracts thereof, e.g., single-cell proteins, to feeding-stuff compositions).

Clearly, the main code C12P7/64 has been maintained over the years. In addition, the codes C12R1/645 and C12N1/12 were also highlighted with 162 published patents each. The

C12R1/645 code is described as follows: C12R (Relating to microorganisms), C12R1 (Microorganisms) and C12R1/645 (Fungi). Likewise, the C12N1/12 code is described as follows: C12N (Microorganisms or enzymes; compositions thereof; propagating, preserving, or maintaining microorganisms; mutation or genetic engineering; culture media), C12N1 (Microorganisms, e.g., protozoa; Compositions thereof; Processes of propagating, maintaining or preserving microorganisms or compositions thereof; Processes of preparing or isolating a composition containing a microorganism; Culture media therefor), C12N1/12 (Unicellular algae; Culture media therefor). This latter code is accompanied by code C12N1/14, which also classifies the use of fungal microorganisms. Therefore, these three codes classify the microorganisms used to produce omega-3 oils as fungi or algae. This may be due to different explanations. The first is that there is still difficulty in taxonomic classification of thraustochytrids and many researchers and institutions use both denominations. The second is that some companies and research centers are using genetic engineering to introduce genes with highly omega-3-producing characteristics from thraustochytrids into fungi such as yeasts. Most yeasts do not produce n-3 LC-PUFAs, such as EPA, DPA and DHA, in large quantities. However, it has been the target of genetic improvement mainly due to the fast-growing ability of yeasts to increase productivity (gram per liter per hour).

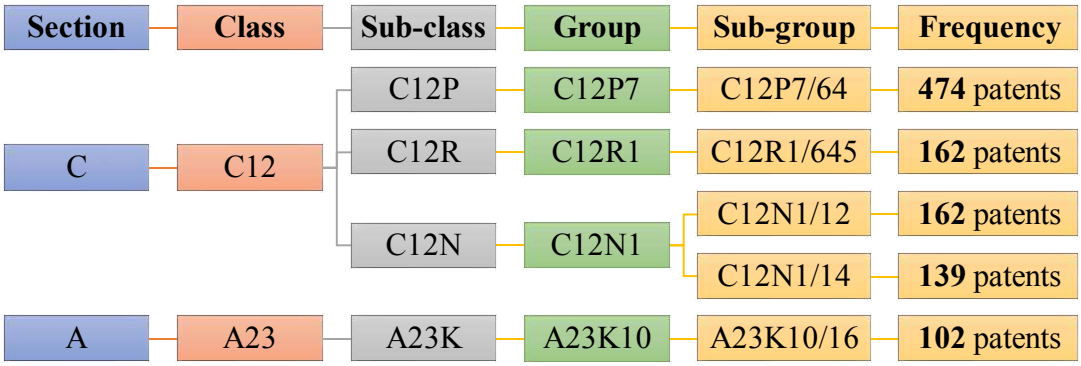

Figure 12. Top 5 codes in the International Patent Classification (IPC) related to omega-3 oils from marine thraustochytrids in the last 20 years (1999–2018).

Thereby, codes on human and animal nutrition have been strongly positioned; this is an indication of how these technologies are reaching the market quickly. Therefore, the production of omega-3 fatty acids from marine thraustochytrids in recent years reveals the tendency of industries in the use of this oil as an ingredient in the enrichment of animal feed. These topics are highlighted in Figure 13. The noun DHA stands out as the main fatty acid produced, where *Schizochytrium* appears as the largest producer. In addition, the noun

Aurantiochytrium became widely used after the taxonomic rearrangement of the *Schizochytrium* genus through some molecular techniques and phylogeny (YOKOYAMA; HONDA, 2007). Glucose has been the main carbon source of *Aurantiochytrium* in the synthesis of omega-3 rich oils. The noun microorganisms highlight some topics such as protists, biomass and lipids. Thus, many taxonomists classify thraustochytrids as marine protists and their ability to produce lipid-accumulating biomass (BENNETT et al., 2017). The noun gene shows prominence in advances in the improvement of PUFA-related molecules and proteins. Likewise, the nouns synthesis and biosynthesis show the relationship of fatty acid metabolism and the enzymes involved, as well as recent studies have tried to elucidate lipid metabolism in thraustochytrids as emerging models to understand the complex pathways in PUFAS synthesis (MORABITO et al., 2019; YANG et al., 2020).

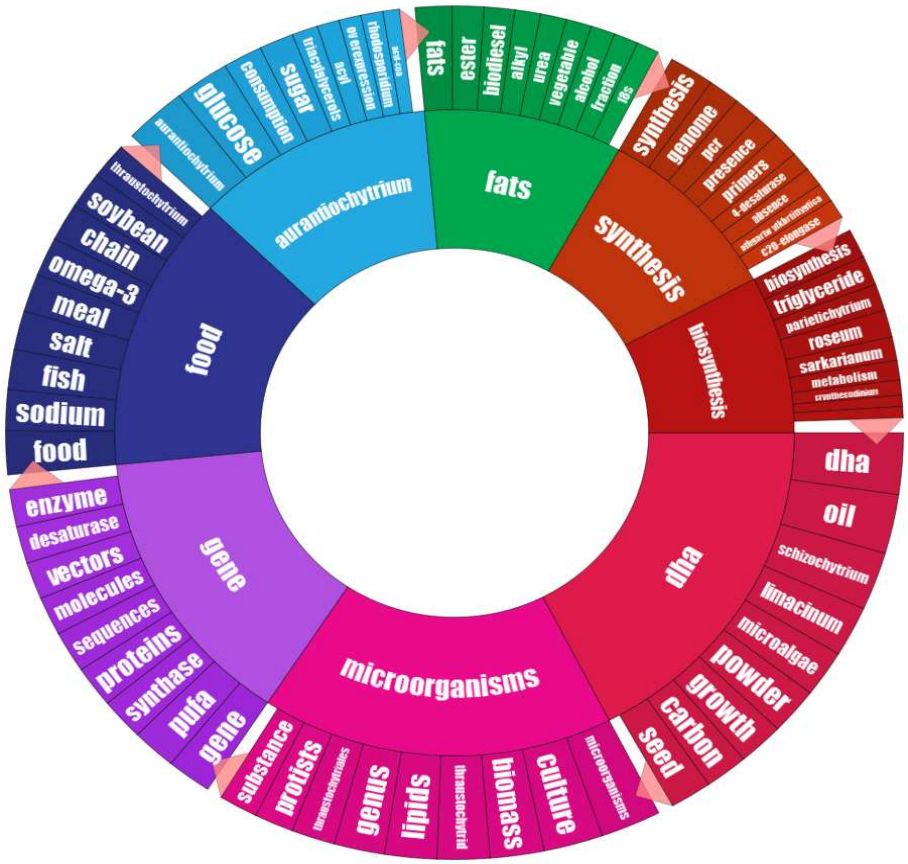

Figure 13. Nouns or highlighted topics in different fields of application related to omega-3 oils from marine thraustochytrids in the last 20 years (1999–2018).

Similarly, functional or nutraceutical food for human or animal consumption is highlighted in noun food, where patents and technologies are suggesting the use of thraustochytrids in aquafeed generating products derived from animal sources highly enriched

with omega-3 fatty acids that reach the end consumer (RAKITSKY; PIECHOCKI; LU, 2018; SCHURR; KUEHNLE, 2018). For example, tilapia is a poor source of n-3 LC-PUFAs compared to the carnivorous marine fish species (e.g., salmon, seabass and, seabream) which naturally provides high levels of EPA and DHA in the human diet. However, a recent study showed rapid growth and weight gain of tilapia, which were fed with *Schizochytrium* meal after 8 weeks, as well as the production of fillets highly enriched with n-3 LC-PUFAs (STONEHAM et al., 2018). Consequently, the main trend for the focus on microorganisms as novel sources of n-3 LC-PUFA in animal feeds has been the decline in the levels of EPA and DHA in farmed fish, as the limited amounts of fish oil are stretched within aquaculture and are supplemented by plant oils that contain no EPA and DHA. Then, several studies have compared the use of *Schizochytrium* sp. oil or meals to fish oil in fish feed (SPRAGUE; BETANCOR; TOCHER, 2017; SPRAGUE; DICK; TOCHER, 2016).

5.3 ORIGIN OF TECHNOLOGIES AND DEVELOPERS

The origin of technology from research and patent researching countries can be seen in Figure 14. The United States (164 patents), China (92 patents), Netherlands (85 patents) and Japan (85 patents) lead the ranking of countries with more innovation in omega-3 fatty acids from thraustochytrids in the last 20 years. These patents obtained from the Patent Inspiration database are related to the following technologies: Genetic engineering, mutation, formulation for animal feed, process optimization in bioreactor, recombinant thraustochytrids growing on xylose, omega-3 PUFA synthase from thraustochytrids in oilseed crops, pharmaceutical formulations, isolation of thraustochytrids omega-3 producers, formulation for aquaculture, formulation for pet food, submerged fermentation using feedstock as cellulose. Likewise, France and Germany occupy an important rank in the development of food solutions with 68 and 53 published patents, respectively. The patent analysis also revealed that in the last 20 years no patents of Latin American and African origin have been published, which highlights United States and Eurasia as market leaders for n-3 LC-PUFAs from thraustochytrids. Clearly, very innovative countries will have a shorter technology cycle, which leads patents to be transformed into products within a company faster than underdeveloped countries.

Moreover, Figure 15 shows the patent office's chosen by the technology-creating countries from Figure 14. These results do not include the 64 patents published by WIPO (World Intellectual Property Organization) with the suffix WO. These countries had as commercial objective to deposit their patents in the offices mainly in China with 222 patents,

the United States with 119 patents and Japan with 71 patents. In addition, the European Patent Office (EPO) has been of commercial interest with 49 patents filed.

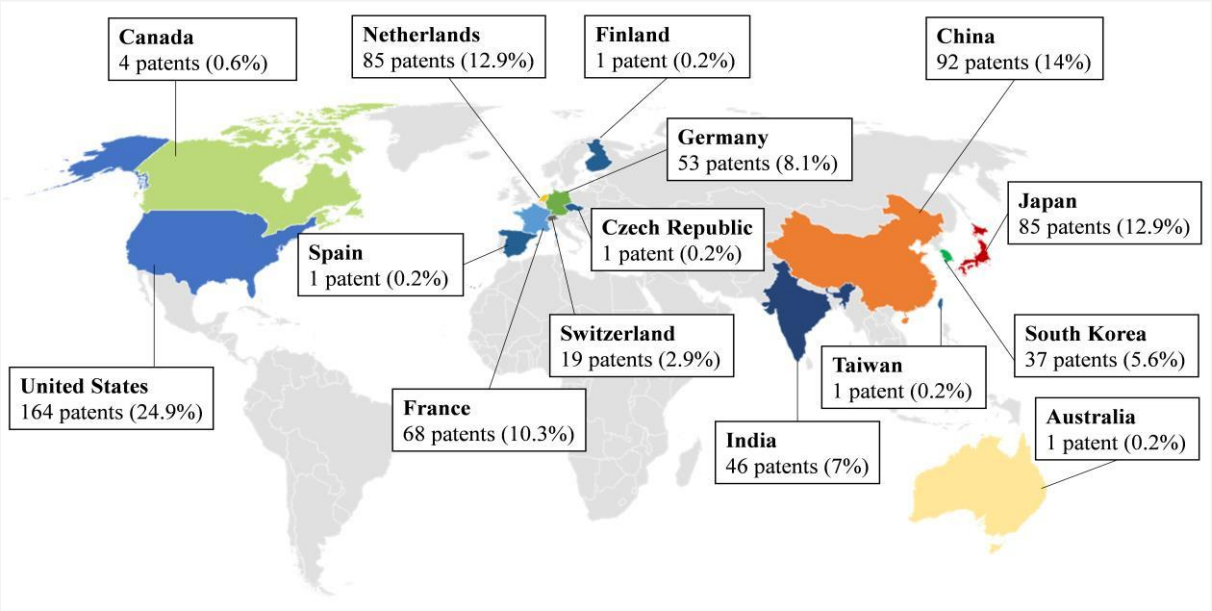

Figure 14. Origin of technologies or applicant countries for omega-3 oils from marine thraustochytrids in the last 20 years (1999-2018).

In Latin America, Brazil has been ranked 1st and 8th in the world among the most searched offices with 14 patents according to Patent Inspiration records. The Brazilian population is increasingly worried about good nutrition in recent years, which makes it possible to be seen by other countries as an important market target. However, Patent Inspiration showed no results where Brazilian companies, universities or research institutes have published patents related to omega-3 fatty acids from marine thraustochytrids between 1999 and 2018. Therefore, most patents published in Brazil belong to foreign companies. These patents are related to the upstream processes (breeding of microorganisms and culture media), midstream (fermentation and process optimization) and downstream (recovery, separation and purification of lipids). The first patent published in the Brazilian patent office was published in 2000 under the number BR9710394A by Japanese companies (Suntory and Nagase). This patent may also be found by the number WO/1998/003671. This patent reports the process of docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) from lipid-producing microorganisms belonging to the genus *Ulkenia* from the thraustochytrids family. The process includes the steps of culturing the microorganism in a liquid medium to produce DHA and DPA as well as the downstream steps for recovery and separation of lipids (TANAKA et al., 2000). On the other hand, the last patent

published in the year 2018 under the number BR112018010884A2 (WO/2017/103421) was deposited by the French company Metabolium. The patent relates to a method of enriching protists in culture medium to produce polyunsaturated fatty acids (PUFAs), which may include, thraustochytrids and labyrinthulids within the genus *Schizochytrium*, *Ulkenia*, *Aurantiochytrium* or *Thraustochytrium* as well as the dinoflagellate microalgae *Crypthecodinium cohnii* (ROMARI, 2018).

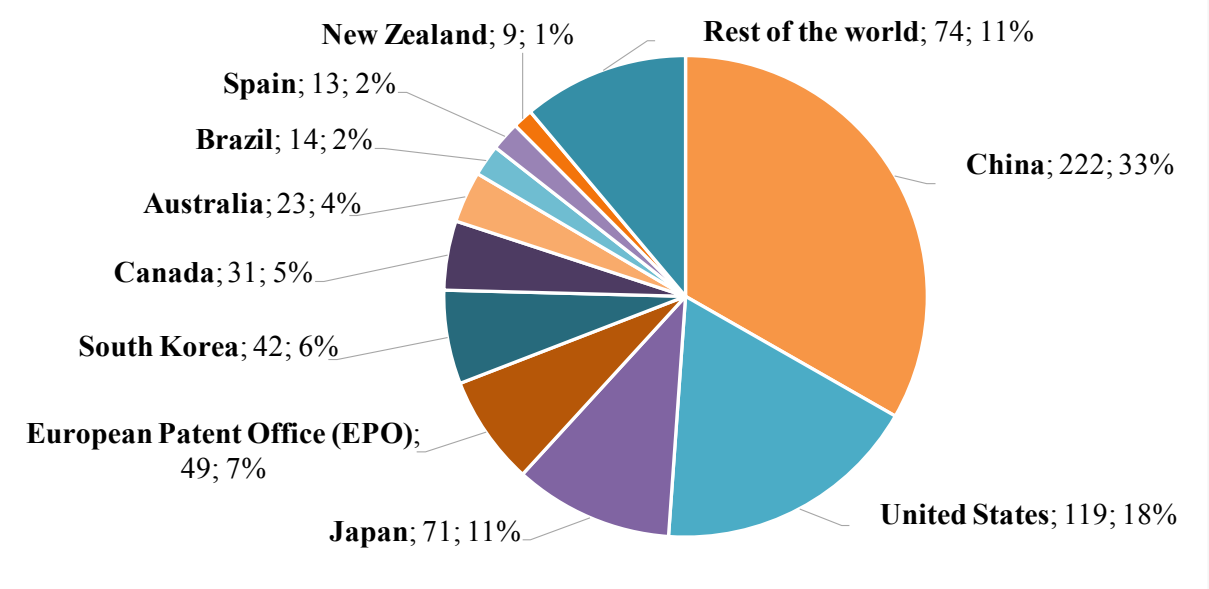

Figure 15. Top 10 commercial interest patent office's for omega-3 oils from marine thraustochytrids in the last 20 years (1999-2018).

Furthermore, few microbial genera from thraustochytrid family have been extensively studied, developed and patented for the commercialization of products, which becomes an innovation. Currently, the largest commercial production of microbial docosahexaenoic acid (DHA) is performed by Schizochytrium species from thraustochytrids family as well as the dinoflagellate microalgae Crypthecodinium cohnii, which have been claimed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) (CHEN; YANG, 2018b; FEDOROVA-DAHMS et al., 2011; NAZIR et al., 2018; WANG et al., 2018a)

Patents related to upstream, midstream, downstream and *in vivo* animal and human testing of omega-3 fatty acids from thraustochytrids have been carried out by different companies over the years (Figure 16). Thus, technology development was led by two companies: Martek Biosciences Corporation and DSM IP Assets BV with 103 and 83 published patents, respectively, in the last 20 years. In 2011, the Dutch company DSM IP Assets BV completed the acquisition of Martek Biosciences Corporation (DSM, 2011) leading to

technology transfer with a total of 186 patents, which corresponds to 25.4% of published patents worldwide. Some omega-3 products from microalgae mainly *Schizochytrium* sp. marketed by DSM with location in United States, including DHAgold™ for animal feed, and Life'sDHA® and Life's™OMEGA for human consumption. Recently, DSM and Evonik established a new company in 2018, Veramaris joint venture, for the production of EPA and DHA from *Schizochytrium* sp. The Veramaris algal oil is produced by fermentation with dextrose obtained from corn, which exceeds a 50% concentration of EPA and DHA. Veramaris is primarily aimed at the animal nutrition industry, specifically aquaculture, and aims to meet 15% of the global omega-3 fatty acid demand for salmon farming. They also aim to contribute to the Global Goals for Sustainable Development, mainly: Goal 2 (Zero hunger), Goal 3 (Good health and well-being), Goal 12 (Responsible consumption and production), Goal 14 (Life below water) and Goal 17 (Partnerships for the goals) (EVONIK, 2018; VERAMARIS, 2020).

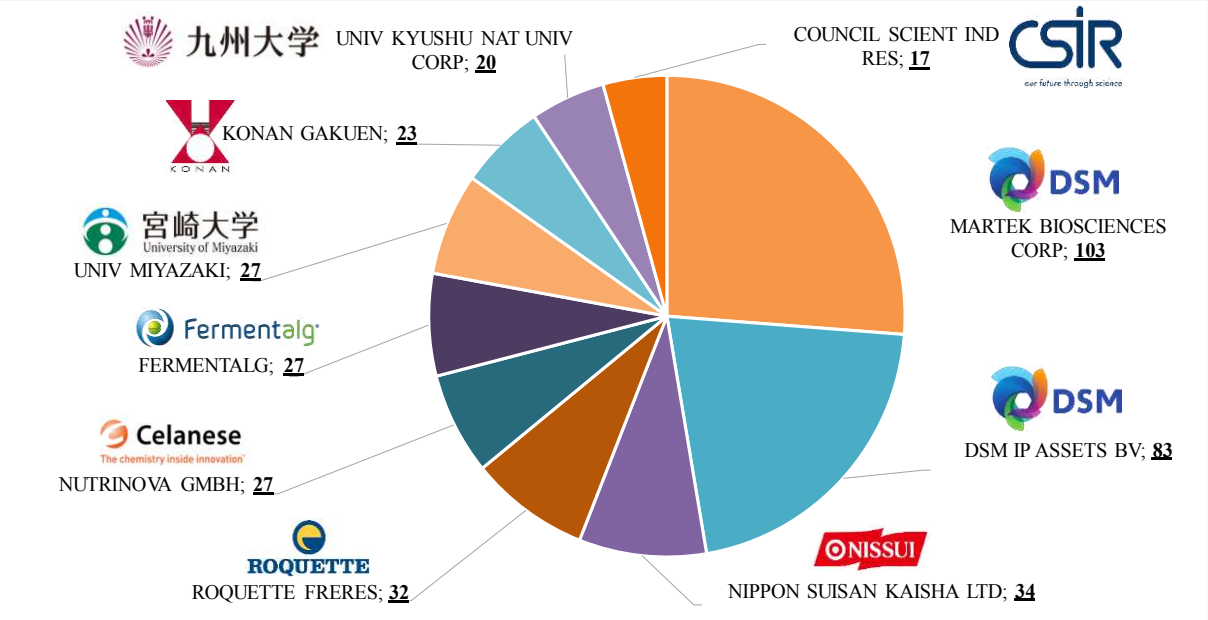

Figure 16. Top 10 companies or assignee names for omega-3 oils from marine thraustochytrids in the last 20 years (1999-2018).

Recently, institutes and universities have become interested in the research and development of this area. However, they still represent a lower percentage compared to the innovation generated by the industries. The first publications conducted by universities was in the year 2007 by the University of Miyazaki and Kyushu University, both of Japanese origin. Moreover, the Japanese company Nippon Suisan Kaisha Ltd. ranks third with 34 published patents worldwide, also it is important to highlight many of these innovations have been

developed in partnership with universities such as the University of Miyazaki with 27 patents, Konan Gakuen University with 23 patents and National University Corporation Kyushu University with 20 patents. Nippon has focused its production on dietary and pharmaceutical supplements in the form of capsules, oil, drinks and powder for human consumption. The Japanese have shown a strong link between academia and industry, the example to follow for many developing countries.

Other important companies in the production of omega-3-rich products are French companies Roquette Frères (32 patents) and Fermentalg (27 patents). Fermentalg is focused on the generation of microalgae-based products, such as DHA ORIGINS 350® and DHA ORIGINS 550®. Likewise, the German-based company Nutrinova is an affiliate of Celanense with 27 published patents in the last 20 years (1999-2018) according to Patent Inspiration database, which has products, such as DHAid™ (DHActive) produced by *Ulkenia* sp. with 35-40% DHA as main PUFA (ROBERTSON et al., 2013). Finally, these companies have focused on improving processes to achieve DHA values greater than 20% within the microalgae accumulated PUFAs, some even exceeding 50% DHA being highly competitive in the nutraceutical food market.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Studies on the safety of new lipid-accumulating species for human and animal consumption are needed. Omega-3-rich microbial oils have been a viable and sustainable alternative to fish oil. In addition, these microbial oils appear as an alternative to vegan diets or people allergic to the proteins of fish and crustaceans. There are many scientific research and papers conducted by universities and institutes on the importance of thraustochytrids in the production of omega-3 and its applications. However, few universities in the world have been able to convert these researches into patents that can generate innovation. Asian universities have been the only ones to patent this type of technology in the world. The Scopus base is an example of the amount of scientific research that has been generated in the last years.

Meanwhile, the technological prospection of patents allowed to study the world situation related to omega-3 fatty acids derived from thraustochytrids. Patent publications have shown a significant increase in recent years between 2014 and 2018 in the world. These patents deal with the isolation of new species, genetic engineering, optimization of processes, reduction of recovery and purification stages, *in vitro* and *in vivo* tests on its health benefits. Thus, the United States and Eurasia lead the world patent market and technology generation. In contrast,

most patents published in the Latin America were made by foreign companies, which means an opportunity for companies and universities of Latin American origin to develop technologies.

Nowadays, despite taxonomic claims about the classification of thraustochytrids, many companies, brands, patents, universities and research articles still use the name algae or microalgae. They are betting on improving production technologies and discovering new application gaps in the market. In addition, the development of projects that seek to prospect new microorganisms employing molecular biology techniques such as new generation sequencing, metagenomics studies or probes for detection has encouraged researchers. Each year new species has been discovered, the pathway to find the best is still long. Moreover, the production of omega-3 fatty acids of microbial origin in bioreactor allows the control of processes in a closed and agitated system reducing possible contaminants as well as the use of agroindustrial residues that can reduce the costs of production are sustainable and renewable solutions.

CHAPTER 3

INTEGRATING METAGENETICS AND HIGH-THROUGHPUT SCREENING FOR BIOPROSPECTING MARINE THRAUSTOCHYTRIDS PRODUCERS OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS

This chapter was published in the journal *Bioresource Technology* (2021). Impact factor: 7.539 (JCR 2019) and doi: 10.1016/j.biortech.2021.125176 (Attachment 2).

ABSTRACT

Omega-3 produced by marine thraustochytrids has appeared as an alternative to fish oil and an eco-friendly solution to overfishing. Herein, an integrative analysis of metagenetics and high-throughput screening was used for bioprospecting marine thraustochytrids from southern Brazil mangrove and coastal seawater. All sampled environments showed biodiversity and abundance of SAR clade. Environmental samples detected with potential lipid-accumulating labyrinthulomycetes were further processed for direct plating and pollen baiting isolation. Microtiter plate system and fluorescence spectroscopy were combined for high-throughput screening of 319 isolates to accumulate lipids. Twenty isolates were selected for submerged cultivation and lipid characterization. Among them, B36 isolate, identified as *Aurantiochytrium* sp. by 18s rRNA sequencing, achieved the highest biomass (25.60 g/l CDW) and lipids (17.12 g/l CDW). This lipid content had a high biological value with 44.37% LC-PUFAs and 34.6% DHA, which can be used as a sustainable source in vegan, seafood-free and animal feed diets.

Keywords: Thraustochytrid; Metagenetics; Bioprospecting; LC-PUFAs; Omega-3.

1. INTRODUCTION

Long-chain polyunsaturated fatty acids (LC-PUFA) has gained great prominence due their nutritional and functional properties. LC-PUFA comprise omega-3 and omega-6 families with more than 18 carbons in their structure. They cannot be produced by humans and should be supplemented through the diet (ABEDI; SAHARI, 2014). The omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), eicosapentaenoic (EPA, 20:5n-3) and

docosahexaenoic (DHA, 22:6n-3) acids, are essential components of a balanced diet, preventing hypertension, cardiovascular diseases, inflammation and cancer (ZHANG et al., 2019). Omega-3 intake can be supplied by the consumption of seafood and fish oils; however, some problems including overfishing, sea pollution and low fish stocks have made it difficult to meet the demands for omega-3 oils. Thus, thraustochytrids has appeared as a sustainable microbial source (OROZCO COLONIA; VINÍCIUS DE MELO PEREIRA; SOCCOL, 2020). Thraustochytrids are eukaryotic stramenopiles found in marine ecosystem. They play an important role as left-over scavengers, using extracellular enzymes to feed on the substrates present in the decomposing sediments (DAMARE, 2019). Additionally, thraustochytrids serve as zooplankton food and can accumulate around 50% of lipids, where DHA constitutes about 25% of these lipids. These heterotrophic microalgae can be grown in bioreactors and also secrete EPS (Extracellular polymeric substances) and SMP (Soluble microbial products) depending on the C/N ratio present in their growth environment (SEPEHRI; SARRAFZADEH, 2018).

Pollen baiting is a recently developed technique for isolation of thraustochytrids which consists of the adherence of thraustochytrids to pine pollen grains due to its ability to metabolize lignocellulosic substrates (GUPTA et al., 2013b). However, there is still difficulty in cultivating them and it may take a long time to find these species in mangroves. The mangrove ecosystem has differences in the biodiversity of its communities and some may not have thraustochytrids inhabiting them or their frequency of occurrence may be low (JARITKHUAN; SUANJIT, 2018b). Thus, next-generation sequencing (NGS) technologies using total DNA from environmental samples have emerged as powerful tool for biotechnological bioprospecting (DHANJAL; SHARMA, 2018). Metagenetics allows to perform a previous screening of a sample, reducing cost and time in isolation of desired microorganisms. Consequently, this approach can be aligned with high-throughput screening (HTS), which uses the Duetz-microtiter plate system in micro-scale cultivation to select and study microbes with biotechnological potential quickly and efficiently (DUETZ, 2007). Thus, HTS appears as a miniaturized alternative to Erlenmeyer flasks, which reduces time and cost and can be applied in microbial screening, drug discovery and in the study of several biological compounds.

This study aimed at the integration of eukaryotic metagenetics, isolation techniques and high-throughput screening to recover thraustochytrids producers of long-chain polyunsaturated fatty acids (LC-PUFAs) from Brazilian mangrove and coastal seawater. The polyphasic selection adopted was used as an efficient bioprospecting tool from a vast number

of microorganisms living in marine environments. A flow diagram of this study is illustrated in Figure 17.

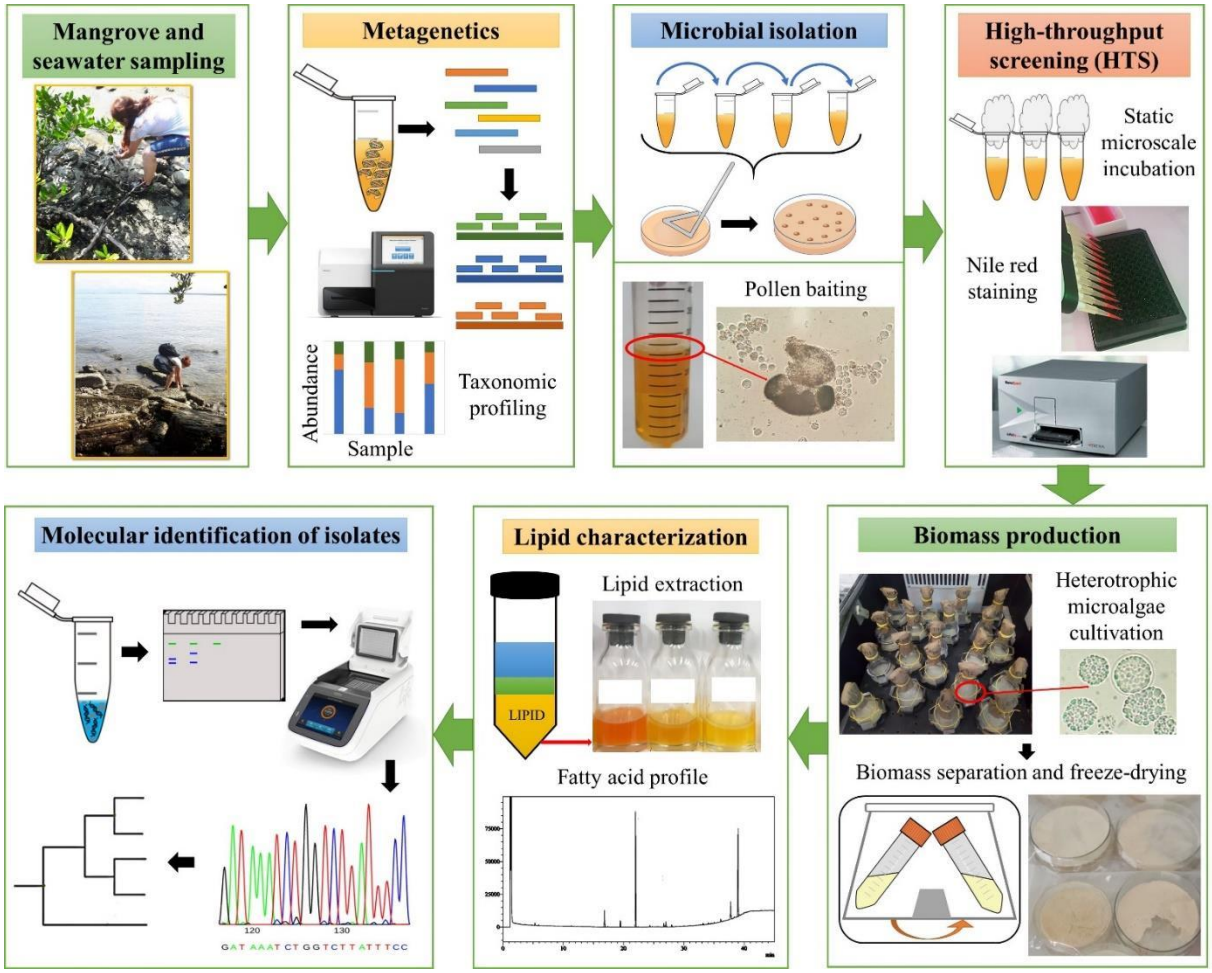

Figure 17. Flow diagram integrating metagenetics and high-throughput screening of thraustochytrids for biomass and lipid production.

2. MATERIALS AND METHODS

2.1 ENVIRONMENTAL SAMPLING

Seven mangrove (M) sediments samples (sludge, decomposing material, stems and leaves) and four coastal seawater (SW) samples were collected in sterile vials from different locations on the Brazilian south coast (Figure 18). Samples from Ilha do Mel (M1, M2 and SW1), Ilha dos Valadares (M3, M4 and SW2) and Antonina (M5, M6 and SW3) were taken in Paranaguá Bay, and samples from Florianópolis (M7 and SW4) were collected at Parque do Manguezal do Itacorubi. Then, the samples were placed in a cooler box and transported to CENBAPAR (Centro de Biotecnologia Agroindustrial e Agroalimentar do Paraná).

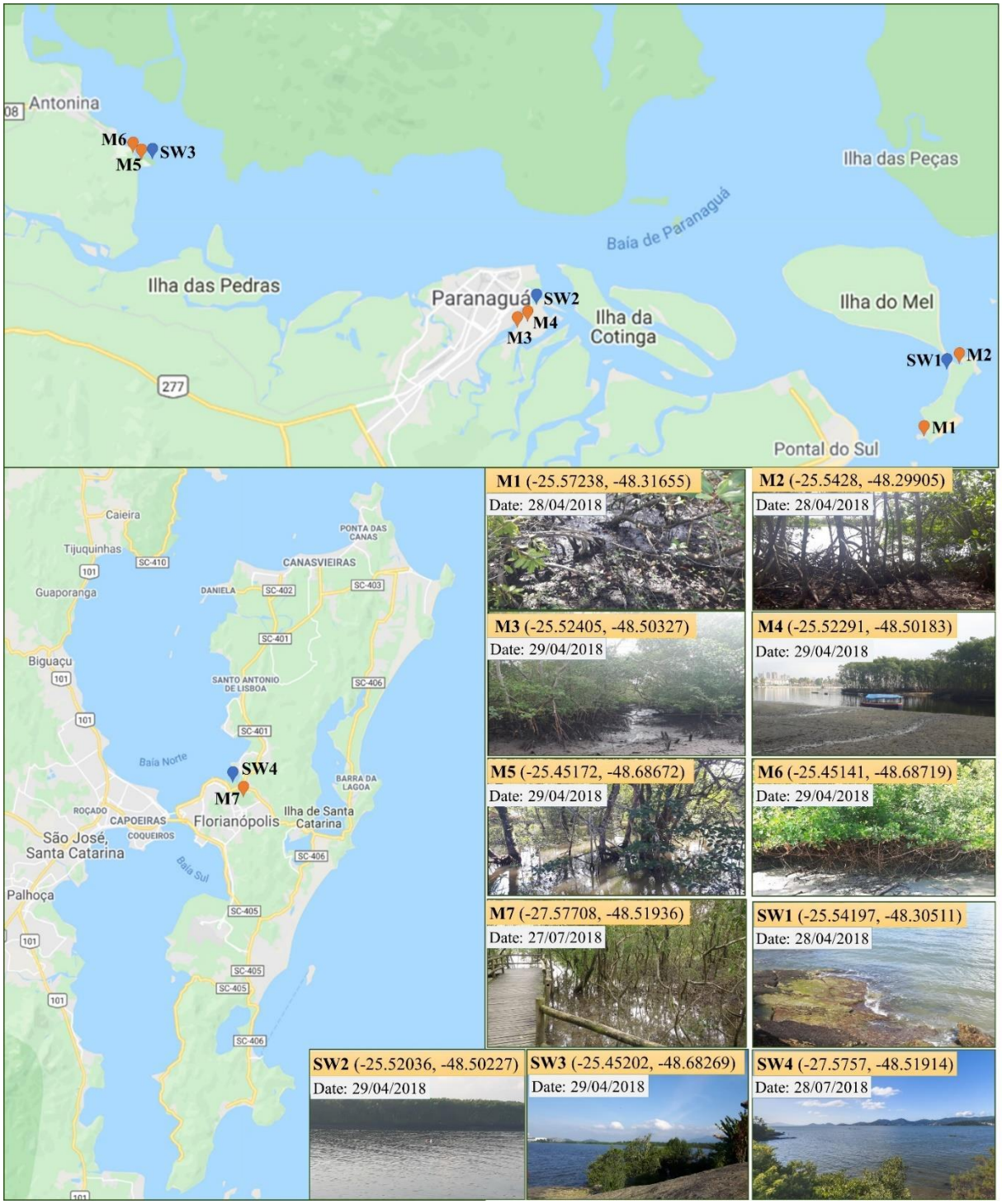

Figure 18. Location and coordinates of mangrove and coastal seawater samples from southern Brazil.

2.2 EUKARYOTIC METAGENETICS

Total DNA extraction and purification from environmental samples were performed with Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research Catalog No. D6010).

The extracted DNA was evaluated by spectrophotometry using NanoDrop™ 2000 (ThermoFisher Scientific). Then, PCR was performed for amplification of the V9 region for the 18S rRNA gene. A DNA concentration of 20 ng/µl was utilized as template. The universal primers for eukaryotes used were 1389F and EukBR (AMARAL-ZETTLER et al., 2009). PCR amplification conditions were as follows: denaturation step (98 °C for 30 sec, followed by 10 cycles at 98 °C for 10 sec), primer annealing and extension (57 °C for 30 sec, 72 °C for 30 sec, 25 cycles at 98 °C for 10 sec, 48 °C for 30 sec, 72 °C for 30 sec) and a final extension (72 °C for 10 min). PCR products were quantified using the Qubit™ dsDNA HS Assay Kit in a Qubit® Fluorometer (ThermoFisher Scientific) and sequenced using the 300V2 Sequencing Kit (Illumina) on an Illumina MiSeq (Illumina). The sequences were analyzed using the QIIME 1.9 (Quantitative Insights Into Microbial Ecology), considering 97% similarity with the 18S SILVA database (QUAST et al., 2013). The metagenetic sequences data used in this study were deposited at GenBank in the NCBI as BioProject acession PRJNA702524 under BioSamples accessions SAMN17970437-SAMN17970447.

2.3 ISOLATION TECHNIQUES OF THRAUSTOCHYTRIDS

Environmental samples with labyrinthulomycetes class detected by metagenetics were processed for strains isolation techniques (GUPTA et al., 2013b). Mangrove sediments (5 g) or coastal seawater (5 ml) samples were added to flasks with 45 ml GPYS broth (10 g/l glucose, 1g/l peptone, 1 g/l yeast extract and 28 g/l sea salts) supplemented with antibiotics solution (500 mg/l penicillin, 500 mg/l streptomycin, 50 mg/l rifampicin, 10 mg/l nystatin). The flasks were incubated 25 °C and 120 rpm for 4 days. Serial dilutions (10^{-3} to 10^{-5}) from flasks were performed in 0.1% peptone salt solution (8.5 g/l NaCl, 1 g/l peptone, pH 7.0) and spread in GYPS agar plates. In addition, decaying leaves were washed with seawater and antibiotic solution, and placed in GYPS agar plates. The plates were incubated at 25 °C for 4 days. Meanwhile, sterile pine pollen grains were added as bait to 50 ml falcon tubes containing 20 ml YPS broth (1 g/l yeast extract and 1 g/l peptone in seawater) with antibiotic solution and incubated at 25 °C for 2 weeks. The presence of thraustochytrids was verified in an optical microscope by the adhesion of the cells to the pollen grains every 24 hours. 20 µl samples containing pollen bait and thraustochytrids were spread on GYPS agar plates containing antibiotics and incubated at 25 °C for 5 days. Colonies proliferated on leaves, pollen bait and plates were isolated and purified. Microscopic analysis were performed according to previously

reported thraustochytrids taxonomy studies (FOSSIER MARCHAN et al., 2018; NAKAI; NAGANUMA, 2015b). The isolates were preserved in 20% glycerol at -20 °C.

2.4 MICROSCALE CULTIVATION

Five microscale cultivations (MC) were performed to select the screening strategy for oleaginous isolates and compared to shaker cultivation (SC) in flasks as control. The strategies are described as follows: (MC1) Static microplate sealed with parafilm and 0.25 ml GYPS broth, (MC2) Static microtube with cotton cap and 1 ml GYPS broth, (MC3) Static microtube with cotton cap and 0.5 ml GYPS broth, (MC4) Stirred microtube with snap cap, 120 rpm and 1 ml GYPS broth, (MC5) Stirred microtube with snap cap, 120 rpm and 0.5 ml GYPS broth and (SC) Stirred Erlenmeyer flask with gauze/cotton cap, 120 rpm and 20 ml GYPS broth. All strategies were grown at 28 °C for 5 days. Five isolates were tested and *Schizochytrium limacinum* SR21 (*Aurantiochytrium limacinum* ATCC® MYA-1381™) was used as standard strain.

2.5 MICROTITER-BASED FLUORESCENCE ASSAY

The fluorescence-based assay was performed according to the protocol adapted for Nile red staining (SITEPU et al., 2012). 150 µl of fermented broth were added in a 96-well black microplate followed by the addition of 20 µl dimethyl sulfoxide (DMSO 40% v/v) and 30 µl Nile red (0.05 mg/l in acetone). Sample and reagent blanks were also analyzed. The microplates were incubated at 40 °C, shaking at 120 rpm, excitation wavelength at 530/25 nm and emission wavelength at 590/35 nm for 10 minutes in a microplate spectrofluorometer reader (Infinite® M200 Pro, Tecan). Likewise, 150 µl of fermented broth were placed in a clear 96-well microplate to determine the optical cell density in a microplate spectrophotometer reader (Synergy™ HTX, BioTek Instruments) at 600nm. The relative fluorescence unit (RFU) was calculated according to Eq. (1).

$$\text{Relative Fluorescence Unit (RFU)} = \frac{\text{Fluorescence intensity}}{\text{Optical density (OD)}} \quad (1)$$

2.6 SUBMERGED CULTIVATION

The isolates were streaked in GYPS agar plates and incubated for 24 hours at 28 °C. The colonies of thraustochytrids were inoculated in a 50 ml flask containing 10 ml GYPS broth.

The inoculum flasks were grown at 120 rpm and 28 °C for 24 hours. Then, 250 ml Erlenmeyer flasks containing 50 ml of sterile fermentation medium (80 g/l glucose, 5 g/l yeast extract, 2 g/l peptone, 5 g/l monosodium glutamate, 28 g/l sea salt and pH 6.0) were inoculated with 10% v/v inoculum of each isolate. The flasks were incubated in a shaker at 120 rpm and 28 °C for 5 days.

2.7 BIOMASS AND LIPID DETERMINATION

The broths were centrifuged at 5000 rpm for 8 minutes at 4 °C to separate the cell biomass. Cell biomasses were frozen in an ultra-freezer at -80 °C and lyophilized for 48 hours. Simultaneously, biomass samples were dried in an oven at 105 °C to constant weight to determine cell dry weight. Then, total lipids were determined gravimetrically (BLIGH; DYER, 1959). 0.2 g of freeze-dried biomass and 0.8 ml of distilled water were added to a 15 ml centrifuge tube and homogenized in the vortex for 10 seconds. 1 ml of chloroform and 2 ml of methanol were added and vortexed for 2 minutes. 1 ml of chloroform was added to the mixture and vortexed for 30 seconds. Then, 1 ml of distilled water was added and vortexed for another 30 seconds. The tubes were centrifuged at 5000 rpm for 6 minutes and 1.4 ml aliquot from lower phase was transferred to a previously weighed flask. The chloroform was evaporated at 45 °C for 3 hours in a vacuum oven. The flasks were cooled for 15 minutes in a glass desiccator and weighed again to determine the lipids according to Eq. (2).

$$\text{Total lipids (\%)} = \frac{\text{Weight of lipid in aliquot (g)} * \frac{\text{Volume of chloroform layer (ml)}}{\text{Volume of aliquot (ml)}}}{\text{Weight of dry biomass sample (g)}} * 100 \quad (2)$$

2.8 DIRECT TRANSESTERIFICATION

Fatty acid methyl esters (FAMEs) were obtained by direct transesterification of lyophilized cell biomass and the protocol was adapted (CHI et al., 2007; INDARTI et al., 2005). 25-40 mg of freeze-dried biomass were weighed into 16 ml glass tubes with PTFE/Teflon cap, where 2.0 ml of 15% H_2SO_4 in methanol were added and vortexed for 30 seconds. Then, 2.0 ml of chloroform was added and vortexed for 10 seconds. The tubes were heated in a water bath at 90 °C for 40 minutes and manually shaken with tweezers every 5 minutes. The tubes were cooled to room temperature. Then, the reaction volumes were transferred to 15 ml centrifuge tubes and 1 ml of milli-Q water was added. The centrifuge tubes were vortexed for

45 seconds followed by centrifugation at 5000 rpm for 5 minutes. The lower phases were dried in clean tubes containing anhydrous sodium sulfate, vortexed for 10 seconds and left to stand for 15 minutes. Then, the organic phases were filtered through cotton to remove possible impurities. FAMEs were stored in sealed chromatography vials at -20 °C until GC analysis.

2.9 GAS CHROMATOGRAPHY ANALYSIS

The fatty acid profiles were analyzed by gas chromatograph (GC-2010 Plus Capillary GC, Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector (FID), an auto injector (AOC-20i) and an SH-RTX™-Wax capillary column (Shimadzu, 30m, 0.32mm ID, 0.25µm). The injection volume was 1 µl and split ratio 1:10. The carrier gas (helium) was controlled at 32.5 cm/sec linear velocity. The injector and FID detector temperature were maintained at 240 °C and 250 °C, respectively. The oven column temperature was set to start at 100 °C for 5 minutes, then raised to 240 °C at a rate of 4 °C/min and held for 5 minutes. The FAMEs were identified and calculated by the retention times and peak areas compared to the Supelco® 37 Component FAME Mix standard (Sigma-Aldrich, Merck). Chromatograms data were analyzed using the GCsolution Workstation Software Version 2.32 (Shimadzu, LabSolutions).

2.10 MOLECULAR IDENTIFICATION OF ISOLATES

The isolates were grown on GYPS agar to form colonies at 25 °C for 48 hours for DNA extraction (PANG et al., 2015). Cell biomass was transferred to 400 ml of extraction buffer (1% CTAB, 1 M NaCl, 100 mM Tris-HCl, 20 mM EDTA) and incubated at 70 °C for 15 min. A volume of 250 µl of phenol, chloroform and isoamyl alcohol (25:24:1) was added, homogenized and centrifuged at 12000 rpm for 10 min. Then, 250 µl of the mixture chloroform and isoamyl alcohol (24:1) was added to the supernatant, homogenized and centrifuged at 12000 rpm for 10 min twice. DNA was precipitated with three volumes of absolute ethanol, incubated for 10 min at room temperature and centrifuged at 12000 rpm for 20 min. The DNA pellet was washed with 1 ml of 80% ethanol, centrifuged at 12000 rpm for 10 min, dried at room temperature and resuspended in 50 µl of sterile ultra-pure water. The extracted DNA was verified by electrophoresis on 0.8% agarose gel and measured on NanoDrop™ 2000 (ThermoFisher Scientific).

Two primer combinations (FA1-RA2 and FA2-RA3) were used for PCR amplification of the 18S rRNA gene (SHENE et al., 2020b). The reaction volume was 25 µl described as

follows: 2 µl DNA (~50 ng/µl), 2.5 µl of 10× PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.75 µl of MgCl2 (50 mM), 0.62 µl of dNTP Mix (10 mM), and 0.5 µl of FA1, FA2, RA2 and RA3 primers (10 mM). Two PCR cycling program for FA1-RA2 and FA2-RA3 were performed. The PCR products were purified and sequenced using an ABI3730 XL automatic DNA sequencer (Applied Biosystems, Paisley, UK). The sequences obtained were analyzed in BioEdit 7.7 software and compared with sequences deposited in GenBank database by BLASTN algorithm. Then, a neighbor-joining phylogenetic tree was constructed using the MEGA X version 10.1 program (KUMAR et al., 2018). The 18S rRNA sequences were deposited under accession numbers MW629370-MW629378.

3. RESULTS AND DISCUSSION

3.1 ALPHA AND BETA DIVERSITY OF ENVIRONMENTAL SAMPLES

Alpha diversity allowed to study the richness and evenness of communities from sequencing data between different environments as shown in Figure 19, where the number of observed operational taxonomic units (OTUs) increases proportionally with the number of sequences per sample. The trends of rarefaction curves grow rapidly as more common species are detected; however, the curves are not stabilized or reach a plateau. The curve stabilization would show the rarest species found in the samples. M2 (Ilha do Mel), M6 (Antonina) and M3 (Ilha dos Valadares) mangrove samples showed a higher number of OTUs at the end of the curve with 91, 85.6 and 84.8 OTUs, respectively. Thereafter, SW2 (Ilha dos Valadares) sample was highlighted among the seawater samples with 75.4 OTUs. These samples had greater coverage in the detection of species by metagenetics. In contrast, M7 (Florianópolis) sample had a lower number of observed OTUs, which indicates a lower coverage compared to other samples.

All coastal seawater samples had a similar pH between 7.0 and 7.5, while the mangrove sediment samples had a slightly acidified pH between 5.9 and 7.0. Salinity was higher for samples collected far from the urban region (M1, M2, M7, SW1 and SW4; salinity between 25 and 30 PPT), while the lowest were found in the samples collected closer to the coast (M3, M4, M5, M6, SW2 and SW3; salinity between 10 and 17 PPT). Likewise, a study of the effects of pH and salinity gradients on the Brazilian mangrove microbiome in Paranaguá Bay had pH means between 5.74 and 6.5, and the samples were grouped by low (<5 PSU) and high (>30 PSU) salinity status (CECCON et al., 2019). They also found that the salinity gradient

had a major influence on the biodiversity of microbial communities than pH gradient. These salinity variations can also be explained by the location of mangroves, where high salinity areas are closer to the open sea and are affected by tidal variations, while low salinity areas (estuarine region) have a high content of organic matter and are affected by freshwater outflow from rivers. Therefore, the number of species and abundance differ between microbial communities depending on the sample type, location, pH, salinity, seasonal rainfall, biogeochemical cycle (carbon, nitrogen, nutrient, water), species growth rate and decomposition (LIN et al., 2019). Thus, the salinity and pH ranges found in this study were ideal to facilitate the development of the microbiome and bioprospecting of most samples.

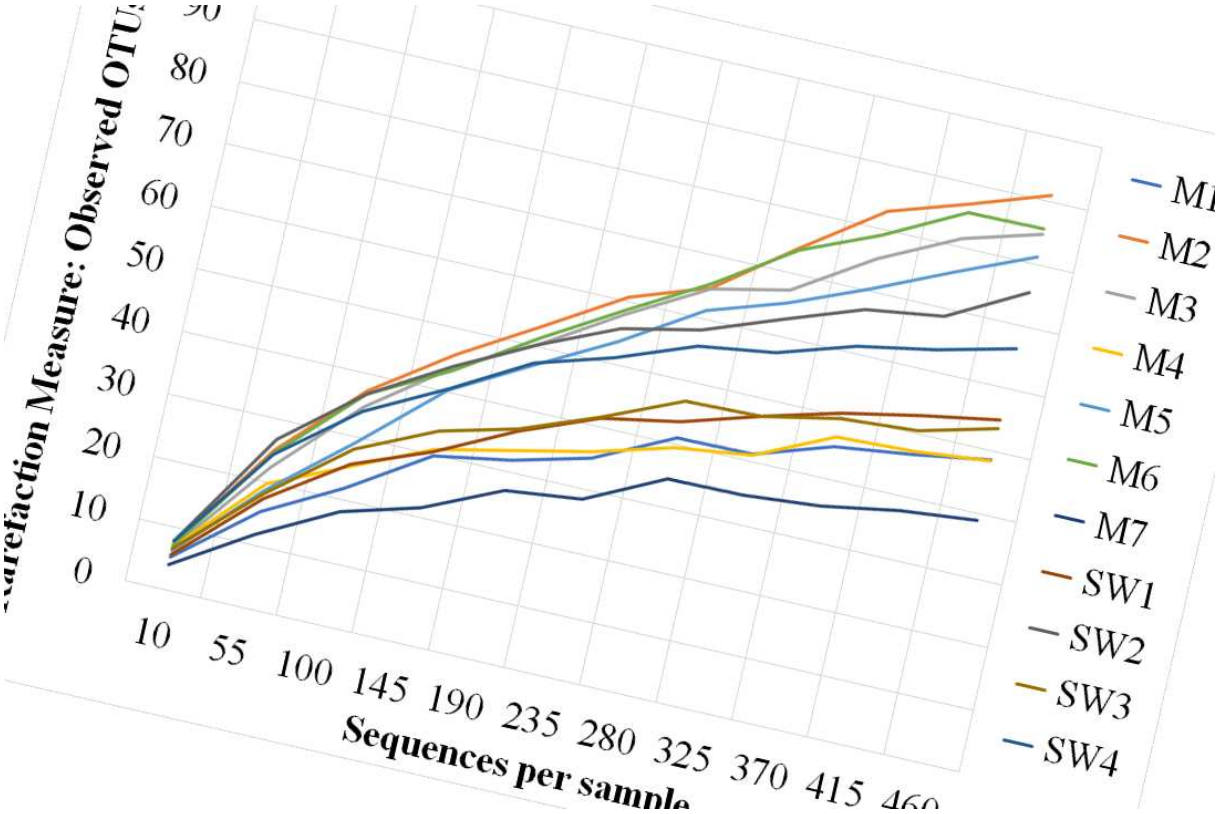

Figure 19. Rarefaction curve for alpha diversity of eukaryotic communities from mangrove and coastal seawater samples.

Furthermore, the principal component analysis (PCA) showed the beta diversity of sediment and seawater samples (Figure 20), where PC1 and PC2 explain 69.51% and 15.52% of the data variability, respectively. The SW1, SW2, SW3 and M2 samples had a positive correlation with each other. In contrast, M1, M3 and M6 samples were inversely correlated to M4, M5, M7 and SW4 samples. The M4 (Ilha dos Valadares), M5 (Antonina) and M7 (Florianópolis) mangrove sediment samples were very close to each other, which suggests

similarity within biological communities despite being from distant locations. Likewise, SW1 (Ilha do Mel) and SW2 (Ilha dos Valadares) seawater samples had similarities in their communities, where both samples were collected in different points of Paranaguá Bay. Thus, the beta diversity allowed to observe the dissimilarity in the composition of the communities between the different locations in the environmental gradient (mangrove and seawater).

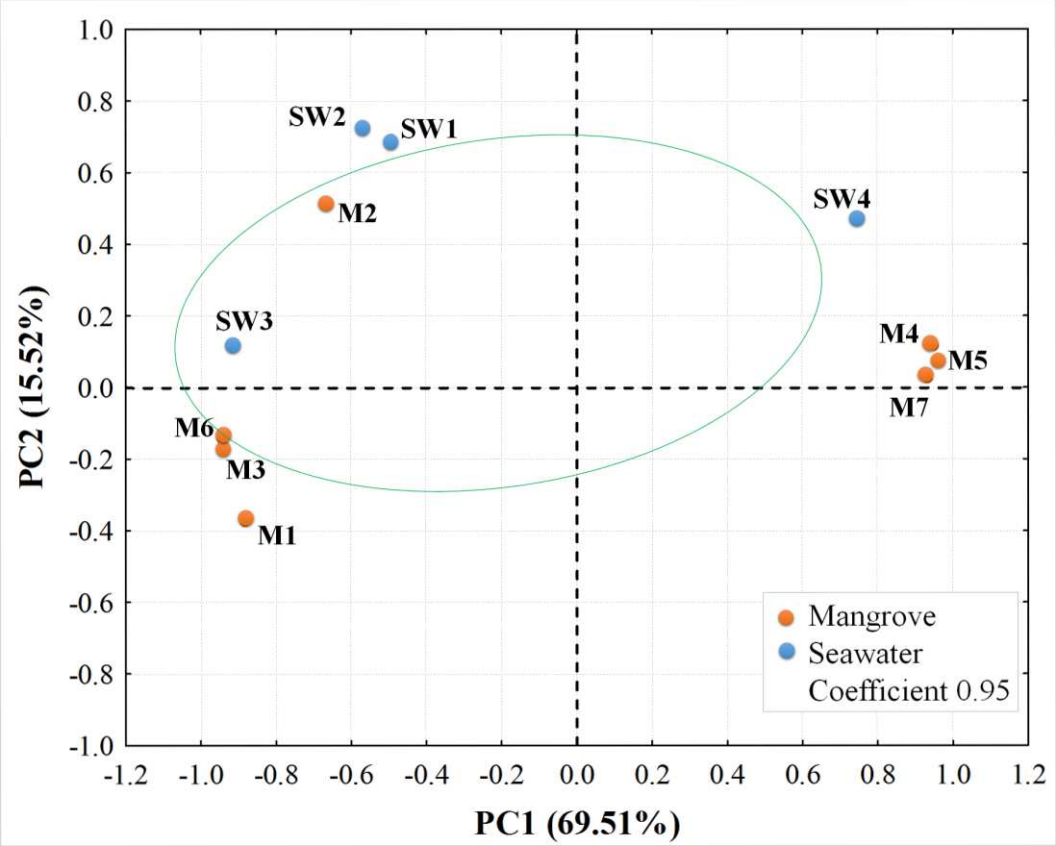

Figure 20. Principal component analysis (PCA) for beta diversity of eukaryotic communities from mangrove and coastal seawater samples.

3.2 ABUNDANCE OF EUKARYOTE COMMUNITIES

The first taxon detected by metagenetics were the supergroups or kingdoms Fungi, Holozoa, Viridiplantae, SAR (Stramenopiles, Alveolata and Rhizaria) (Figure 21). In addition, the classifications of other SAR, other eukaryota and groups with less than 1% incidence included Excavata, Rhodophyta, Hacrobia, Apusozoa, Amoebozoa, and other ophistokonta. All samples showed abundance in the protist kingdom, especially SAR or Harosa supergroup commonly found in marine environments. Hence, the samples with highest abundance of stramenopiles were M1 (94.5%), M3 (61.4%) and M6 (60.5%). Stramenopiles, also known as heterokonts, include heterotrophic and photosynthetic organisms, diatoms, unicellular and

multicellular microalgae (YOON et al., 2009). These heterokonts are originated by the secondary endosymbiosis between some red algae and a flagellated heterotrophic organism. They are characterized by presenting two flagella and some have chloroplasts for their photosynthesis, which are abundant in the marine ecosystem as reported in community studies based on metagenetics (SIERACKI et al., 2019). Otherwise, the alveolata and rhizaria groups were less prevalent within the SAR supergroup. The alveolates were more abundant in SW1 (15.4%) and SW2 (13.1%) seawater samples. The alveolates include phyla ciliophora, apicomplexa and dinoflagellates. They are known for the formation of blooms in algae and the production of toxins that contaminate seafood, as well as some are predators of other microorganisms or animal parasites (SIMPSON; EGLIT, 2016). Thereafter, the rhizaria group showed the highest abundance in M7 sample (5.4%). The rhizaria are characterized by the amoebic form, some having pseudopods for their food and flagella for locomotion (LOVEJOY, 2020).

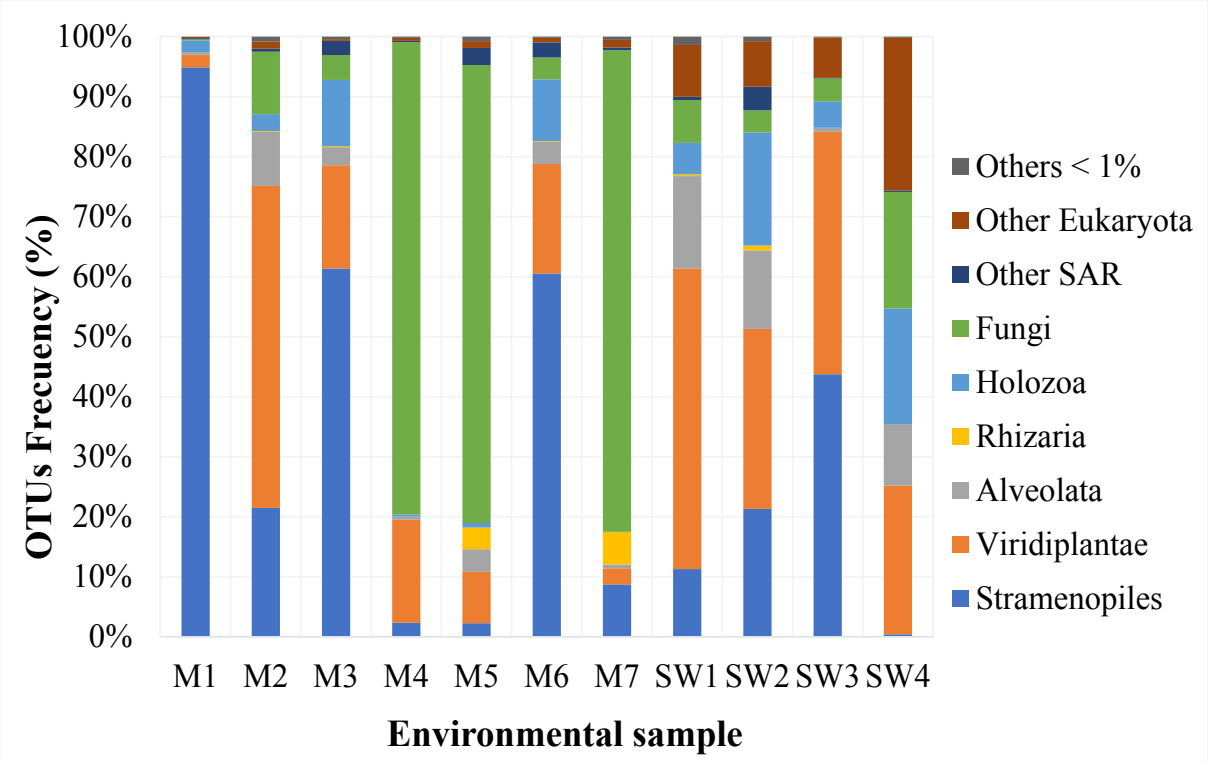

Figure 21. Supergroups and kingdoms within eukaryotic communities detected by metagenetics from environmental samples.

Likewise, the viridiplantae was present in all samples (Figure 21), where M2 (53.7%) and SW1 (50.1%) samples had a higher taxonomic frequency. Viridiplantae (green plants) are a monophyletic clade that include aquatic green algae and terrestrial plants. Green algae

originated from primary endosymbiosis with cyanobacteria (chloroplasts) and have cellulose on their cell wall. Thus, terrestrial or land plants emerged through adaptation and evolution from the aquatic environment (SIMPSON, 2010). Moreover, the fungi kingdom was detected in all samples and showed great prominence in M4 (78.7%) and M7 (80.2%) mangrove samples. Fungi can be saprophages feeding on decaying dead organisms or predators. Mycotoxin-producing fungi can be harmful to human, but many have been studied and used widely in the food and pharmaceutical industry. Beneficial fungi impart organoleptic, functional and nutritional properties to various products such as cheeses, wines and breads (MORETTI; SARROCCO, 2016). Consequently, the other eukaryote group was highlighted in all water samples with a frequency of species that have not yet been studied, cultivated or detected by metagenetics.

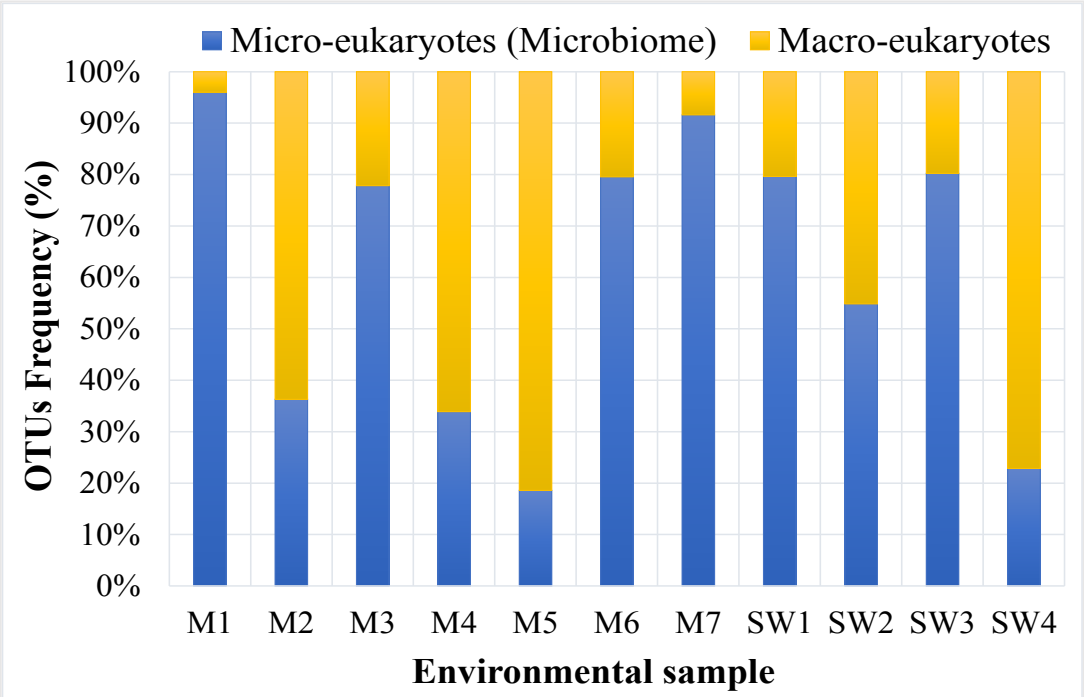

Figure 22. Micro-eukaryotes and macro-eukaryotes communities detected by eukaryotic metagenetics from environmental samples.

On the other hand, communities were grouped by macro-eukaryotes and micro-eukaryotes (Figure 22). Micro-eukaryotes corresponded to microalgae from viridiplantae, protists from SAR supergroup, filamentous fungi (molds) and yeasts from fungi kingdom. Macro-eukaryotes corresponded to the holozoa group, mushrooms (Agaricomycetes) from fungi, terrestrial plants (Embryophyta) and green macro-algae from viridiplantae. M1 (96%) and M7 (91.6%) samples showed the highest microbial abundance among mangrove samples.

Then, M3 (77.8%), M6 (79.6%), SW1 (79.6%) and SW3 (80.2%) samples had similar abundances in the microbiome. In contrast, M2, M4, M5 and SW4 samples showed low abundance in their microbiome when compared to the detected macro-eukaryotes. Thus, marine diversity has been little studied and, even though the samples are from coastal areas close to the mangroves, there is still a lack of mechanisms that allow to know or find new species.

3.3 CLASS-LEVEL EUKARYOTIC MICROBIOME

The microbial class diversity allowed to visualize the similarities between the samples (Figure 23). The M1 and M2 mangrove sediment samples showed a similar abundance profile in the diatoms (Bacillariophyceae, Mediophyceae) and Labyrinthulomycetes classes. M3 and M6 samples showed similar abundances with the classes of diatoms (Mediophyceae, Coscinodiscophyceae, Bacillariophyceae), photosynthetic green microalgae (Trebouxiophyceae, Prasinophyceae) and heterotrophic microalgae (Labyrinthulomycetes). However, M4 sample did not present a similar profile to the M3 even though they were from nearby locations (Ilha dos Valadares). In the same way, M5 and M6 samples from Antonina had different abundances and diversities. Thus, M4 and M5 samples had similar abundances, especially Eurotiomycetes (Fungi) and Trebouxiophyceae (green microalgae) classes. Eurotiomycetes class was also reported in microbiome studies from Indian mangrove with high-throughput sequencing (SANKA LOGANATHACHETTI; POOSAKKANNU; MUTHURAMAN, 2017). M4 and M5 samples had similarity to SW4 seawater sample with a high abundance of Dinophyceae (dinoflagellate unicellular algae). In contrast, the SW3 sample was closer to the M3 and M6 samples mainly in the Mediophyceae and Trebouxiophyceae classes.

Then, SW2 seawater sample showed greater diversity and abundance of Trebouxiophyceae, Syndiniales, Mediophyceae, Bacillariophyceae and Labyrinthulomycetes, and was close to SW3 sample. The Syndiniales classes correspond to dinoflagellates commonly found in ocean waters and they are parasites of other organisms, such as fish, crustaceans, algae and protists (BRANDT et al., 2020). The wide diversity of SW2 sample can also be explained by the proximity of the collection site to the mangroves in Ilha dos Valadares (Paranaguá Bay), where SW1 had similarities to SW2. Finally, M7 sample showed a unique diversity compared to other samples with a greater abundance of the fungal classes Microbotryomycetes and protists from SAR supergroup (Imbricatea, Labyrinthulomycetes and Bacillariophyceae). These micro-eukaryotes such as labyrinthulomycetes, diatoms and dinophyceae were also

prevalent in studies of European mangrove sediments and coastal waters (MASSANA et al., 2015). Likewise, fungi and the labyrinthulomycetes dominated biomass on bathypelagic marine snow in the deep ocean, this can be attributed to the fact that both groups are important in the degradation of organic matter (BOCHDANSKY; CLOUSE; HERNDL, 2017).

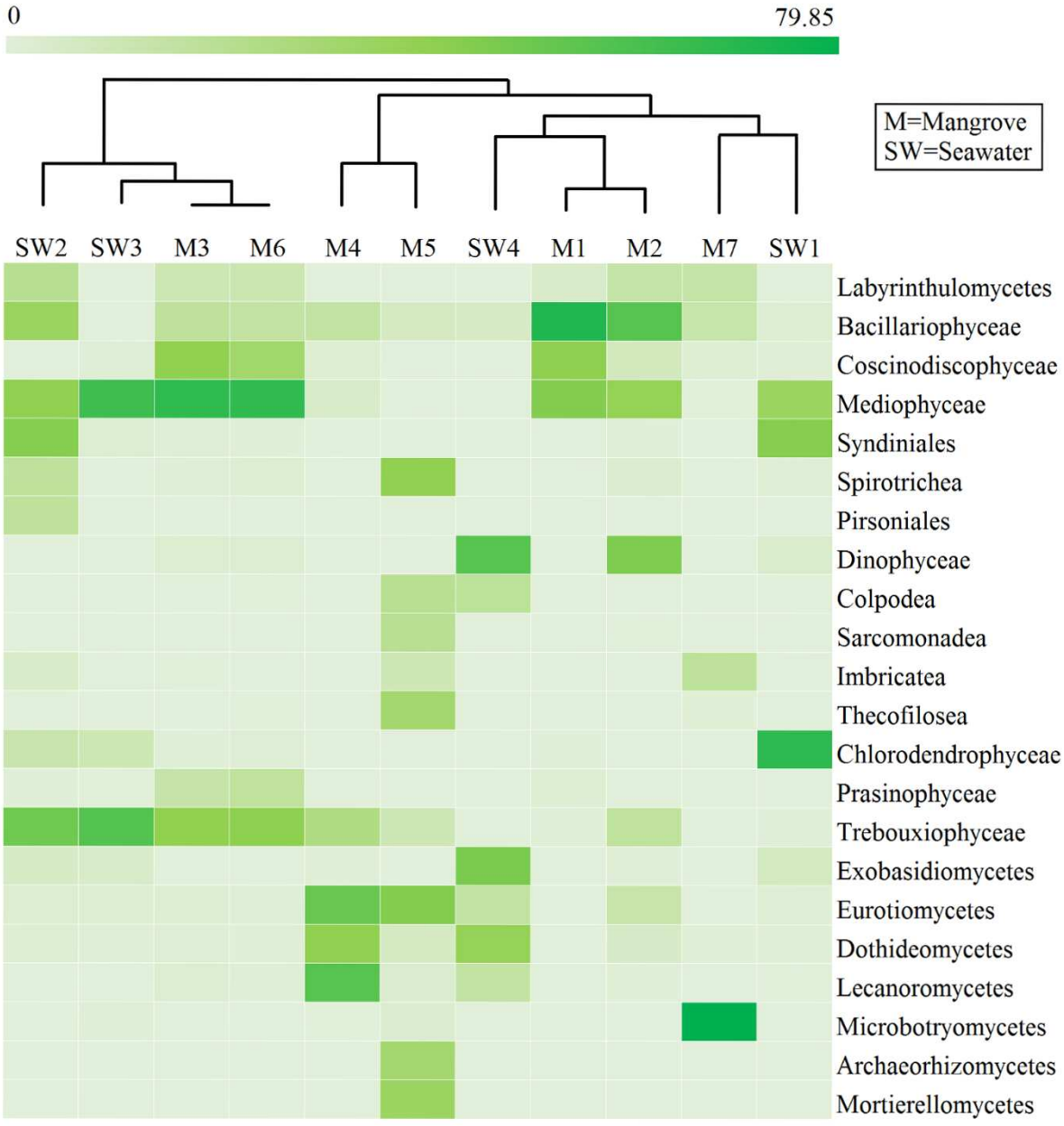

Figure 23. Hierarchical clustering heatmap of eukaryotic microbiome by class level.

3.4 GENUS-LEVEL EUKARYOTIC MICROBIOME

The microbial genera were classified by cluster according to the NCBI taxonomy tool (Figure 24). The *Aspergillus* genus from ascomycetes cluster was highlighted in almost all

samples. The cercozoans cluster had a high prevalence in M5 mangrove sample; and no genus was detected in seawater samples. On the other hand, chytrids were highlighted in M2 sample with *Nowakowskiella* and*, Synchytrium* genus. The ciliates cluster was prevalent in M2, SW1 and SW2 samples.

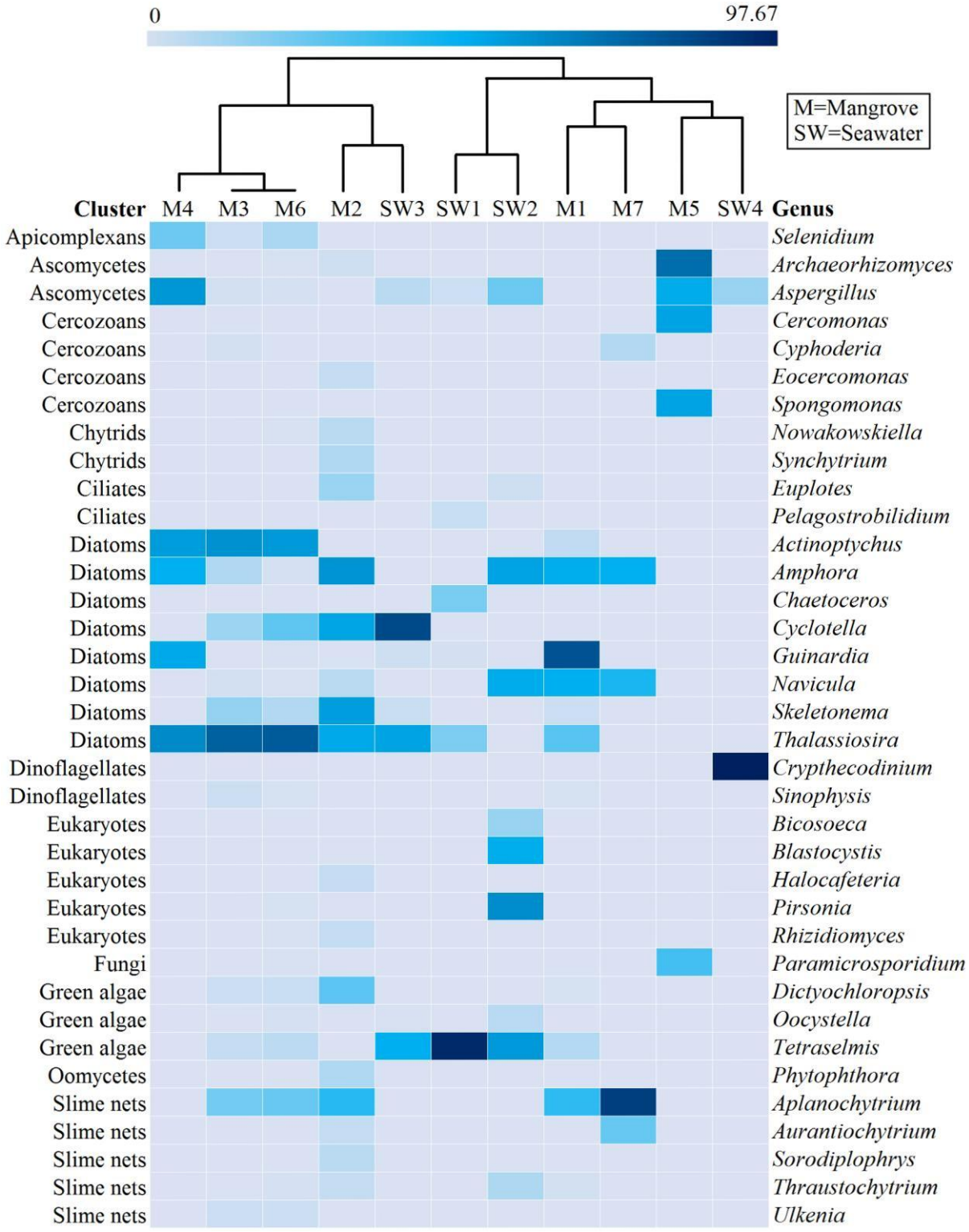

Figure 24. Microbial genera reveled by eukaryotic metagenetics from environmental samples.

As expected, diatoms were the cluster with the greatest abundance and diversity found in most samples, except M5 and SW4 samples, where *Actinoptychus, Amphora, Chaetoceros, Cyclotella, Guinardia, Navicula, Skeletonema* and *Thalassiosira* genus were prevalent. The dinoflagellates genus highlighted was *Crypthecodinium* (97.67%) in SW4 sample. In contrast, some genera did not have a specific cluster classification in the NCBI, which were grouped as eukaryotes and fungi. The eukaryotes cluster comprised heterokonts from the SAR clade and fungi cluster comprised *Paramicrosporidium* genus. The green algae cluster appeared in almost all samples, where *Dictyochloropsis* stood out in mangrove samples and *Tetraselmis* had a high OTUs frequency in all except SW4 seawater sample. Finally, the slime nets cluster was prevalent from labyrinthulomycetes class, including *Aplanochytrium* (M1, M2, M3, M6 and M7)*, Aurantiochytrium* (M2 and M7)*, Sorodiplophrys* (M2)*, Thraustochytrium* (M1, M2, M6 and SW2) and *Ulkenia* (M3 and M6).

Most of the microbial clusters detected by eukaryotic metagenetics have reported biotechnological potential for production of high-value lipids, mainly LC-PUFAs. The Thraustochytriaceae (Labyrinthulomycetes) and Crypthecodiniaceae (Dinophyceae) families have been highlighted by high production of docosahexaenoic acid (DHA) (FOSSIER MARCHAN et al., 2018; KARNAOURI et al., 2020). Moreover, thraustochytrids from labyrinthulomycetes class were detected in most of the marine environments studied. However, not all samples had a prevalence of them. Therefore, eukaryotic metagenetics was a powerful tool for bioprospecting thraustochytrids without prior isolation, which facilitated subsequent isolation and high-throughput screening from the desired samples.

3.5 HIGH-THROUGHPUT SCREENING (HTS)

The isolation techniques allowed to obtain 391 isolates after microscopy evaluation from M1 (n=55), M2 (n=20), M3 (n=72), M6 (n=25), M7 (n=191) and SW2 (n=28) samples. Despite the great richness of marine samples shown by the metagenetics, seawater had a reduced number of microbial colonies due to difficulty in similar this natural habitat. Thus, the major limitation of classical cultivation techniques is to drastically underestimate the number and microbial composition in the samples under study (AL-AWADHI et al., 2013).

The high-throughput screening (HTS) was carried out by analyzing the relative fluorescence unit (RFU) (Figure 25A). The MC1 static microplate strategy was not very efficient due to the difficulties of avoiding cross-contamination among the isolates; however, the isolates classification sequence was the same as the traditional shaker cultivation (SC),

which suggests that the MC1 has potential and could be improved in the future. This strategy is also known as microtiter plate (MTP) and has been studied as mini-bioreactors for the production of several metabolites, suggesting well-closed systems with orbital shaking and headspace air to improve gas–liquid oxygen-transfer (BACK et al., 2016; DUETZ, 2007).

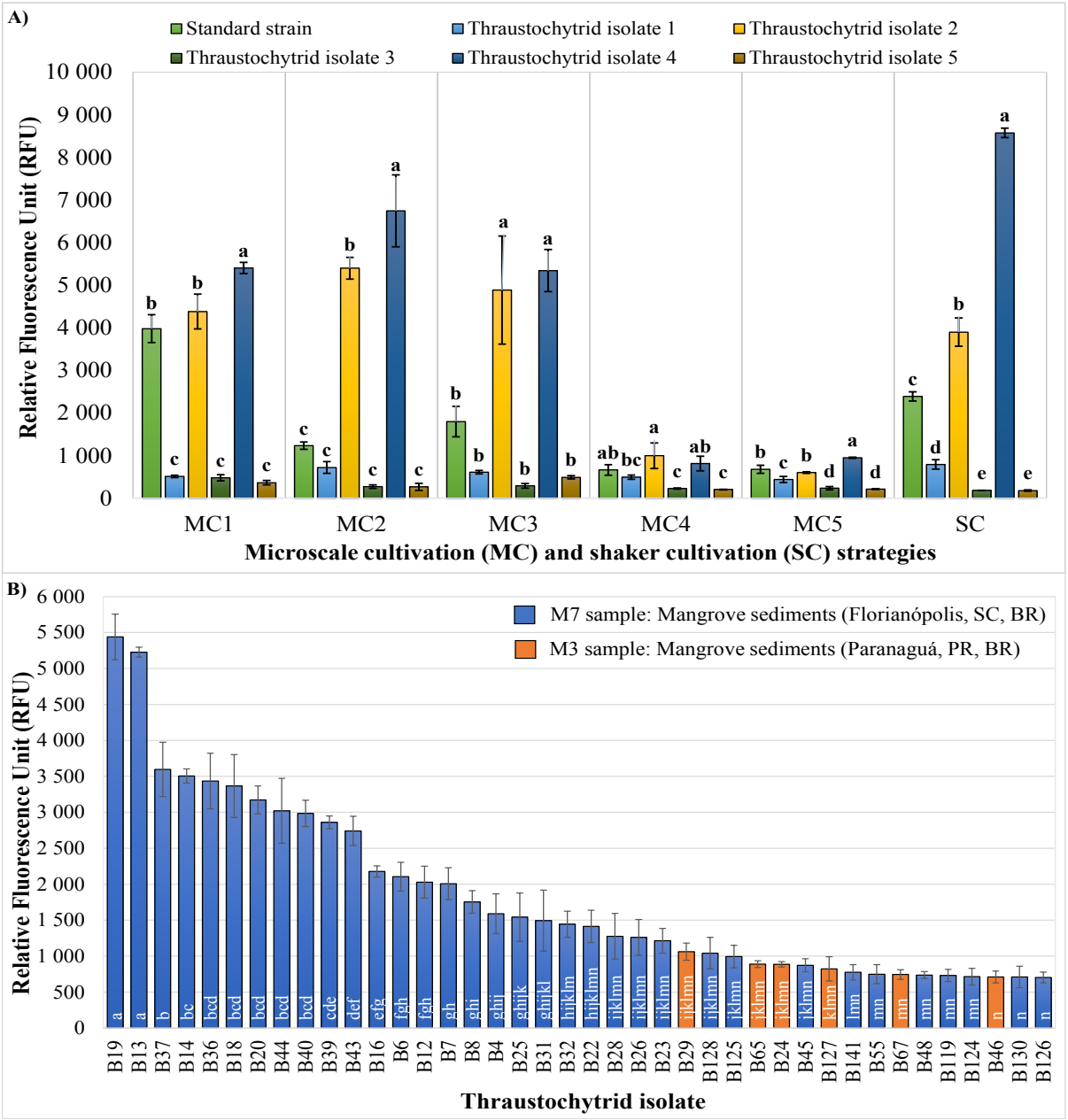

Figure 25. High-throughput screening (HTS) of thraustochytrids as lipid accumulators. A) Microscale cultivation strategies compared to standard strain (*Schizochytrium limacinum* SR21). B) Thraustochytrids isolates and post hoc analysis by Tukey HSD test for homogenous groups.

In contrast, the MC2 and MC3 strategies in microtubes and cotton caps were efficient because they allowed the exchange of oxygen even though it was a static culture, and the isolates classification order was the same as SC. On the other hand, MC4 and MC5 strategies

had very low fluorescence and did not have the same classification order when compared to the SC; this may be due to the fact that the microtubes were sealed and even with agitation they had little oxygen exchange. Thus, MC2 strategy was selected for bioprospecting 391 isolates with a lower standard deviation than MC3.

Several authors have reported detection of lipids by fluorescence with Nile red in microalgae, protists and fungi with values around 700 RFU (ALEMÁN-NAVA et al., 2016; SITEPU et al., 2012). Thus, RFU>700 was used as selection criterion in the tested isolates. Figure 25B shows forty isolates with RFU> 700, where six isolates were from M3 mangrove sample (Ilha dos Valadares) and thirty-four isolates were from M7 mangrove sample (Florianópolis). The isolates B19 (5438.39f ± 317.26 RFU) and B13 (5226.27 ± 69.09 RFU) had a high fluorescence emitted in comparison to cell biomass. These results were even similar and higher than the fluorescence intensities reported in microorganisms such as *Aurantiochytrium* sp. KRS101 (>1000) (VELMURUGAN et al., 2014), *Schizochytrium* sp. (>5000) (XIU-HONG et al., 2019), *Pseudochlorococcum* sp. (>600), *Scenedesmus dimorphus* (>400) and *Chlorella zofingiensis* (>700) (CHEN; SOMMERFELD; HU, 2011), *Neochloris oleoabundans* UTEX 1185 (>1000) (POPOVICH et al., 2012) and yeast strains (>1000) (SITEPU et al., 2012). Then, twenty isolates were selected according to Tukey post variance analysis for batch submerged cultivation, which did not have the last homogeneous group (n).

3.6 PRODUCTION OF BIOMASS AND LIPIDS

The twenty isolates had a biomass growth or cell dry weight (CDW) between 20.80 and 31.20 g/l, total lipid between 7.86 and 24.05 g/l, and lipid content between 34.21 and 85.00% after 5 days of cultivation in shake flasks at 28 °C (Table 4). Likewise, (PATEL et al., 2020c) reported 25.59 g/l CDW and 11.91 g/l lipids by *Schizochytrium limacinum* SR21 grown in shake flasks at 25 °C for 5 days with glucose. Studies have shown that thraustochytrids can accumulate between 30 and 50% of lipids in their cell biomass (MORABITO et al., 2019). Subsequently, gas chromatography analysis allowed to quantify the total fatty acids (TFA) in these lipids after transesterified. The isolates with highest TFA in CDW were B12 (56.12%), B40 (49.86%) and B36 (46.30%). It can be observed that the TFA levels are lower than lipid content, which may be due to the fact that TFA are derived from lipid biomolecules such as storage lipids (triglycerides or TAGs), membrane lipids (phospholipids, lipoproteins, glycolipids) and some derived lipids (pigments, fat-soluble vitamins, etc.) (MITCHELL; FLIGHT; MOSELEY, 2020).

Furthermore, the LC-PUFAs produced by isolates corresponded to omega-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA) and omega-6 long-chain polyunsaturated fatty acid (n-6 LC-PUFA) as shown in Table 4. The isolates highlighted by LC-PUFAs content in TFA were B36 (44.37% LC-PUFA, 36.18% n-3 LC-PUFA, 8.20% n-6 LC-PUFA) and B40 (40.45% LC-PUFA, 32.83% n-3 LC-PUFA, 7.62% n-6 LC-PUFA). These results could be further improved for human and animal consumption. Researchers at the University of California-Davis used the spray-dried cells *Schizochytrium* sp. (45.3% n-3 LC-PUFA and 17.6% n-6 LC-PUFA in TFA) as a sustainable substitute for fish oil, which improved the deposition of n-3 LC-PUFA levels in Nile tilapia fillets (SARKER et al., 2016).In addition, B36 and B40 isolates showed biotechnological potential for docosahexaenoic acid (DHA) production in bench-scale submerged cultivation (Table 1). B36 isolate produced 4.10 g/l DHA (0.16 g/g_{CDW}) and B40 isolate produced 3.69 g/l DHA (0.15 g/g_{CDW}). Likewise, some studies have reported similar DHA production in shake flasks by *Aurantiochytrium* sp. T66 with 2.15 g/l DHA (0.15 g/g_{CDW}) (PATEL et al., 2020b), and 2.07 g/l DHA (0.17 g/g_{CDW}) (PATEL et al., 2020a). Even more, DHA production after media optimization by central composite design (CCD) and response surface methodology (RSM) have been reported with 4.75 g/l DHA (0.25 g/g_{CDW}) by *Aurantiochytrium* SW1 cultivated in shake flasks at 30 ºC for 4 days with fructose (NAZIR et al., 2018). Similarly, the Indian isolate *Thraustochytrium* sp. T01 had 6.1 g/l DHA (0.23 g/g_{CDW}) after media optimization in shake flasks at 25 ºC for 3 days with glucose (CHANDRASEKARAN; ROY; CHADHA, 2018).

3.7 FATTY ACID COMPOSITION

The fatty acid (FA) profiles showed a great diversity among the isolates, some had similar and very different compositions (Figure 26). The B36 and B40 isolates were highlighted by docosahexaenoic acid (DHA, C22:6 n-3) and docosapentaenoic acid (DPA, C22:5 n-6) with 34.6% and 31.1% DHA content respectively, followed by 6.3% and 7.0% DPA content, respectively; however, B36 isolate had a greater diversity of fatty acids compared to B40 isolate. Likewise, B32, B7, B12, B8 and B43 isolates had similar DHA content (12.7-19.9%), followed by B25 and B31 isolates with DHA content between (4.5-7.7%) and low DPA content. Another cluster with similar profiles was presented by B44, B13, B20, B39, B18, B37 and B19 isolates with low DHA content (0.7-1.8%) and no DPA content. In contrast, B16 isolate had a varied profile when compared to all isolates with n-3, n-6 and n-9 fatty acids.

Table 4. Production of cell biomass, lipids and polyunsaturated fatty acids (PUFAs) by thraustochytrids isolates in batch submerged cultivation.

Thraustochytrid isolates	Cell dry weight (CDW)	Total lipid	DHA	DHA yield ($Y_{P/X}$)	Lipid content	Total fatty acids (TFA)	LC-PUFA	n-3 LC-PUFA	n-6 LC-PUFA	n-3:n-6 PUFA ratio
	g/l	g/l	g/l	mg/g CDW	% of CDW	% of CDW	% of TFA	% of TFA	% of TFA	
B4	27.4	10.33	0	0	37.71	22.89	0.68	0.68	0	-
B6	20.8	11.02	0	0	53	23.35	0	0	0	-
B7	23.52	10.03	1.06	45.22	42.64	33.4	18.06	15.22	2.84	5.4
B8	22.68	9.28	1.07	47.09	40.93	29.76	20.56	17.33	3.23	5.4
B12	28.4	19.03	2.36	83.06	67	56.12	19	16.01	2.99	5.4
B13	31.2	24.05	0.08	2.68	77.07	36.53	3.35	3.35	0	-
B14	26.4	17.67	0	0	66.93	28.04	2.11	2.11	0	-
B16	29.8	15.84	0.07	2.23	53.14	34.61	6.22	3.08	3.14	1
B18	27.2	17.87	0.08	2.85	65.71	29.49	3.25	3.25	0	-
B19	26.8	22.78	0.13	4.98	85	27.08	3.51	3.51	0	-
B20	23.8	15.37	0.04	1.83	64.57	20.34	3.02	3.02	0	-
B25	26.41	9.51	0.24	9.1	36	20.05	8.58	6.56	2.02	3.2
B31	24.8	8.77	0.33	13.43	35.36	17.37	11.49	9.7	1.79	5.4
B32	22.98	7.86	0.82	35.83	34.21	28.17	19.29	15.49	3.81	4.1
B36	25.6	17.12	4.1	160.19	66.86	46.3	44.37	36.18	8.2	4.4
B37	26.8	18.19	0.1	3.92	67.86	26.14	3.78	3.78	0	-
B39	22.04	12.07	0.06	2.74	54.79	29.69	2.11	2.11	0	-
B40	23.81	14.62	3.69	154.86	61.43	49.86	40.45	32.83	7.62	4.3
B43	24.6	13.28	1.77	71.77	54	36.07	25.07	21.28	3.79	5.6
B44	25	15.55	0.06	2.3	62.21	33.28	2.4	2.4	0	-

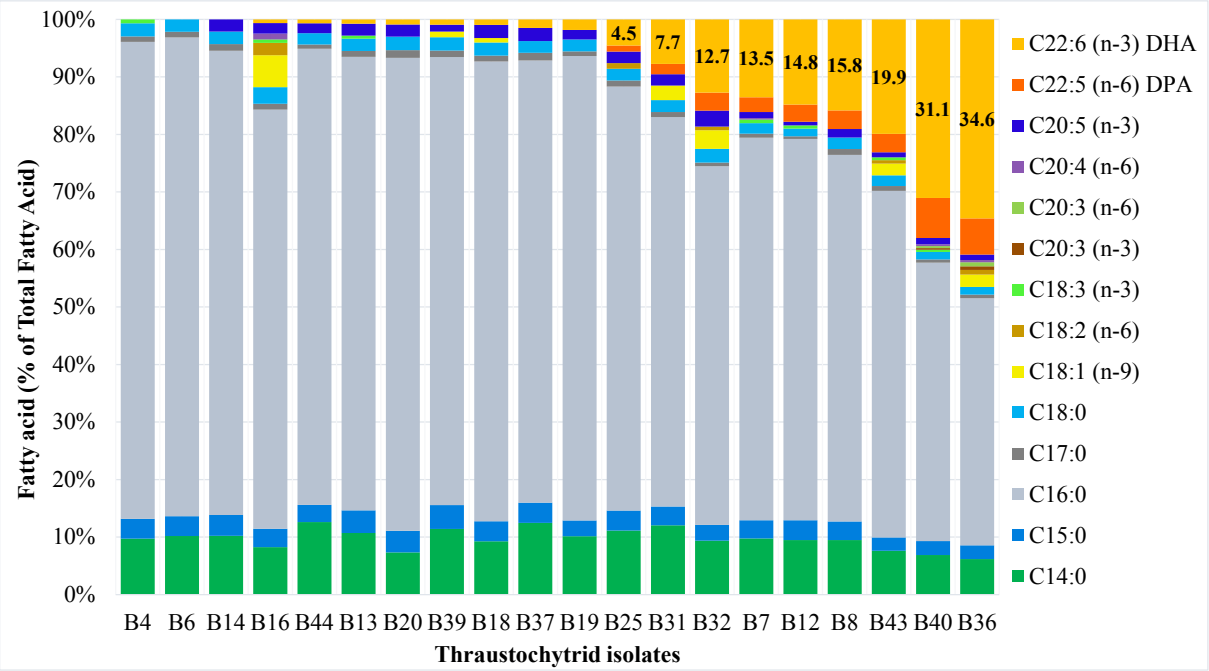

Figure 26. Fatty acid profiles of thraustochytrids isolates detected by gas chromatography.

Moreover, this study showed that even though some isolates produced high amounts of biomass and lipids, they did not produce LC-PUFAs (Table 4). Palmitic acid (C16:0) and DHA (22:6) are predominant in the FA composition of thraustochytrids, however, B4, B6 and B14 isolates did not present DHA content. This can be explained due to the metabolic pathway for high or low production of LC-PUFAs depend on different factors such as cultivation conditions, the complexities of nutrients, nitrogen limitation, starvation, stress, among others (MORABITO et al., 2019; SAJJADI et al., 2018). The DHA synthesis in thraustochytrids can take place in two different and independent pathways: the aerobic pathway (fatty acid synthase or FAS) and the anaerobic pathway (polyketide synthases, PKS or PUFA synthase). The FAS pathway requires oxygen in the desaturation and elongation steps by introducing double bonds. The PKS pathway involves dehydration and isomerization reactions without requiring dissolved oxygen. Likewise, studies have shown that aeration in both shaker and bioreactor cultivation can influence the FA composition. A high rate of oxygen transfer tends to rapidly promote cell biomass growth by consuming substrate and a low oxygen rate increases lipid synthesis in later stages (MORABITO et al., 2019; PATEL et al., 2020c). However, the role of oxygen in increasing DHA synthesis has not been fully clarified and seems to vary between thraustochytrids species. This difference between the isolates can also be explained by the

diversity of thraustochytrids (Order: Labyrinthulomycetes, Cluster: Slime nets) found in marine environments revealed by metagenetics (Figure 24).

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3.8 PHYLOGENETIC ANALYSIS OF LC-PUFA PRODUCERS

The tree of phylogenetic analysis is shown in Figure 27. Most isolates were grouped by their proximity to *Aurantiochytrium mangrovei* and *Aurantiochytrium limacinum* genus, however the fatty acid profiles showed differences between strains (Figure 26). In addition, B12 and B36 isolates showed proximity to *Aurantiochytrium acetophilum* genus. *Aurantiochytrium acetophilum* HS-399 is a new species recently identified after studies of whole nuclear genome, microscopy, carbon assimilation and fatty acid profile (GANUZA et al., 2019).

4. CONCLUSIONS

The high-performance sequencing tool provided the study of eukaryotic communities from mangrove and seawater in southern Brazil, as well as a reduction in the bioprospecting time of marine thraustochytrids. In addition, the high-throughput screening (HTS) was promising due to the rapid analysis to select lipid accumulators. Thus, metagenetics combined with the HTS reduced the number of isolates to be studied on a bench scale, which allowed a detailed analysis of fatty acid profiles. Finally, Brazilian thraustochytrid isolates had a high biomass and lipid production rich in LC-PUFA content. Future studies to optimize media and bioprocess variables are needed to increase the production in bioreactors for applications in food and pharmaceutical industry.

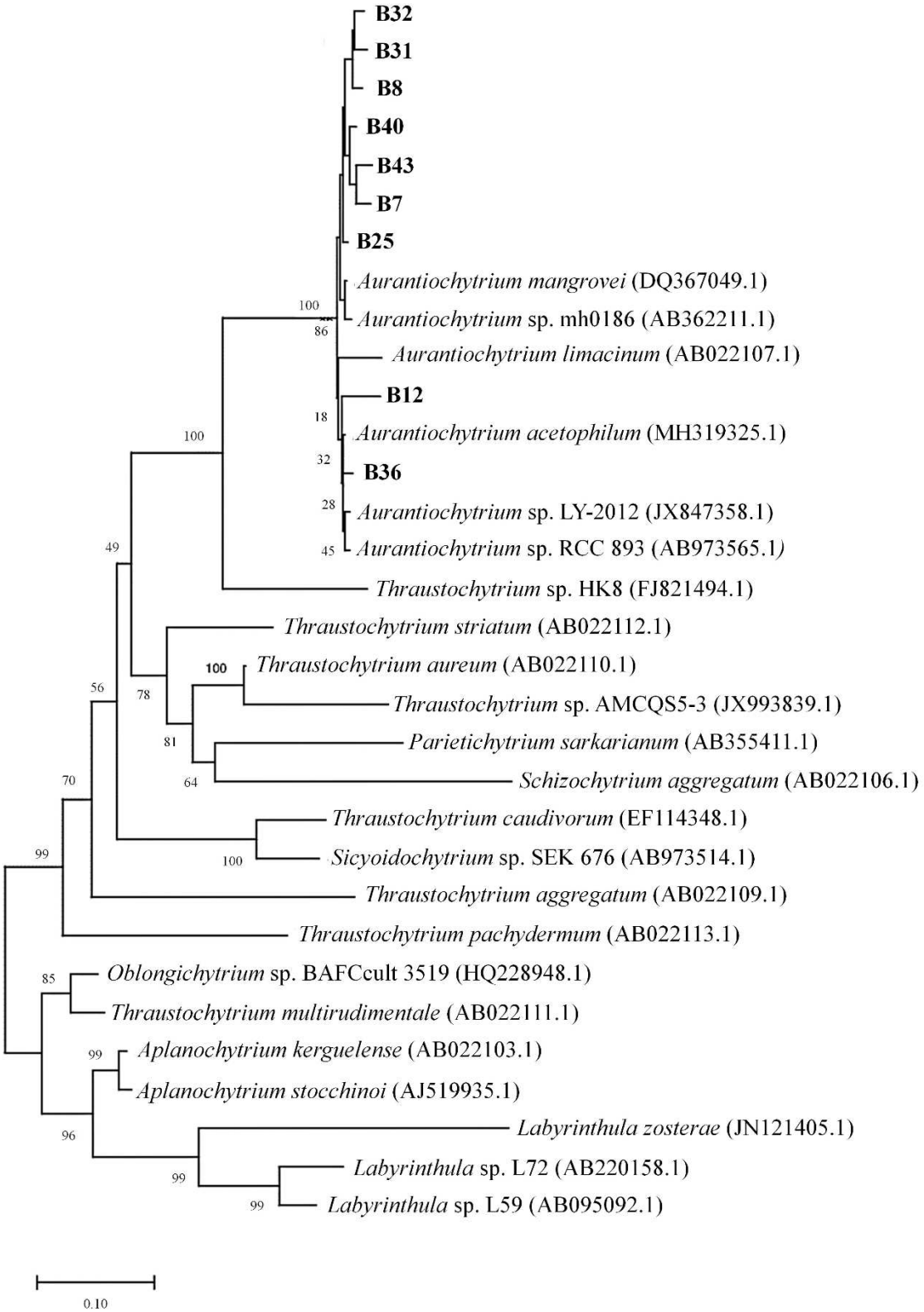

Figure 27. Molecular phylogenetics of LC-PUFA-producing thraustochytrids isolates.

CHAPTER 4

THRAUSTOCHYTRID IMPROVEMENT FOR PRODUCTION OF LIPID-RICH BIOMASS: BY-PRODUCTS VALORIZATION, BIOPROCESS OPTIMIZATION AND BIOREACTOR SCALE-UP

The results obtained in the *Scale-up in stirred-tank bioreactor (STBR)* of this chapter were deposited under patent number BR102020026212-2 at INPI (Instituto Nacional de Propriedade Industrial) on December 21, 2020 (Attachment 3).

ABSTRACT

Thraustochytrids are an alternative source of biomass rich in high-value lipids, which can be developed in fermentation processes through the use of low-cost agro-industrial by-products. Here, a complete study of thraustochytrid improvement by ultraviolet mutation combined with a microtiter-based fluorescence assay was performed to select highly lipid accumulating mutants. Then, M29 mutant and wild-type were cultivated to produce lipid-rich biomass, going through three steps of bioprocess optimization with sugarcane molasses, corn steep liquor and residual yeast cream as recovered by-products. After optimization in shake flasks, the biomass, lipid content and lipid production by M29 mutant increased by 1.24, 1.56 and 1.96-fold, respectively. Likewise, biomass, lipid content and lipid production by wild-type increased by 1.12, 1.72 and 1.92-fold, respectively. Thus, the scale-up in stirred-tank bioreactor by wild-type had increments of 2.27, 1.50 and 3.40-fold in biomass, lipid content and lipids, respectively, when compared to shaker optimization. The highest biomass, lipids and lipid-biomass yield ($Y_{P/X}$) in bioreactor were 39.29 g/l (0.64 g/l.h), 14.98 g/l (0.35 g/l.h) and 0.45 g/g CDW, respectively. The lipid-rich thraustochytrids biomass showed great biotechnological potential for future large-scale production for applications in animal and human nutrition.

Keywords: Thraustochytrid; Microbial lipids; Ultraviolet mutagenesis; By-product valorization; Bioprocess optimization; Bioreactor scale-up.

1. INTRODUCTION

High-value lipids such as omega-3 have stood out for their nutritional properties and health benefits such as promoting brain development, bone and eye health, reducing inflammation, preventing hypertension, cancer, stress cardiovascular and Alzheimer's disease (SHAHIDI; AMBIGAIPALAN, 2018). These lipids are long-chain polyunsaturated fatty acids (LC-PUFAs), where fish oil and fish meal have been the traditional sources in human and animal food. However, this source has been questioned due to the risk of contaminants, pollutants, overfishing and the non-supply of global demand. Thus, the levels of predation in the seas increase each time, which occurs at such a fast speed that the ocean cannot replace marine species in time, which leads to their extinction and scarcity (KAR; MATSUDA, 2006). Thraustochytrid biomass has emerged as a sustainable and renewable alternative to high-value lipids. They are marine protists also known as heterotrophic microalgae, which can accumulate more than 50% of their biomass as intracellular lipids, where more than 25% is equivalent to high-value lipids such as omega-3 (FOSSIER MARCHAN et al., 2018). They have advantages such as they are not pathogenic or toxic, are a low-cost sustainable source, can grow under controlled fermentation conditions, are a vegan source, they can be consumed by people allergic to fish or seafood, are less susceptible to environmental contamination by chemicals, have oxidative stability, have high lipid yields compared to fish and produce several important secondary metabolites (e.g., enzymes, astaxanthin, canthaxanthin, antioxidants and squalene) (FINCO et al., 2017; MORABITO et al., 2019; OROZCO COLONIA; VINÍCIUS DE MELO PEREIRA; SOCCOL, 2020).

Moreover, animals and humans are not able to efficiently synthesize omega-3s in their metabolism, requiring their consumption in diets. Microalgae such as thraustochytrids are the primary producers of omega-3s in the marine environment, and fish contain omega-3s because they largely consume other fish or zooplankton that feed on these thraustochytrids (VENTURA et al., 2017). Thus, lipid-rich thraustochytrid biomass can be used for human and animal nutrition, including fortified foods and beverages, dietary supplements, infant formulas, clinical nutrition and medical foods, pharmaceuticals, animal feed and pet foods (ROBERTSON et al., 2013). Several studies have reported positive effects on the use of thraustochytrid biomass in animal feed, where inclusion values around 5% in aquafeed such as Nile tilapia and Atlantic salmon increased weight gain, protein level and omega-3 levels in fillets (DOS SANTOS et al., 2019; LEE CHANG et al., 2020). The inclusion of 3% thraustochytrids biomass in hen diets has also been evaluated by increasing the docosahexaenoic acid (DHA) content from 248 to

776 mg per 100 g of egg yolk (AO et al., 2015). These results showed the biotechnological potential of thraustochytrid biomass as a substitute for fish oil or fish meal in animal feed.

On the other hand, Agri-food industries generate a significant amount of liquid and solid waste. These residues, when untreated, can cause major environmental, economic and social problems. However, their use in the generation of new products makes them viable, reducing industrial waste and converting them into by-products with economic value. Brazil is the world's largest producer of sugar and the world's second largest producer of ethanol, generating by-products such as sugarcane molasses and residual yeast cream. Sugarcane molasses (SCM) s is a brown viscous liquid agro-industrial by-product rich in sugars (54-59.2%) and pH between 5.7 and 5.9 resulting from the centrifugation step in the manufacture of sugar. Approximately 40 to 60 kg of molasses are generated per ton of processed sugarcane and its main application is in ethanol production plants. Currently, it is also used as a raw material in the biotechnology, pharmaceutical, chemical and food industries (OLIVEIRA et al., 2019). Likewise, residual yeast cream (RYC) is an agro-industrial by-product generated from the biotechnological production of industrial ethanol and beer. After the alcoholic fermentation ends, the yeast cells are naturally decanted, where about 75-85% have cell viability and can be reused in future fermentations. Thus, approximately 20% of the remaining yeasts make up the by-product known as residual cream yeast with high protein content (33-50%). The RYC is washed, desalted and spray dried (equivalent to 2.5-3 kg of dry yeast per 100 liters of ethanol produced). Thus, dry cane yeast is used in animal feed, while dry brewer's yeast is used as a nutritional supplement in human food (CABALLERO-CÓRDOBA; PACHECO; SGARBIERI, 1997; MATHIAS et al., 2015; POVEDA PARRA et al., 2013).

In addition, Brazil is the world's third largest producer of corn and its derivatives, generating corn steep liquor as a by-product. Corn steep liquor (CSL) is an agro-industrial by-product with a light to dark brown color, ensiled odor, low pH (3.7-3.86) due to its high lactic acid content and high protein content (31.6-49.0%). It is obtained by wet milling in corn processing, where the corn kernels are soaked in heated water while being successively macerated. Recently, CSL has been used in animal feed and as a substitute for yeast extract in fermentation processes. (BUYONDO; JAQUESS, 2017; CHOVATIYA; BHATT; SHAH, 2011; ECKERLE et al., 2012; MAHR-UN-NISA et al., 2006; SANTOS et al., 2012).

Furthermore, UV mutagenesis is a strain improvement technique which uses direct exposure of cells to ultraviolet (UV) radiation. Non-ionizing UV light induces mutation or variation in genes and interferes with the binding between DNA nucleotides forming pyrimidine dimers and causing distortion in the DNA molecule (LIU et al., 2020). UV radiation often leads

to cell death in most cells. However, a wrong base may be incorporated into the position of the strand dimer in the next DNA replication in a minority of cells, affecting transcription and promoting changes in cell biochemistry and metabolism. Thus, the survival of the obtained mutants depends on the dose of the mutagenic agent and the exposure time, which are key factors for maintaining cell viability. Some strategies used to select highly lipid-producing mutants include Sudan Black B, Nile Red and BODIPY staining, metabolites production, cerulenin stress, butanol tolerance, iodoacetate and malonic acid resistance (DUAN et al., 2019; LI et al., 2017; LIAN et al., 2010; PARK et al., 2018a; PATEL et al., 2020a; TAKAKU et al., 2021; TAPIA V et al., 2012; YAMADA et al., 2017).

Therefore, the objective of this work was to produce lipid-rich thraustochytrids biomass seeking to reuse agro-industrial by-products. These by-products appear as low-cost substrates, where CSL and RYC act as the main sources of nitrogen and favor the growth and cellular development of biomass. SCM acts as the main source of carbon and contributes to the transformation and accumulation of high-value lipids in the cell. This study comprises strain improvement by UV mutagenesis integrated with microtiter-based fluorescence assay, bioprocess optimization through the design of experiments (DOE) methodology and scale-up in a bioreactor for fermentative kinetic studies (Figure 28)

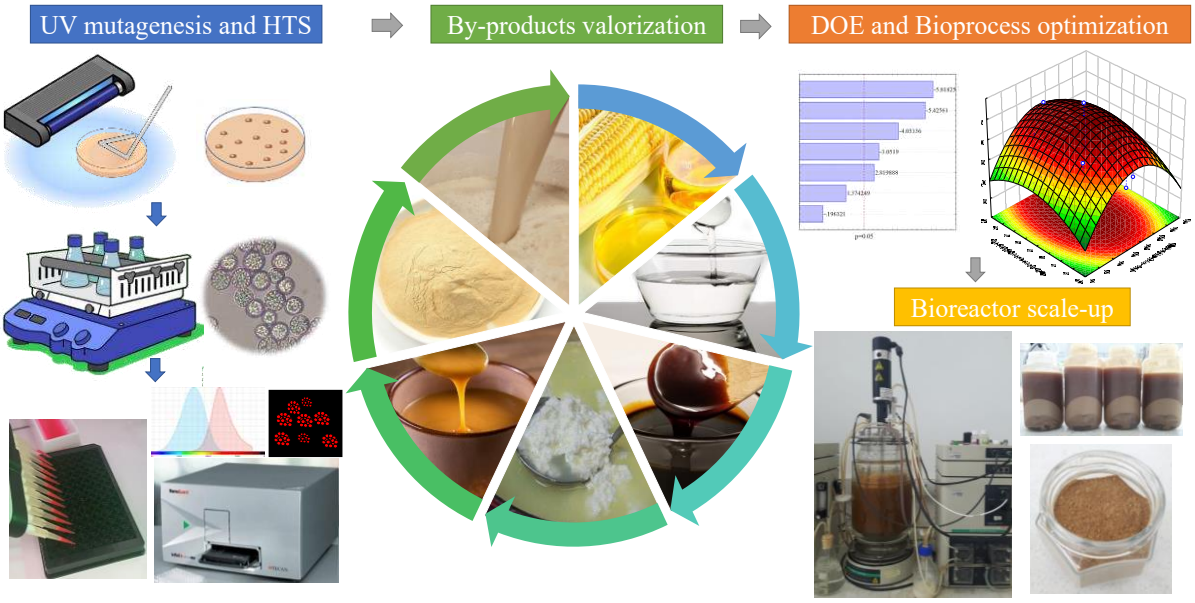

Figure 28. Flow diagram of thraustochytrid improvement for production of lipid-rich biomass.

2. MATERIALS AND METHODS

2.1 UV MUTAGENESIS

Aurantiochytrium limacinum SR21 (ATCC® MYA-1381™) was used for random selective pressure by ultraviolet (UV) irradiation. The cells were grown in shaken flasks containing 10 ml of GPYS broth (10 g/l glucose, 1 g/l peptone, 1 g/l yeast extract and 28 g/l sea salts) for 72 hours. Then, 100 µl of the cell broth was transferred and spread on GYPS agar plates. The plates were irradiated with ultraviolet C (UVC) light at 254 nm, 15W (1.5 mW/cm^2) and a distance of 30 cm. The exposure time was adjusted to obtain a survival rate of approximately 5%. After mutagenesis, the plates were incubated at 25 °C in the dark (LI et al., 2017). Approximately 100 mutant colonies were selected to assess cell growth and lipid accumulation. The lethality rate (%) and the survival rate (%) were calculated according to Eq. (1) and Eq. (2). The strains were cryopreserved at -80 °C in 20% glycerol.

$$\text{Lethality rate (\%)} = \frac{\text{control colonies} - \text{survival colonies}}{\text{control colonies}} * 100 \qquad (1)$$

$$\text{Survival rate (\%)} = 100 - \text{Lethality rate (\%)} \qquad (2)$$

2.2 SCREENING OF MUTANT LIPID ACCUMULATORS

The mutant colonies and the wild type strain were streaked on GYPS agar plates. Thereafter, an inoculum rate of 10% was added to 250 ml Erlenmeyer flasks containing 50 ml of fermentation medium (80 g/l glucose, 5 g/l yeast extract, 2 g/l peptone, 5 g/l monosodium glutamate, 28 g/l sea salt and pH 6.0). Submerged culture was performed in a shaker at 120 rpm and 28 °C for 5 days. Then, the microtiter-based fluorescence assay with Nile red was used to evaluate the accumulation of lipids emitted by fluorescence in relation to cell growth of mutants according with previous work (COLONIA et al., 2021). The relative fluorescence unit (RFU) was calculated according to Eq. (3).

$$\text{Relative Fluorescence Unit (RFU)} = \frac{\text{Fluorescence intensity (FI)}}{\text{Optical density (OD)}} \qquad (3)$$

2.3 BIOPROCESS OPTIMIZATION AND DESIGN OF EXPERIMENTS (DOE)

2.3.1 STEP 1: STUDIES OF NITROGEN AND CARBON SOURCES

The mutant strain M29 and the parent strain (wild-type) were selected for submerged cultivation. Agro-industrial by-products and chemically defined sources were used in complex and synthetic media. Both strains were grown by varying nitrogen sources (Whey, residual yeast cream, corn steep liquor, yeast extract, sodium nitrate, monosodium glutamate and urea) and fixing glucose as a carbon source. After that, the nitrogen source was selected and fixed in the culture medium, where the carbon sources were varied. The carbon sources used were cane syrup, cane molasses, high fructose corn syrup (HFCS), soft drink wastewater, granulated sugar, glycerol and glucose. The composition of the fermentation media was 100 g/l carbon source, 10 g/l nitrogen source and 28 g/l sea salts. The fermentation was performed in 250 ml Erlenmeyer flask containing 50 ml of media and pH 5.5. The inoculum rate was 10% v/v and the flasks were incubated in a shaker at 120 rpm and 28 ºC for 5 days. The data obtained were submitted to Analysis of Variance (ANOVA) and the Tukey post-variance test with 5% significance to determine the difference between the means using the software STATISTICA 7 (StatSoft).

2.3.2 STEP 2: PLACKETT–BURMAN (PB) DESIGN

A screening of fermentation variables and culture medium was carried out on a bench scale using agitated flasks. Seven different variables were tested in a Plackett–Burman design (PB12, 4 center points and -1.0, 0 and +1.0 levels) to select the significant variables in the production of biomass and lipids (Table 5). These variables were sugarcane molasses (50, 75 and 100 g/l), corn steep liquor (10, 20 and 30 g/l), residual yeast cream (2, 4 and 6 g/l), yeast extract (0, 2.5 and 5 g/l), sea salts (10, 20 and 30 g/l), pH of culture media (4.0, 6.0 and 8.0) and inoculum rate (5, 10 and 15%). The fermentation was performed in 250 ml Erlenmeyer flask containing 50 ml of media and incubated in a shaker at 120 rpm and 28 ºC for 5 days. The significant variables were selected in Pareto charts from ANOVA at 5% significance.

2.3.3 STEP 3: CENTRAL COMPOSITE DESIGN (CCD)

The variables evaluated in the Central Composite Design (CCD 2^3, 4 center points, -1.68, -1.0, 0, +1.0, +1.68 levels) to optimize the production of biomass and lipids were sugarcane molasses (66, 80, 100, 120 and 134 g/l), corn steep liquor (2, 8, 17, 26 and 32 g/l)

and residual yeast cream (2.6, 4, 6, 8 and 9.4 g/l) (Table 6). The fixed variables were inoculum rate (10%), pH of the culture medium (5.5), sea salt (10 g/l), agitation (120 rpm) and temperature (28 ºC). The cultivation took place in 250 ml flasks containing 50 ml of culture medium and incubated in a shaker for 5 days. The data was analyzed in the software STATISTICA 7 (StatSoft) using the Pareto charts from ANOVA, observed values vs. predictive values and response surface methodology (RSM) with a significance of 5%. These studies generated mathematical models for desirability profiling and optimization.

Table 5. Plackett–Burman design (PB12) for screening of significant variables in the production of biomass and lipids by wild type and mutant strain M29.

Independent variables	Levels		
	-1	0	+1
Sugarcane molasses (g/l)	50	75	100
Corn steep liquor (g/l)	10	20	30
Residual yeast cream (g/l)	2	4	6
Yeast extract (g/l)	0	2.5	5
Sea salts (g/l)	10	20	30
pH of culture media	4.0	6.0	8.0
Inoculum rate (%)	5	10	15

Table 6. Central Composite Design (CCD 23) for optimization of biomass and lipid production by wild type and mutant strain M29.

Independent variables	Levels				
	-1.68	-1	0	+1	+1.68
Sugarcane molasses (g/l)	66	80	100	120	134
Corn steep liquor (g/l)	2	8	17	26	32
Residual yeast cream (g/l)	2.6	4	6	8	9.4

2.4 SCALE-UP IN STIRRED-TANK BIOREACTOR (STBR)

The pre-inoculum was prepared in a 250 ml flask containing 70 ml of culture broth (50 g/l glucose, 5 g/l yeast extract, 2 g/l peptone and 15 g/l sea salts) and pH 6.0. The flask was incubated in a shaker at 120 rpm and 25 °C for 24 hours. Then, the inoculum was prepared in a 2 L Erlenmeyer flask containing 630 ml of culture medium (50 g/l sugarcane molasses, 10 g/l

corn steep liquor, 2 g/l residual yeast cream and 10 g/l sea salts) and pH adjusted to 6.0. The 70 ml pre-inoculum (equivalent to a 10% inoculum rate) was added to the culture medium to produce the inoculum and incubated at 120 rpm and 28 °C for 48 hours.

The submerged batch cultivation was developed on a bench scale in a 10L stirred-tank bioreactor (STBR, New Brunswick Scientific BioFlo® 110 Fermentor, Eppendorf) with a working volume of 7L (30% headspace). The bioreactor fermentation medium was 6.3 L containing 90 g/l sugarcane molasses, 25 g/l corn steep liquor, 15 g/l residual yeast cream, 10 g/l sea salts and 2 g/l monosodium glutamate. The initial pH of the culture medium was adjusted to 5.5 with KOH 5M. The bioreactor vessel was sterilized at 121 °C and 1 atm for 30 minutes. The inoculum rate was 10% (equivalent to 700 ml). The bioprocess was controlled at 25-28 °C, aeration rate at 0.5-1.0 vvm and agitation speed at 400-800 rpm to maintain a 30% dissolved oxygen (DO) for 120 hours. The foam generation was controlled by addition of food grade antifoam. Samples containing 15 ml of the culture broth were taken from the bioreactor every 12 hours and centrifuged at 5000 rpm for 10 min. The substrate consumption (S), biomass production (X) and lipid production (P) were determined and plotted for studies of batch growth kinetics as described in Table 7.

Table 7. Equations for kinetic determinations in batch bioreactor.

Kinetic determinations	**Equation**
Maximum specific growth rate (h^{-1})	$\mu_{max} = \frac{lnX_2 - lnX_1}{t_2 - t_1}$
Doubling time of cells (h)	$\tau_d = \frac{ln2}{\mu_{max}}$
Maximum biomass productivity ($g \cdot l^{-1} \cdot h^{-1}$)	$r_{X\,max} = \frac{\Delta X}{\Delta t} = \frac{X_2 - X_1}{t_2 - t_1}$
Total biomass productivity ($g \cdot l^{-1} \cdot h^{-1}$)	$r_{X\,total} = \frac{X_f - X_i}{t_f - t_i}$
Overall biomass productivity ($g \cdot h^{-1}$)	$r_{X\,overall} = r_{X\,total} * V$
Maximum substrate consumption productivity($g \cdot l^{-1} \cdot h^{-1}$)	$r_{S\,max} = \frac{\Delta S}{\Delta t} = \frac{S_1 - S_2}{t_2 - t_1}$
Total substrate consumption productivity ($g \cdot l^{-1} \cdot h^{-1}$)	$r_{S\,total} = \frac{S_i - S_f}{t_f - t_i}$
Overall substrate consumption productivity ($g \cdot h^{-1}$)	$r_{S\,overall} = r_{S\,total} * V$

Maximum product formation productivity ($g \cdot l^{-1} \cdot h^{-1}$)	$r_{P\,max} = \frac{\Delta P}{\Delta t} = \frac{P_2 - P_1}{t_2 - t_1}$
Total product formation productivity ($g \cdot l^{-1} \cdot h^{-1}$)	$r_{P\,total} = \frac{\Delta P}{\Delta t} = \frac{P_f - P_i}{t_f - t_i}$
Overall product formation productivity ($g \cdot h^{-1}$)	$r_{P\,overall} = r_{P\,total} * V$
Specific substrate consumption rate (h^{-1})	$Q_{S\,max} = r_S * \frac{1}{X}$
Specific product formation rate (h^{-1})	$Q_{P\,max} = r_P * \frac{1}{X}$
Biomass-substrate yield ($g_{biomass} \cdot g_{substrate}{}^{-1}$)	$Y_{\frac{X}{S}} = \frac{\Delta X}{\Delta S} = \frac{X_f - X_i}{S_i - S_f}$
Product-substrate yield ($g_{product} \cdot g_{substrate}{}^{-1}$)	$Y_{\frac{P}{S}} = \frac{\Delta P}{\Delta S} = \frac{P_f - P_i}{S_i - S_f}$
Product-biomass yield ($g_{product} \cdot g_{biomass}{}^{-1}$)	$Y_{\frac{P}{X}} = \frac{\Delta P}{\Delta X} = \frac{P_f - P_i}{X_f - X_i}$

2.5 BIOMASS AND LIPID DETERMINATION

Biomass or dry cell weight was determined by drying in an oven at 105 °C until constant weight. The production of lipids was determined by the cold extraction method adapted in previous work (COLONIA et al., 2021). Biomass and lipids were calculated according to Eq. (4), (5) and (6).

$$\text{Biomass (g/l)} = \frac{\text{Weight of dry biomass (g)}}{\text{Volume of fermented broth (l)}} \qquad (4)$$

$$\text{Lipid content (\%)} = \frac{\text{Weight of lipid in aliquot (g)} * \frac{\text{Volume of chloroform layer (ml)}}{\text{Volume of aliquot (ml)}}}{\text{Weight of dry biomass sample (g)}} * 100 \qquad (5)$$

$$\text{Lipids (g/l)} = \text{Biomass (g/l)} * \text{Lipid content (\%)} \qquad (6)$$

2.6 REDUCING SUGARS DETERMINATION

The supernatant was used to determine the substrate consumption (S) in bioreactor. The reducing sugars was determined by the DNS method (3,5-dinitrosalicylic acid) previously adapted (MILLER, 1959). 100 µl of the supernatant was diluted in 900 µl of distilled water

(equivalent to a dilution factor of 10). The assay was performed in 2 ml microtubes, where 25 µL of the diluted supernatant and 25 µL of the DNS reagent were added. The microtubes were boiled at 100 °C for 5 minutes. Then, the microtubes were cooled in an ice bath and 330 µl of distilled water was added. Aliquots of 300 µl were transferred in a 96-well transparent microplate with a flat bottom and absorbance was measured at 540 nm in a microplate spectrophotometer reader (Synergy™ HTX, BioTek Instruments). The concentrations of reducing sugars or substrate consumption (g/l) were determined according to the regression equation generated by a standard glucose curve (0.0-5.0 g/l).

3. RESULTS AND DISCUSSION

3.1 STRAIN IMPROVEMENT BY UV MUTAGENESIS

The UV irradiation survival and lethality rates were determined at different exposure times (0s, 10s, 15s, 20s, 30s, 60s, 90s, 150s and 210s) as shown in Figure 29. Thus, it is possible to observe a significant drop in the first 30 s of UV irradiation with 30.1% survival rate and 69.9% lethality rate. After this time, both rates increase and decrease slowly every 30 s until reaching 95.1% lethality rate and 4.9% survival rate at 150 s. This trend is similar to that reported by a recent UV irradiation study of *Aurantiochytrium limacinum* SR21, where the lethality rate increased drastically up to 40 s and between 40 - 100 s there was a slow tendency to increase up to 100% lethality rate (DUAN et al., 2019). Likewise, the mutant strain *Schizochytrium* sp. 2010-0321 (CCTCC M2011024) obtained by UV irradiation with exposure time between 70 and 90 s had a lethality rate between 60 and 80%, which allowed increase biomass (>3.0%) and DHA (>10%) production compared to wild-type (PORA; JIE, 2012).

The mutant colonies (n = 103) were recovered and cultivated for the production of biomass and lipids, among which six mutants had relative fluorescence unit (RFU) superior to the wild-type as demonstrated in Figure 30. Additionally, the post-hoc test allowed to select promissory mutants such as M29 and M14 with 6528.64±562.20 and 6279.17±725.22 RFU, respectively, compare to wild-type with 4924.36±270.17 RFU. In addition, the mutants M13, M19, M22 and M40 had RFU values higher than the wild-type, but they did not show significant statistical differences between them and the wild-type when grouped in the Tukey HSD test at 5% significance.

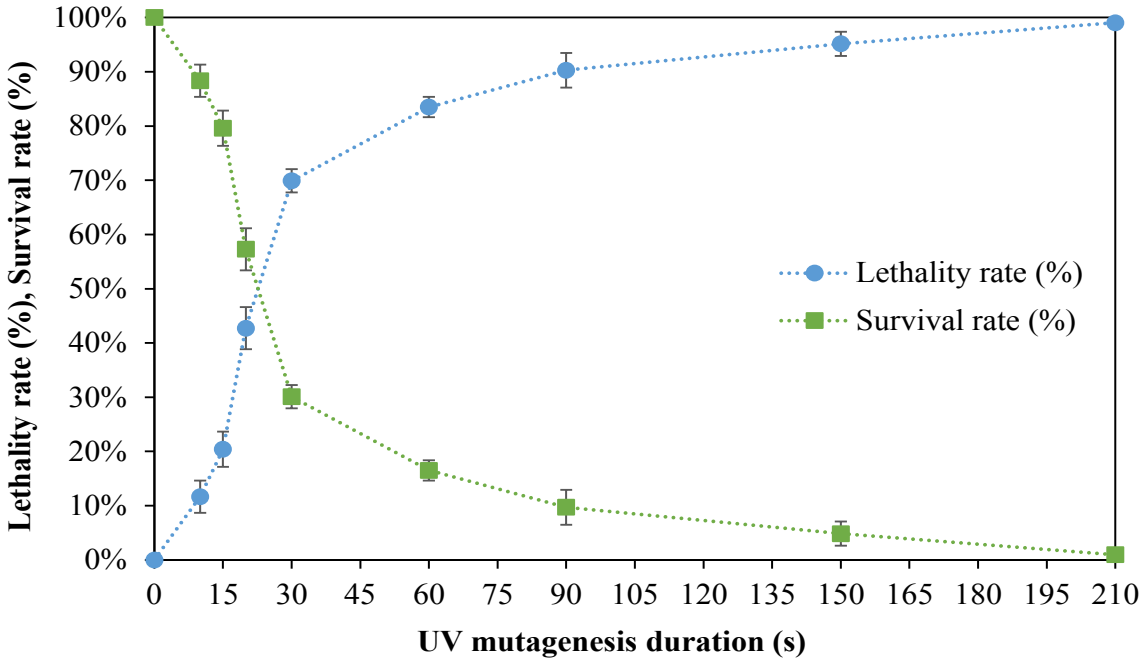

Figure 29. Lethality and survival rate curves of *Aurantiochytrium limacinum* SR21 under various UV mutagenesis time.

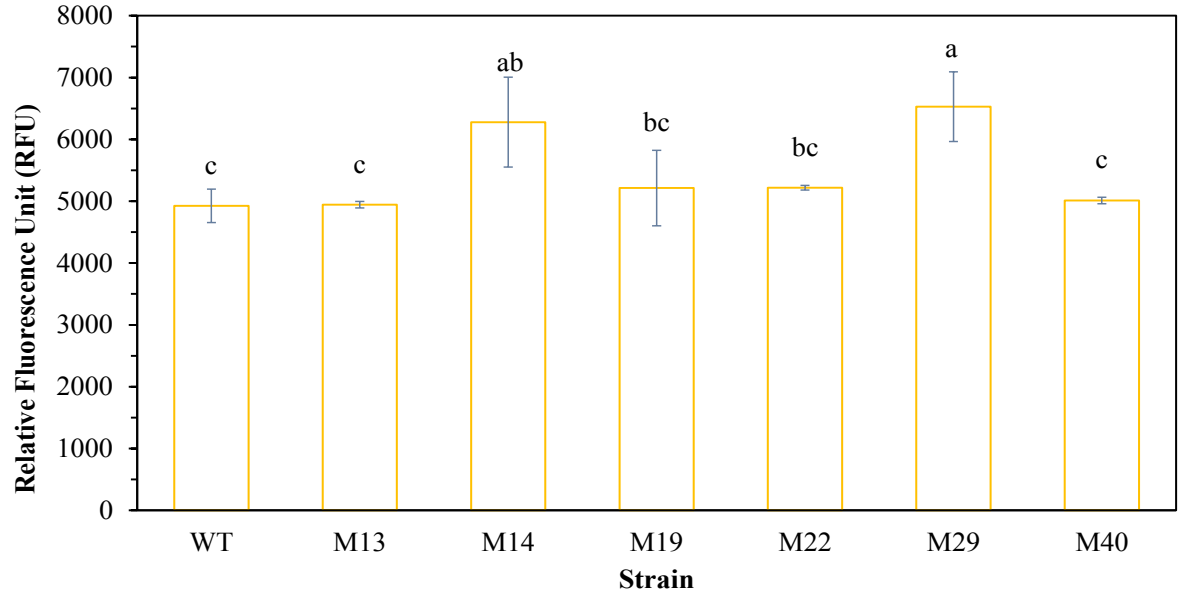

Figure 30. Selection of mutants obtained from *Aurantiochytrium limacinum* SR21 (WT: Wild Type) by microtiter-based fluorescence assay.

The relative increments in lipid accumulation (FI: Fluorescence intensity), biomass production (OD: Optical density) and lipid yield in relation to biomass ($Y_{P/X}$: FI/OD) were compared to the wild-type (Figure 31). Mutants M29 and M22 had an increase of 26% (1.26-fold) and 23% (1.23-fold) in the accumulation of lipids (FI), respectively. On the other hand, no mutant had an increase greater than 20% (>1.2-fold) in the production of biomass (DO). In

addition, lipid yield in relation to biomass (FI/OD) had mutants M29 and M14 highlighted with 33% (1.33-fold) and 28% (1.28-fold) increase, respectively. Both mutants were obtained with a UV mutagenesis dose of 15W (1.5 mW/cm^2) and 30 s. These results could be explained by the effect of UV mutagenesis on genes related to the accumulation of lipids. Likewise, UV irradiation at 50 W for 30 s was selected as the indicated mutagenesis dose for *Aurantiochytrium* sp. PKU#Mn16 to obtain mutant colonies with increased biomass (1.53-fold), lipids (1.79-fold) and DHA (1.90-fold) (LIU et al., 2020). They also studied important genes related to the polyketide synthase (PKS) pathway and lipid accumulation through comparative transcriptomic analysis, where they observed positive regulation of these genes in the mutant strain when compared to negative regulation in the wild type.

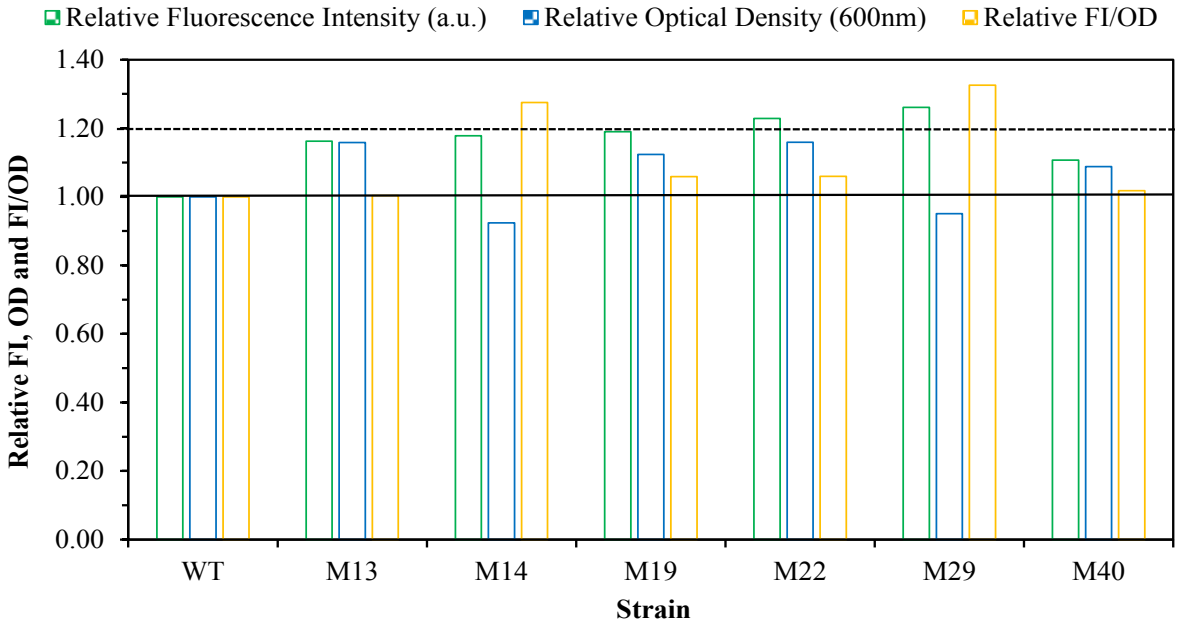

Figure 31. Relative fluorescence intensity (FI), optical density (OD) and FI/OD of mutants. Relative values for wild-type (*Aurantiochytrium limacinum* SR21) were set to 1.0 (solid line) and relative values with more than 20% increase were set to 1.2 (dotted line).

Moreover, UV mutagenesis was also applied to *Schizochytrium limacinum* B4D1 (CGMCC 8313), where the mutants did not obtain significant differences in the production of biomass and lipids, however the mutant strain F6 had differences in the fatty acid profile with decreased SFAs (saturated fatty acids) and increased PUFAs (polyunsaturated fatty acids). These increases in PUFAs were reflected in the 11.2% (1.11-fold) increase in DHA in the F6 mutant compared to the wild-type (LI et al., 2017). In addition, chemical-physical mutagenesis (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine coupled with UV radiation) was applied to the parental

strain *Schizochytrium* sp. (CCTCC M209059), where increments of 34.84% in lipids and 38.88% in DHA were obtained by the mutant strain *Schizochytrium* sp. HX-308M (LIAN et al., 2010).

On the other hand, recent physical mutagenesis techniques such as low-energy ion implantation, heavy-ions irradiation and atmospheric and room-temperature plasma (ARTP) have been applied to *Aurantiochytrium* sp. and *Schizochytrium* sp. seeking to improve strains. Low-energy N^+ ion implantation was applied to *Schizochytrium* sp. ATCC20888, where four mutant colonies had increments greater than 20% (> 1.20-fold) in biomass, lipids and DHA (FU et al., 2016). Similarly, heavy-ion irradiation with carbon ion was applied to *Aurantiochytrium* sp. CGMCC 6208, where the T-99 mutant increased DHA productivity (g/l.h) by 50% and DHA yield (g/l) by 30% in 5L fed-batch fermentation (CHENG et al., 2016). Otherwise, ARTP mutagenesis was used in *Schizochytrium* sp. S31 (ATCC 20888), where the lethality rate reached 80% in 15s and the mz-17 mutant had an increase of 31% in the lipid content and 26% in the DHA content compared to the wild-type (ZHAO et al., 2018).

3.2 EFFECT OF NITROGEN AND CARBON SOURCE

The study of the effects of the nitrogen source was carried out by fixing glucose as a carbon source and varying the different alternative sources of nitrogen (Figure 32A). Both the M29 mutant and the wild-type showed higher biomass production with corn steep liquor (CSL) and residual yeast cream (RYC). The accumulation and production of lipids was mainly favored by yeast extract, followed by CSL and RYC. Then, CSL and RYC were fixed as nitrogen sources by varying carbon sources (Figure 32B). Glucose and sugarcane molasses (SCM) had the best biomass production by the M29 and wild-type, showing no significant differences. Glucose and yeast extract have been the most used sources of carbon and nitrogen in thraustochytrids culture medium for the production of high-value lipids (SHENE et al., 2020c; SOHEDEIN et al., 2020a). Glucose was more prominent in the accumulation of lipids for both strains with 25.22 and 20.05% lipid content in M29 and wild-type respectively, followed by SCM. This can be explained by the fact that glucose is fully fermentable sugars easily converted in lipid biosynthesis metabolism by thraustochytrids (MORABITO et al., 2019). In contrast, raw sugarcane molasses, being a more complex source, presents reducing sugars (glucose and fructose) and non-reducing sugars (sucrose), as well as a low protein content (around 3%) and ash (about 10%) (CARIOCA; LEAL, 2011; NIKODINOVIC-RUNIC et al., 2013). Therefore, M29 and wild-type had 16.11 and 15.44 g/l biomass, 17.50 and 14.85% lipid content, and 2.82

and 2.30 g/l lipids, respectively, when cultivated with SCM, CSL and RYC. Thus, these cheaper alternative sources had promising results to replace glucose and yeast extract.

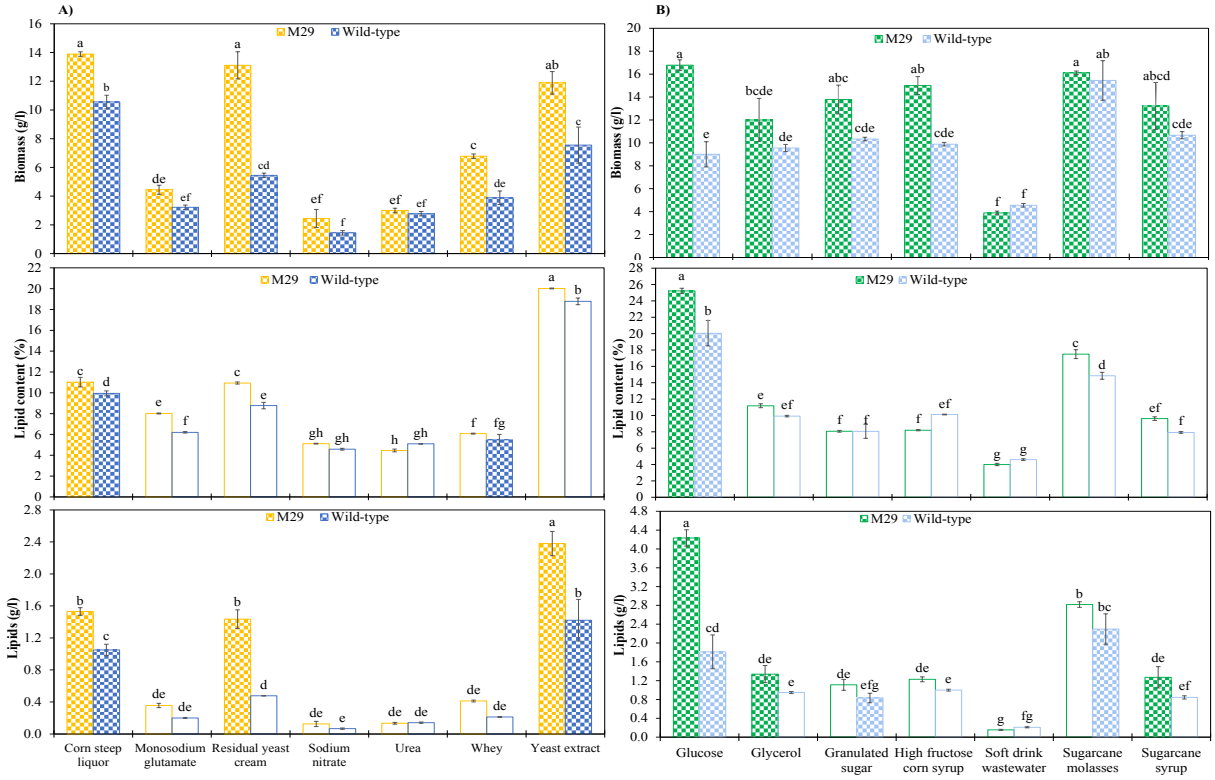

Figure 32. Biomass and lipid production by the mutant strain M29 and wild-type using agro-industrial by-products and synthetic sources. A) Nitrogen source. B) Carbon source.

3.3 SCREENING OF BIOPROCESS VARIABLES

Seven variables were evaluated in Plackett–Burman design in order to select the significant variables in the production of biomass and lipids (Table 8). Concentrations of SCM, CSL, RYC and sea salts, pH variations and inoculum rates were evaluated. Yeast extract was also evaluated in order to test the possible inclusion or not in the fermentation. The maximum production values by the M29 mutant were 32.20 g/l of biomass (Run 6), 37.07% lipid content (Run 12) and 5.36 g/l of lipids (Run 5). Otherwise, the wild type had its maximum values with 27.60 g/l of biomass (Run 7), 28.71% lipid content (Run 1) and 3.04 g/l of lipids (Run 1).

Additionally, Figure 33 shows the significant variables highlighted in the Pareto chart. The biomass produced by the mutant strain M29 showed CSL (+1), RYC (+1), SCM (+1) and sea salts (+1) as significant variables. However, the lipids of the M29 mutant strain had only the pH of the culture medium (-1) as a significant variable. At the same time, wild-type biomass presented CSL (+1) and pH (+1) as significant variables. The wild-type lipid content had pH (-

1), CSL (-1), sea salt (-1), yeast extract (-1) and SCM (+1) as significant variables. However, the production of lipids by the wild-type showed that only the SCM (+1) was significant.

It can be observed that the significant values and their increasing or decreasing trends differ depending on the metabolic route, whether for biomass production or lipid accumulation. Thus, the manipulation of cell metabolism through nutrients and fermentation conditions are important in the production of lipid-rich biomass. Pareto graphs suggest increases in CSL to promote cell growth or biomass production and a decrease in pH along with an increase in SCM in lipid production. This can be explained by the fact that the cell uses high concentrations of sugars to promote cell weight gain through the accumulation of lipids under nitrogen-limited conditions.

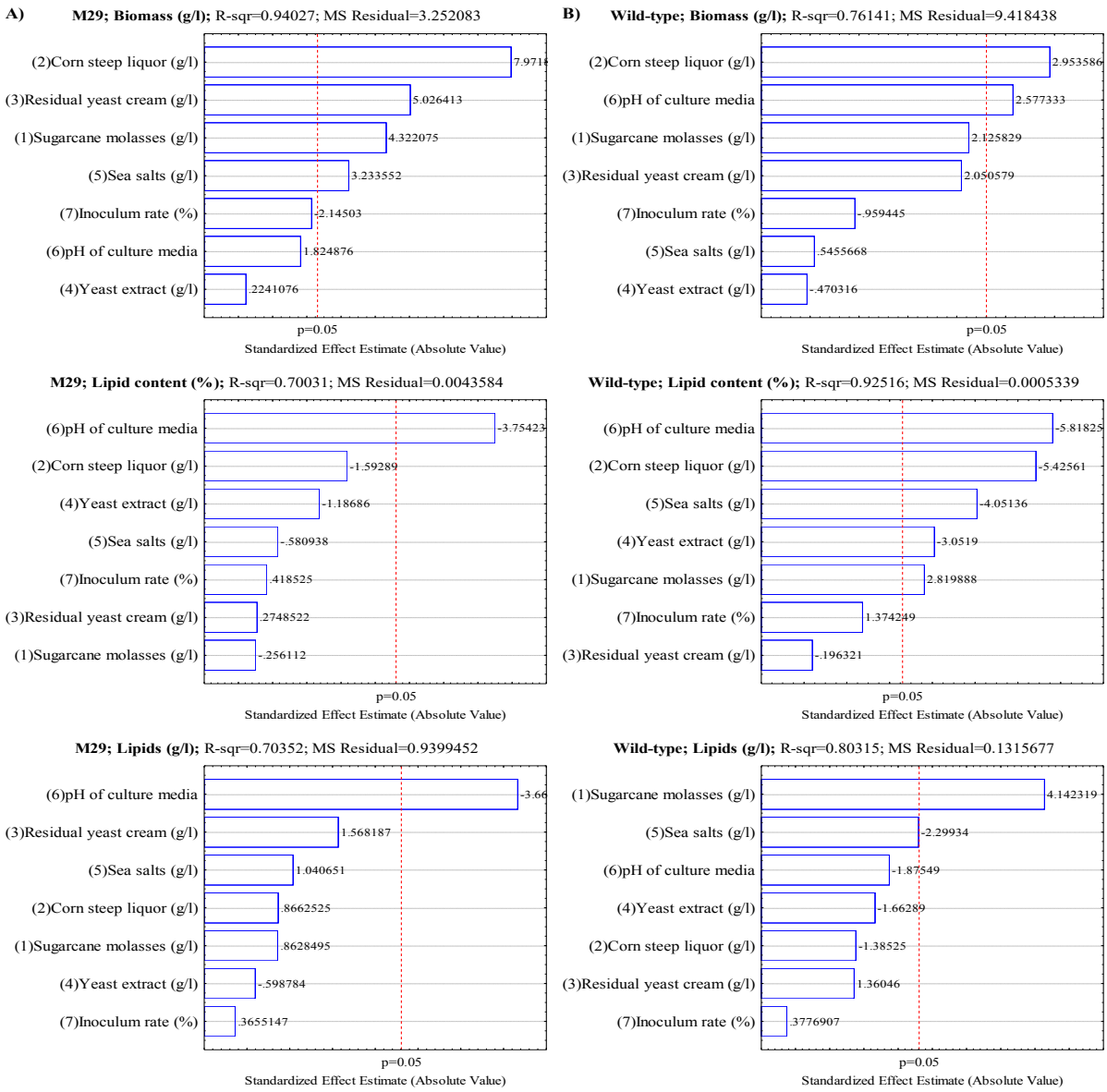

Figure 33. Pareto chart of standardized effects of Plackett – Burman design (7 factor screening) for biomass and lipid production. A) Mutant strain M29. B) Wild-type strain.

Table 8. Plackett – Burman design (PB12 + 4 center points) for the production of biomass and lipids by the mutant strain M29 and wild-type.

Run	Independent variables							Dependent variables					
								M29			WT		
	Sugarcane molasses (g/l)	Corn steep liquor (g/l)	Residual yeast cream (g/l)	Yeast extract (g/l)	Sea salts (g/l)	pH of culture media	Inoculum rate (%)	Biomass (g/l)	Lipid content (%)	Lipids (g/l)	Biomass (g/l)	Lipid content (%)	Lipids (g/l)
1	100.0	10.0	6.0	0.0	10.0	4.0	15.0	14.20	30.00%	4.26	10.60	28.71%	3.04
2	100.0	30.0	2.0	5.0	10.0	4.0	5.0	22.40	11.86%	2.66	12.80	14.57%	1.87
3	50.0	30.0	6.0	0.0	30.0	4.0	5.0	25.80	19.36%	4.99	12.00	8.79%	1.05
4	100.0	10.0	6.0	5.0	10.0	8.0	5.0	21.00	11.07%	2.33	16.00	13.07%	2.09
5	100.0	30.0	2.0	5.0	30.0	4.0	15.0	22.60	23.71%	5.36	14.40	13.14%	1.89
6	100.0	30.0	6.0	0.0	30.0	8.0	5.0	32.20	10.21%	3.29	27.60	7.79%	2.15
7	50.0	30.0	6.0	5.0	10.0	8.0	15.0	23.60	11.57%	2.73	17.00	8.64%	1.47
8	50.0	10.0	6.0	5.0	30.0	4.0	15.0	16.20	22.57%	3.66	9.40	13.93%	1.31
9	50.0	10.0	2.0	5.0	30.0	8.0	5.0	12.20	7.29%	0.89	9.60	6.14%	0.59
10	100.0	10.0	2.0	0.0	30.0	8.0	15.0	18.40	11.86%	2.18	11.60	15.71%	1.82
11	50.0	30.0	2.0	0.0	10.0	8.0	15.0	15.60	6.71%	1.05	13.60	7.07%	0.96
12	50.0	10.0	2.0	0.0	10.0	4.0	5.0	10.40	37.07%	3.86	8.80	25.86%	2.28
13 (C)	75.0	20.0	4.0	2.5	20.0	6.0	10.0	19.80	12.93%	2.56	16.40	11.92%	1.96
14 (C)	75.0	20.0	4.0	2.5	20.0	6.0	10.0	21.00	9.21%	1.94	16.40	13.00%	2.13
15 (C)	75.0	20.0	4.0	2.5	20.0	6.0	10.0	21.60	8.79%	1.90	17.20	12.43%	2.14
16 (C)	75.0	20.0	4.0	2.5	20.0	6.0	10.0	19.00	9.93%	1.89	16.80	11.79%	1.98

3.4 BIOPROCESS MODELLING AND OPTIMIZATION

Variables such as inoculum rate (10%), medium pH (5.5) and sea salt (10 g/l) were fixed. The yeast extract variable was not statistically significant in most Pareto charts; therefore, it was not included in the CCD. Thus, the most relevant variables such as SCM, CSL and RYC were evaluated in the central composite design (CCD 2^3) to optimize the production of biomass and lipids (Table 9).

The M29 mutant strain had the best results with 21.22 g/l biomass production (run 8), 35.14% lipid content (run 11) and 5.40 g/l lipids (run 9). The wild-type strain had the best biomass production with 17.60 g/l (run 3), the lipid content with 29.14% (run 9) and the lipid production with 4.49 g/l (run 9). Likewise, researchers obtained 25 g/l biomass and 25.5% lipid content (equivalent to 6.38 g/l lipids) after optimization by CCD with 70 g/l SCM, 30 g/l CSL and 30 g/l sodium glutamate grown in 500 mL shake flask, 165 rpm and temperature shifted from 28 to 20 ºC for 4 days by the mutant strain *Schizochytrium* sp. SHG104 (PARK et al., 2018a).

Pareto graphs show that increasing CSL and RYC to promote biomass production has significant linear effects on both strains (Figure 34). A justification for the SCM not being more significant in wild-type biomass is the fact that it reached optimal concentrations, contrary to the production of lipids where the decrease in SCM (linear effect) is significant even with a quadratic effect significant positive. This behavior suggests that high SCM inputs could cause cell inhibition by the substrate, but they are important to increase lipid accumulation by the cell.

On the other hand, the linear effect of RYC was not significant on wild-type lipid accumulation and the decrease in CSL has a significant quadratic effect on lipid accumulation. In contrast, the decrease of the three variables (CSL, RYC and SCM) would be beneficial for the accumulation of lipids in the mutant strain M29, which suggests that optimal production regions have not yet been found. Thus, it is possible to observe the differences between M29 and wild-type in the different nutrient requirements for biomass and lipid production.

Table 9. Central Composite Design (CCD 2^3 + 4 center points) for the production of biomass and lipids by the mutant strain M29 and wild-type.

Run	Independent variables			Dependent variables					
				M29			WT		
	X_1 Sugarcane molasses (g/l)	X_2 Corn steep liquor (g/l)	X_3 Residual yeast cream (g/l)	Y_1 Biomass (g/l)	Y_2 Lipid content (%)	Y_3 Lipids (g/l)	Y_1 Biomass (g/l)	Y_2 Lipid content (%)	Y_3 Lipids (g/l)
1	80.0	8.0	4.0	10.60	23.14%	2.45	6.00	9.07%	0.54
2	80.0	8.0	8.0	14.20	22.14%	3.14	11.00	21.29%	2.34
3	80.0	26.0	4.0	18.60	24.29%	4.52	17.60	15.79%	2.78
4	80.0	26.0	8.0	20.00	17.57%	3.51	15.40	14.93%	2.30
5	120.0	8.0	4.0	13.00	23.79%	3.09	6.60	7.79%	0.51
6	120.0	8.0	8.0	16.20	21.29%	3.45	8.80	6.93%	0.61
7	120.0	26.0	4.0	18.80	23.00%	4.32	14.40	14.50%	2.09
8	120.0	26.0	8.0	21.22	17.36%	3.68	17.40	10.79%	1.88
9	66.0	17.0	6.0	16.80	32.14%	5.40	15.40	29.14%	4.49
10	134.0	17.0	6.0	15.60	22.00%	3.43	16.00	24.07%	3.85
11	100.0	2.0	6.0	12.33	35.14%	4.33	6.40	14.71%	0.94
12	100.0	32.0	6.0	20.00	18.00%	3.60	17.40	13.29%	2.31
13	100.0	17.0	2.6	11.20	21.79%	2.44	13.00	24.07%	3.13
14	100.0	17.0	9.4	16.60	18.36%	3.05	16.20	14.07%	2.28
15 (C)	100.0	17.0	6.0	14.80	18.71%	2.77	14.00	18.79%	2.63
16 (C)	100.0	17.0	6.0	14.60	18.36%	2.68	15.00	19.64%	2.95
17 (C)	100.0	17.0	6.0	14.20	19.79%	2.81	14.60	18.14%	2.65
18 (C)	100.0	17.0	6.0	14.20	19.36%	2.75	15.60	17.71%	2.76

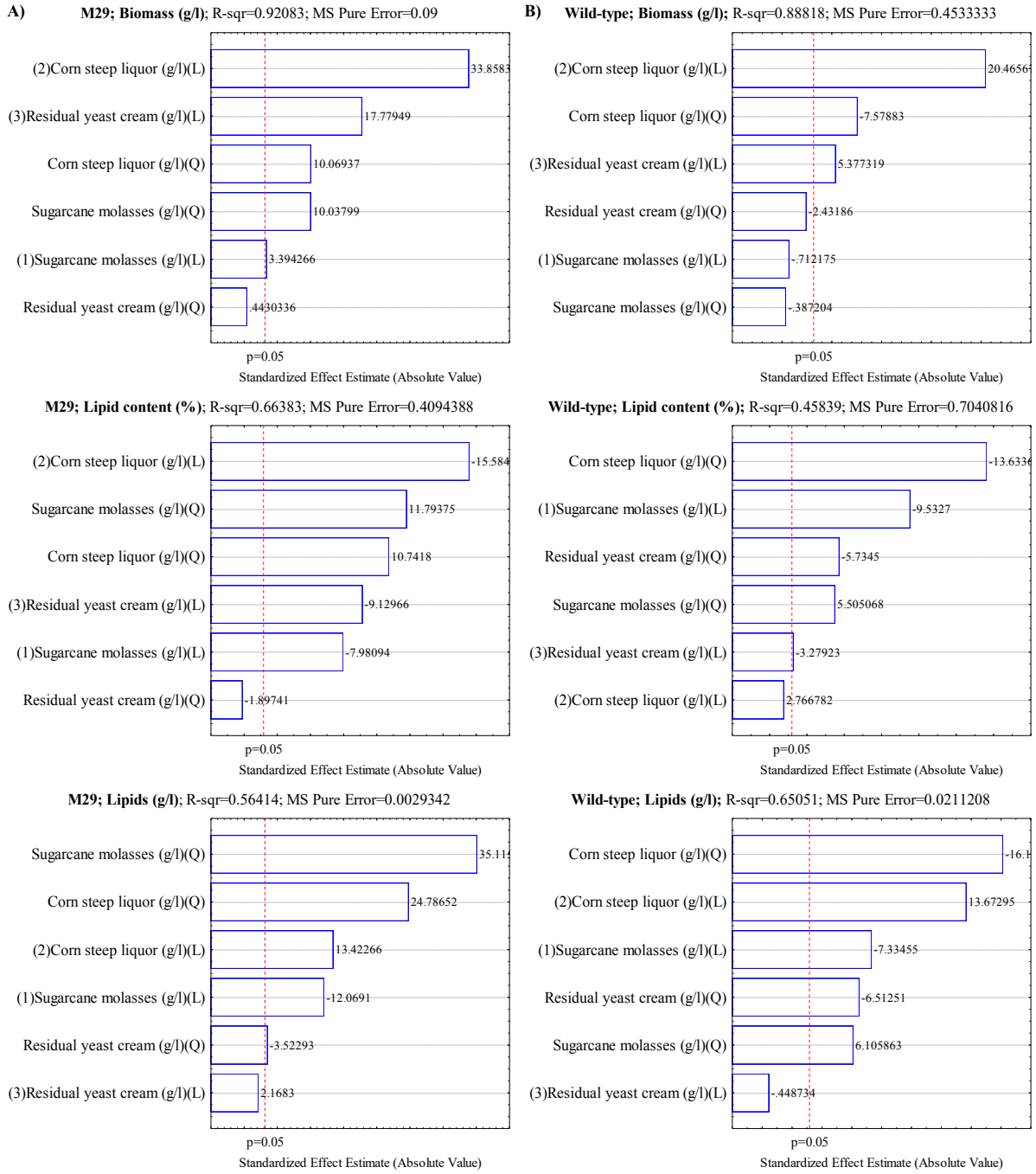

Figure 34. Pareto chart of standardized effects in the central composite design (CCD) for biomass and lipid production. A) Mutant strain M29. B) Wild-type strain

Moreover, the CCD allowed the generation of mathematical regression models for both strains that best fit the response variables (Table 10). Four models were evaluated according to R^2, adjusted R^2 and F-test for lack of fit. The linear/quadratic model with main effects was selected for the three response variables (Y_1, Y_2 and Y_3) and represented by Eq. (7).

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_1^2 + \beta_3 X_2 + \beta_4 X_2^2 + \beta_5 X_3 + \beta_6 X_3^2 \quad (7)$$

Table 10. Regression coefficients of linear/quadratic main effects model for prediction of biomass and lipid production by mutant strain M29 and wild-type.

Independent variable (X)	Regression coefficient	M29		
		Dependent variable (Y)		
		Y_1: Biomass (g/l)	Y_2: Lipid content (%)	Y_3: Lipids (g/l)
Mean/Intercept	β_0	27.7359	92.0557	17.2163
X_1: Sugarcane molasses (g/l)	β_1	-0.4026	-1.1120	-0.2718
X_1^2: Sugarcane molasses (g/l)	β_2	0.0021	0.0052	0.0013
X_2: Corn steep liquor (g/l)	β_3	-0.0558	-1.1253	-0.1391
X_2^2: Corn steep liquor (g/l)	β_4	0.0107	0.0242	0.0047
X_3: Residual yeast cream (g/l)	β_5	0.6082	0.2202	0.1741
X_3^2: Residual yeast cream (g/l)	β_6	0.0092	-0.0839	-0.0132
R^2		0.921	0.664	0.564
Adjusted R^2		0.878	0.480	0.326
Independent variable (X)	Regression coefficient	Wild-type		
		Dependent variable (Y)		
		Y_1: Biomass (g/l)	Y_2: Lipid content (%)	Y_3: Lipids (g/l)
Mean/Intercept	β_0	-5.5503	38.9751	4.6544
X_1: Sugarcane molasses (g/l)	β_1	0.0296	-0.7463	-0.1370
X_1^2: Sugarcane molasses (g/l)	β_2	-0.0002	0.0032	0.0006
X_2: Corn steep liquor (g/l)	β_3	1.0278	1.4421	0.3412
X_2^2: Corn steep liquor (g/l)	β_4	-0.0180	-0.0404	-0.0083
X_3: Residual yeast cream (g/l)	β_5	1.8458	3.6205	0.7763
X_3^2: Residual yeast cream (g/l)	β_6	-0.1132	-0.3326	-0.0654
R^2		0.888	0.458	0.651
Adjusted R^2		0.827	0.163	0.460

3.5 PREDICTION AND DESIRABILITY PROFILING

These models generated predictive values compared to the observed ones (Figure 35). Biomass points by M29 and wild-type were close to the trend line and within the regression limits, and few values were outside the confidence area. This can be explained by the fact that the R^2 was higher in biomass production and had a better fit to the data obtained. However, the lipid points became more spread out and some even left the regression area. The R^2 of lipids were lower, but with statistically significant predictors. Some justifications such as errors in the optimization and experimentation process, variability in cell duplication, cell stress or changes in lipid metabolism could explain this behavior.

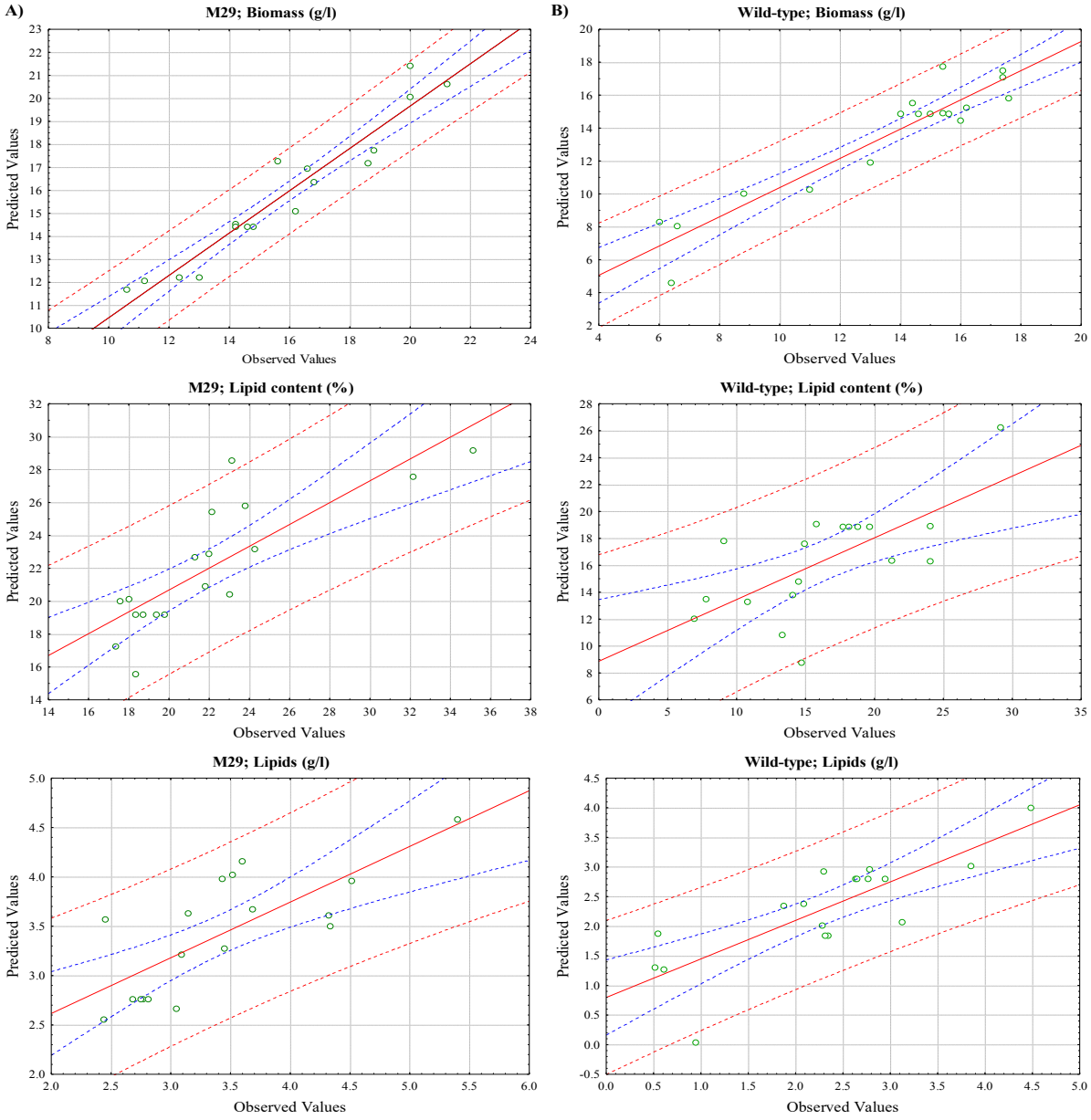

Figure 35. Predicted vs. observed values for biomass and lipids production with 5% confidence level (blue dotted line) and prediction level (red dotted line). A) Mutant strain M29. B) Wild-type strain.

The desirability index (DI) was evaluated considering possible combinations for multicriteria optimization (Table 11). It is possible to observe that considering only the biomass variable (Y_1) in the optimization, high values of SCM (X_1), CSL (X_1) and RYC (X_3) would reach the highest production by the predictive model with DI of 1.0 for both strains. However, this criterion does not take into account the production of lipids (Y_2), which, if analyzed individually, suggests low values of SCM, SCL and RYC to promote the accumulation of lipids with DI of 1.0 and 0.87 for M29 and wild-type, respectively. On the other hand, considering the criteria (Y_1 and Y_2) the DI is 0.84 and 0.87 for M29 and wild-type, respectively. This last

result coincides with the multicriteria of Y_1, Y_2 and Y_3 using the same predictive values for both strains. Therefore, the multicriteria Y_1, Y_2 and Y_3 would be ideal for considering the three response variables for biomass (g/l), lipid content (%) and lipids (g/l) with an overall desirability of 0.89 and 0.87 for M29 and wild-type, respectively.

Table 11. Analysis of possible combinations of response variables with optimized values obtained from prediction and desirability profiles for mutant strain M29 and wild-type strain.

Parameters	Response variables combinations						
Mutant strain M29	Y_1	Y_2	Y_3	Y_1, Y_2	Y_1, Y_3	Y_2, Y_3	Y_1, Y_2, Y_3
X_1: Sugarcane molasses (g/l)	134.0	66.0	66.0	66.0	66.0	66.0	66.0
X_2: Corn steep liquor (g/l)	32.0	2.0	32.0	32.0	32.0	2.0	32.0
X_3: Residual yeast cream (g/l)	9.4	7.7	9.4	2.6	9.4	6.0	2.6
Predicted Biomass (g/l)	26.82	-	-	21.00	25.89	-	21.00
Predicted Lipid content (%)	-	35.95	-	30.21	-	37.53	30.21
Predicted Lipids (g/l)	-	-	5.88	-	5.88	5.32	5.77
Desirability index	1.00	1.00	1.00	0.84	1.00	0.99	0.89
Wild-type strain	Y1	Y2	Y3	Y1, Y2	Y1, Y3	Y2, Y3	Y1, Y2, Y3
X_1: Sugarcane molasses (g/l)	83.0	66.0	66.0	66.0	66.0	66.0	66.0
X_2: Corn steep liquor (g/l)	32.0	17.0	17.0	24.5	24.5	17.0	24.5
X_3: Residual yeast cream (g/l)	7.7	6.0	6.0	6.0	6.0	6.0	6.0
Predicted Biomass (g/l)	17.62	-	-	17.00	17.00	-	17.00
Predicted Lipid content (%)	-	26.23	-	24.48	-	26.23	24.48
Predicted Lipids (g/l)	-	-	3.99	-	3.98	3.99	3.98
Desirability index	1.00	0.87	0.88	0.87	0.91	0.87	0.87

The prediction profile shows the desirability for the selected multicriteria (Y_1, Y_2 and Y_3) of the M29 mutant (Figure 36). Thus, the best combination to meet multiple responses would be an optimized media with SCM (66 g/l), CSL (32 g/l) and RYC (2.6 g/l) that would achieve a desirability of 0.89. The predicted biomass value would be 21.00 g/l with prediction limits between 19.31 and 22.70 g/l. The predicted lipid content value would be 30.21 % with prediction limits between 26.60 and 33.82%. The predicted lipid value would be 5.77 g/l with prediction limits between 5.46 and 6.08 g/l. Thus, it would be necessary to obtain biomass values of 21.22 g/l, lipid content of 35.14% and lipid production of 5.40 g/l for the three variables to reach DI of 1.0.

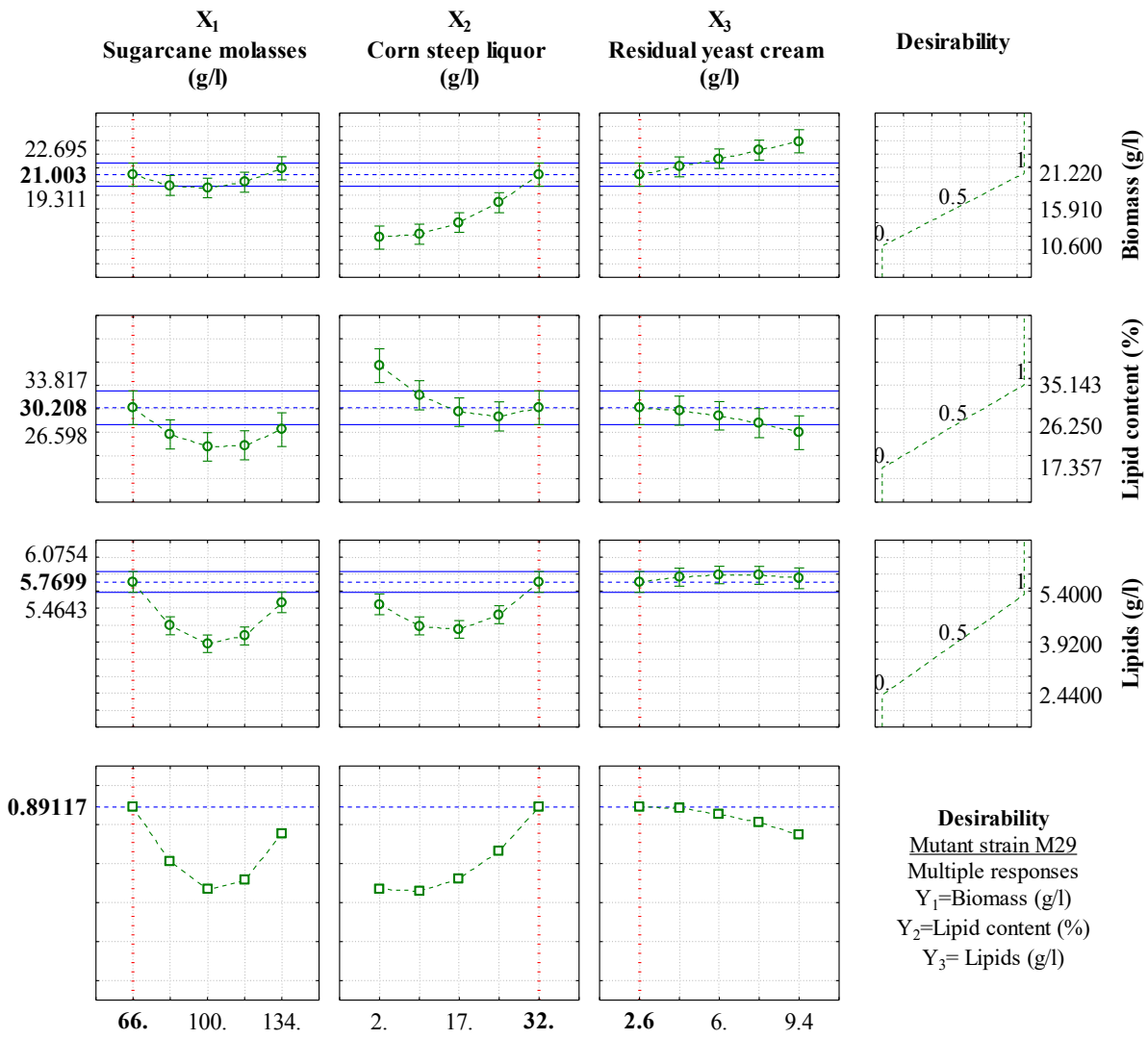

Figure 36. Prediction profiler and desirability for optimization of multiple responses (Y_1, Y_2, Y_3) by mutant strain M29.

The wild-type prediction profile shows the desirability for the selected multicriteria (Y_1, Y_2 and Y_3) presented an DI of 0.87 (Figure 37). Thus, the best combination to meet multiple responses would be an optimized media with SCM (66 g/l), CSL (24.5 g/l) and RYC (6 g/l). The predicted biomass value would be 17.00 g/l with prediction limits between 14.24 and 19.75 g/l. The predicted lipid content value would be 24.49% with prediction limits between 21.06 and 27.92%. The predicted lipid value would be 4.00 g/l with prediction limits between 3.39 and 4.57 g/l. Thus, for the three variables to reach DI of 1.0, it would be necessary to obtain biomass values of 17.60 g/l, lipid content of 29.14% and lipid production of 4.49 g/l.

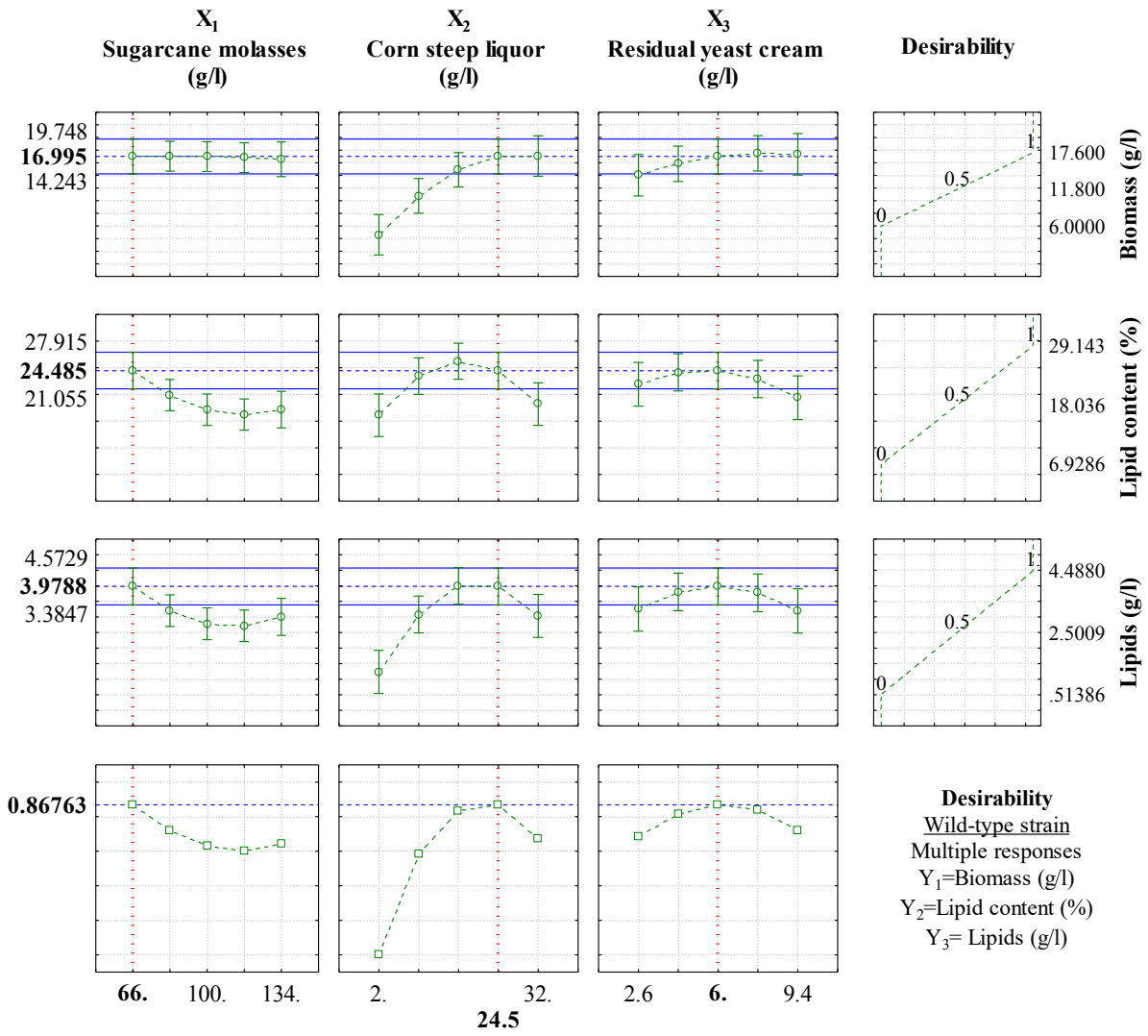

Figure 37. Prediction profiler and desirability for optimization of multiple responses (Y_1, Y_2, Y_3) by wild-type strain.

Subsequently, the response surfaces were generated once the optimized values of the culture medium that met the multiple responses of the M29 mutant strain were selected and fixed on the predicted values (Figure 38). The trend of biomass production would be to increase while the concentration of CSL and RYC in the medium decreases. On the other hand, the SCM even fixed at 66 g/l covers the entire surface area between the minimum and maximum values tested, this is confirmed when the desirability analysis shows that considering only the biomass (Y_1) the SCM concentration should be 134 g/l as shown in Table 11. In addition, the lipid response surface shows a tendency to increase when SCM and CSL decrease. The surfaces show that the production of biomass and lipids has not yet reached an optimal production region, suggesting further optimization steps to find these optimal points.

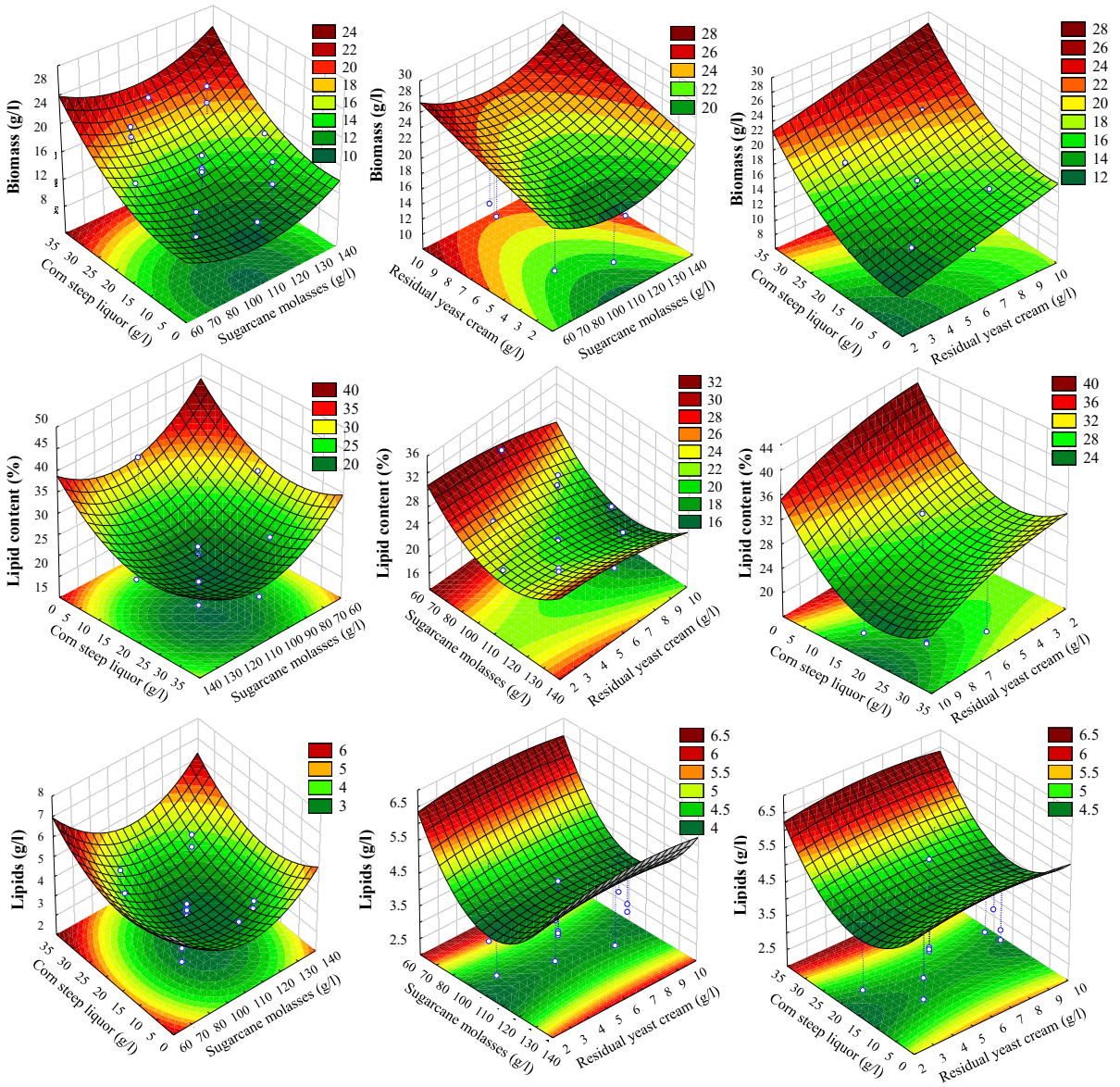

Figure 38. Response surfaces generated by mathematical regression models from the central composite design (CCD 2^3) for bioprocess optimization by the M29 mutant strain. Sugarcane molasses (X_1: 66 g/l), Corn steep liquor (X_2: 32 g/l) and Residual yeast cream (X_3: 2.6 g/l) were fixed at the optimized values according to the prediction and overall desirability profiles for multiple responses.

Furthermore, the response surfaces generated by wild-type regression models favored the fixed predictive values allowing visualization of optimized regions (Figure 39). The range of tested SCM values covered the entire optimal biomass production area, which is why the Pareto chart (Figure 34) showed that SCM was not a significant variable as it was already within the optimal region. Otherwise, the SCM set at 66 g/l allowed to generate a more optimized response surface for biomass production with the CSL and RYC variables within the optimal values. The same phenomenon happened with the surfaces generated for the lipids with well

demarcated optimized areas. However, the surfaces still show a tendency to increase lipids with decreasing SCM and allowed to visualize the beginning of the optimal region.

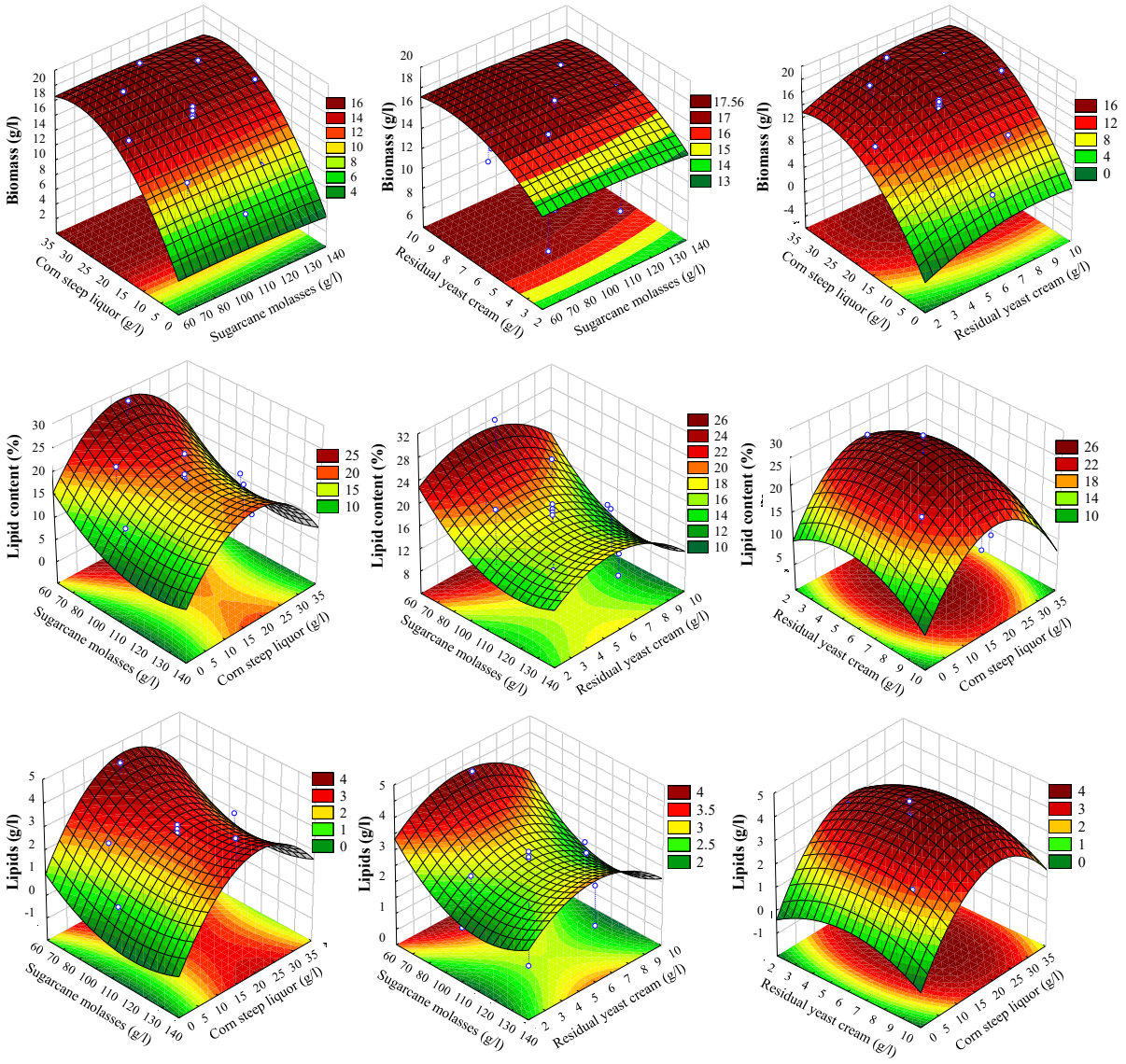

Figure 39. Response surfaces generated by mathematical regression models from the central composite design (CCD 2^3) for bioprocess optimization by the wild-type strain. Sugarcane molasses (X_1: 66 g/l), Corn steep liquor (X_2: 24.5 g/l) and Residual yeast cream (X_3: 6 g/l) were fixed at the optimized values according to the prediction and overall desirability profiles for multiple responses.

The predictive values of multiple responses were experimentally verified in culture in 250 ml shake flasks for both strains (Table 12). All experimental values obtained were within the prediction intervals determined by the regression model. The prediction intervals of the M29 mutant are even higher than that of the wild-type. However, it is possible to observe that despite the mutant strain having stood out over the wild-type throughout the optimization process, the

experimental values obtained show a great proximity between them. The increase in M29 was more predominant in biomass production (1.15-fold), but the lipid content (1.07-fold) was not much different with the wild-type. The increase in lipids (1.25-fold) seemed promising, but it is highly influenced by the multiplication of biomass by the lipid content. This can also be explained by the fact that the wild-type is in the optimized region, but the M29 mutant would need other steps to increase its production.

Table 12. Experimental data obtained compared to predictive data after selection of optimized variables of SCM, CSL and RYC for mutant M29 and wild-type.

Strain	Parameters	Biomass (g/l)	Lipid content (%)	Lipids (g/l)
M29	Measured values	19.98	27.25	5.51
	Predicted values	21.00	30.21	5.77
	-95% Confidence	19.61	27.23	5.52
	+95% Confidence	22.40	33.19	6.02
	-95% Prediction	19.31	26.60	5.46
	+ 95% Prediction	22.70	33.82	6.08
	Pure error	0.09	0.41	0.00
Wild-type	Measured values	17.30	25.49	4.41
	Predicted values	17.00	24.49	3.98
	-95% Confidence	15.27	22.33	3.61
	+95% Confidence	18.72	26.64	4.35
	-95% Prediction	14.24	21.05	3.38
	+ 95% Prediction	19.75	27.92	4.57
	Pure error	0.45	0.70	0.02

The increments generated between the start of the optimization step (step 1) and the final optimization step (step 3) are described in Table 13. The M29 mutant had increases in biomass, lipid content and lipids of 24.01% (1.24-fold), 55.76% (1.56-fold) and 95.51% (1.96-fold), respectively. Likewise, the wild-type had increases in biomass, lipid content and lipids by 12.01% (1.12-fold), 71.66% (1.72-fold) and 91.98% (1.92-fold), respectively. It is important to highlight that only the steps of optimization of carbon/nitrogen sources, process variables and culture medium allowed to considerably increase the accumulation and production of lipids by the wild-type without the need for any genetic improvement. Thus, the wild-type had good yields in an optimized bioprocess using agro-industrial by-products, being appropriate for later steps of scale-up.

Table 13. Increments and folds generated between step 1 and step 3 of bioprocess optimization by the M29 mutant and the wild-type.

Response variable	M29		Wild-type	
	Increase (%)	Fold	Increase (%)	Fold
Biomass (g/l)	24.01	1.24	12.01	1.12
Lipid content (%)	55.76	1.56	71.66	1.72
Lipids (g/l)	95.51	1.96	91.98	1.92

3.6 BATCH KINETICS STUDIES OF BIOREACTOR SCALE-UP

The wild-type was selected for scale-up in a 10L bioreactor and fermentation kinetic studies (Table 14). Optimized shaker medium concentrations were adapted through various bioreactor fermentations to meet the requirement of about 30% dissolved oxygen by varying the impeller agitation speed and aeration. The SCM concentration was increased to 90 g/l as the process would be a batch and no feed would be added during fermentation. Thus, the consumption of substrate or residual reducing sugars by the cell could be evaluated, which would increase the weight gain caused by the accumulation of fat and induced by nitrogen limitation. The concentration of CSL remained close to the optimized value in a shaker with 24.5 g/l. RYC concentration increased to 15 g/l to promote cell growth and biomass production in the first hours of fermentation.

Additionally, 2 g/l monosodium glutamate (MSG) was added to the bioreactor as a precursor of fatty acid biosynthesis. In this study, biomass production, lipid content and lipid production were 39.29 g/l, 38.13% and 14.98 g/l in the batch bioreactor at 120 hours (Figure 40A-B). These values had an increase of 2.27-fold, 1.50-fold and 3.40-fold, respectively, when compared to cultivation in 250ml flasks. Researches have addressed strategies such as adding MSG and malic acid to boost the supply of NADPH in the culture medium. Thus, MSG is a nitrogen source that can simulate aerobic glycolysis by increasing the activity of the enzyme glucose-6-phosphate dehydrogenase (G6PDH) providing more NADPH, which depends on the pentose phosphate cycle in the cytosol of non-photosynthetic cells. Thus, NADPH is an essential reducer of the enzyme complex responsible for the elongation and introduction of double bonds for the production of polyunsaturated fatty acids (PUFAs) (MORABITO et al., 2019). REN et al., (2013) added MSG in medium containing treated cane molasses and managed to increase biomass and DHA content from 29.65 g/l and 38.71% to 36.6 g/l and 42.39%, respectively, by *Schizochytrium* sp. CCTCC M209059. Another study containing 80 g/l glucose, 10 g/l yeast extract, 10 g/l MSG, 15 g/l sea salt, 2 g/l $MgSO_4 \cdot 7H_2O$, and 1 g/l KH_2PO_4 in the cultivation of *Schizochytrium* sp. ATCC 20888 obtained 55.83 g/l of biomass,

34.97% lipid content, 19.50 g/l of lipids and 35.68% of DHA in TFAs (JIANG et al., 2017). They also observed that by varying MSG concentrations it was possible to regulate lipid accumulation, where high MSG concentrations decrease lipid content and low MSG concentrations increase DHA content. Similarly, *Aurantiochytrium* sp. SW1 produced 24.46 g/l biomass and 38.46% lipid content (9.40 g/l lipids) with optimized medium containing 60 g/l glucose, 2 g/l yeast extract, 24 g/l monosodium glutamate and 6 g/l sea salt in a 5L bioreactor at 30 ºC, aeration at 1vvm and impeller speed of 200 rpm for 96 hours (MANIKAN; KALIL; HAMID, 2015). Thus, enzymatic assays can be performed to study enzymes important in the metabolism of lipid synthesis at different stages of fermentation such as: ATP citrate lyase (ACL), malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH) and $NADP^+$-isocitrate dehydrogenase (IDH) to regulate the acetyl-CoA and NADPH supply (REN et al., 2009). Another approach sought to overexpress G6PDH in *Aurantiochytrium* sp.SD116 resulting in changing the fatty acid profile and increasing the proportion of PUFAs (CUI et al., 2016; HEGGESET et al., 2019).

Table 14. Media and fermentation conditions tested in 250 mL shake flask and 10L STBR by wild-type strain.

Media composition	250 mL shake flask	10 L STBR
SCM: Sugarcane molasses (g/l)	66	90
CSL: Corn steep liquor (g/l)	24.5	25
RYC: Residual yeast cream (g/l)	6	15
SS: Sea salts (g/l)	10	10
MSG: Monosodium glutamate (g/l)	-	2
Fermentation conditions		
Operation mode	Batch	Batch
Working volume	50 ml	7L
Headspace volume	200 ml	3L
Agitation speed (rpm)	120	400-800
Impeller type	-	Rushton
Aeration rate (vvm)	-	0.5-1.0
Dissolved oxygen (DO)	-	30%
Temperature (ºC)	28	25-28
pH of liquid media	6	5.5-6.0
Inoculum rate (%)	10	10
Cultivation time (h)	120	120

Otherwise, the logarithmic cell growth curves of the culture in 250 ml shake flasks and in a 10L bioreactor presented similar profiles (Figure 40C). However, the maximum specific

growth rate (μ) went from 0.59 day^{-1} in shaker to 0.66 day^{-1} in the bioreactor, consequently the cell doubling time also decreased from 28.41 to 25.11 hours (Table 15). The maximum yields also increased with 0.64 g/l.h of biomass ($r_{X\ max}$) and 0.35 g/l.h of lipids ($r_{P\ max}$). The substrate consumption productivity was 0.74 g/l.h ($r_{S\ max}$), where 90 g/l of initial SCM was equivalent to 60.25 g/l of reducing sugars and at the end of fermentation this concentration dropped to 8.25 g/l of reducing sugars. Thus, the specific substrate consumption rate ($Q_{S\ max}$) reached 0.12 and 0.09 h^{-1} at 24 hours for shake flask and bioreactor cultivation, respectively (Figure 40D). The specific product formation rate ($Q_{P\ max}$) was 0.01 h^{-1} at 72 hours in shake flasks and 0.02 h^{-1} at 60 hours in the bioreactor. This shows that the consumption of substrate in the first 24 hours is essential to promote cell growth, but cultivation in the bioreactor begins to rapidly accumulate lipids within 60 hours of fermentation, where reducing sugars are found at 37.15 g/l.

Table 15. Kinetics in batch cultivation for lipid-rich biomass production by wild-type thraustochytrid in shake flasks and bioreactor using agro-industrial by-products.

Kinetic parameters	250 mL shake flask	10 L STBR
μ_{max} (day^{-1})	0.59	0.66
τ_d (h)	28.41	25.11
$r_{X\ max}$ (g/l.h)	0.23	0.64
$r_{X\ total}$ (g/l.h)	0.12	0.28
$r_{X\ overall}$ (g/h)	0.01	1.94
$r_{S\ max}$ (g/l.h)	0.51	0.74
$r_{S\ total}$ (g/l.h)	0.33	0.43
$r_{S\ overall}$ (g/h)	0.02	3.03
$r_{P\ max}$ (g/l.h)	0.09	0.35
$r_{P\ total}$ (g/l.h)	0.04	0.12
$r_{P\ overall}$ (g/h)	0.002	0.87
$Q_{S\ max}$ (h^{-1})	0.12	0.09
$Q_{P\ max}$ (h^{-1})	0.01	0.02
X_i: Initial biomass (g/l)	2.36	5.98
X_f: Final biomass (g/l)	17.30	39.29
ΔX: Biomass (g/l)	14.94	33.31
S_i: Initial substrate (g/l)	43.27	60.25
S_f: Final substrate (g/l)	3.65	8.25
ΔS: Residual substrate (g/l)	39.62	52.00
P_i: Initial product (g/l)	0.08	0.10
P_f: Final product (g/l)	4.41	14.98
ΔP: Product (g/l)	4.33	14.88
$Y_{X/S}$ ($g_{biomass}/g_{substrate}$)	0.38	0.64
$Y_{P/S}$ ($g_{product}/g_{substrate}$)	0.11	0.29
$Y_{P/X}$ ($g_{product}/g_{biomass}$)	0.29	0.45

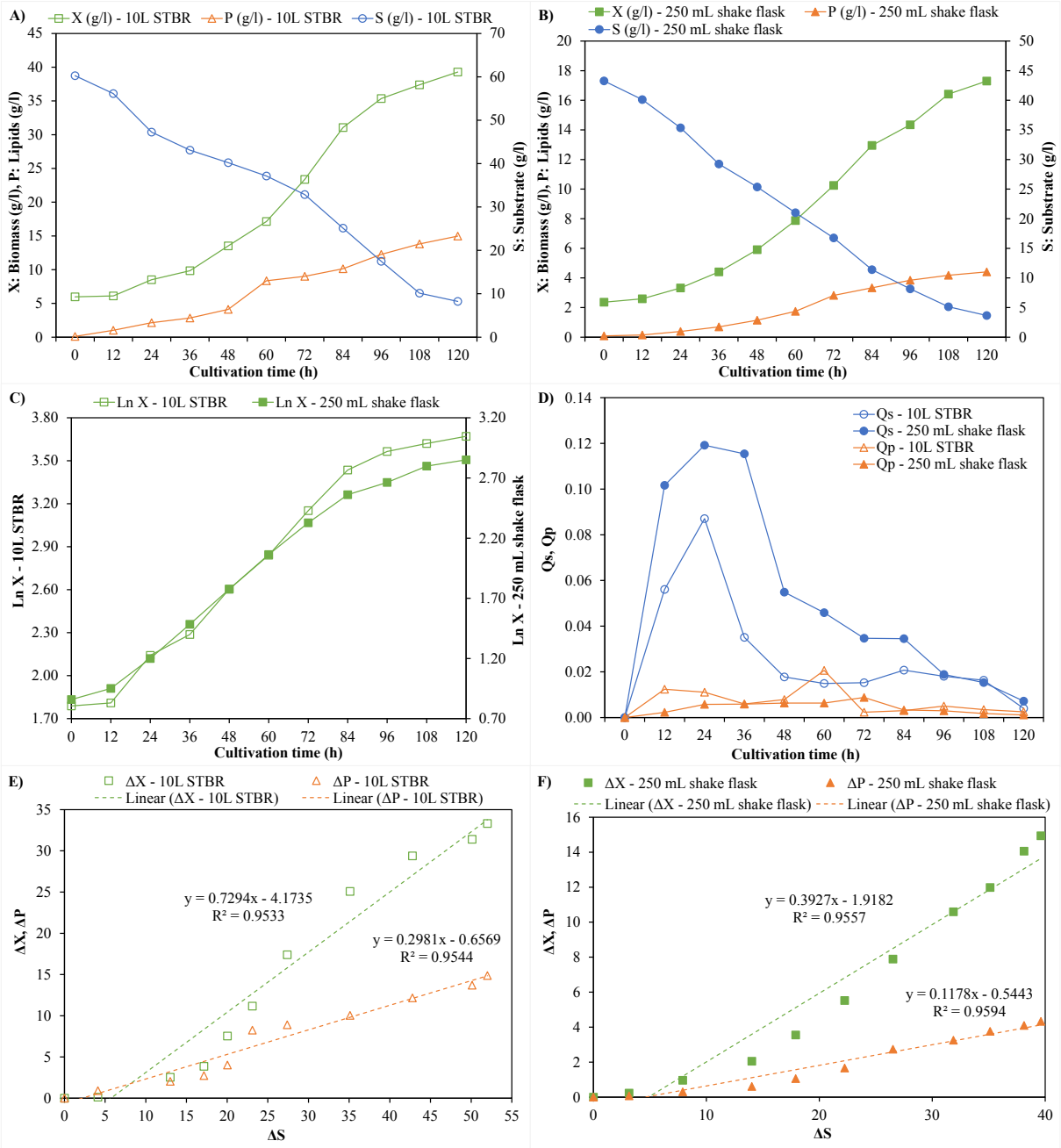

Figure 40. Kinetic curves of bioprocess by wild-type thraustochytrid using agro-industrial by-products as substrate. A) Scale-up in stirred-tank bioreactor (STBR). B) 250 mL shake flask. C) Logarithmic cell growth (Ln X). D) Substrate consumption rate (Qs) and product formation rate (Qp). E) Actual yield evolution curve for ΔX, ΔP and ΔS variations in 10L STBR. F) Actual yield evolution curve for ΔX, ΔP and ΔS variations in 250 mL shake flask.

The biomass-substrate ($Y_{X/S}$) and product-substrate ($Y_{P/S}$) yields in the bioreactor were 0.64 $g_{biomass}/g_{substrate}$ and 0.29 $g_{lipids}/g_{substrate}$, respectively. These values were similar to those graphed and obtained by the equation (Figure 40E-F). The increase in $Y_{X/S}$ and $Y_{P/S}$ yields in the bioreactor compared to the culture in shaken flasks shows a better use of nutrients present in the culture medium by wild-type and its efficiency in converting them into biomass and

product through cell metabolism. Product-biomass yield ($Y_{P/X}$) increased from 0.29 $g_{lipid}/g_{biomass}$ in shaker to 0.45 $g_{lipid}/g_{biomass}$ in bioreactor.

Recently, *Aurantiochytrium* sp. SW1 was evaluated in medium containing agro-industrial by-products (100 g/l MD-2 pineapple extract, 14 g/l MSG, 50% salinity and 6 g/l yeast extract) in a 5L batch bioreactor at 28 °C, 500 rpm and 1 vvm for 120 hours. They managed to reach maximum values of biomass (31.87 g/l), lipids (18.85 g/l) and DHA (8.20 g/l) with MD-2 extract as a potential substitute for commercial glucose and fructose (NAZIR et al., 2020). Factors such as aeration and agitation speed significantly contribute to increasing process yields. They improve the homogenization of nutrients in the medium, increase the concentration of dissolved oxygen, improve mass and heat transfer, and improve the suspension of cells in the liquid medium (BANDAIPHET; PRASERTSAN, 2006).

Moreover, *A. limacinum* BUCHAXM 122 produced 43.05 g/l biomass and a DHA yield of 0.142 g/g CDW in GYP medium (60 g/l glucose, 10 g/l yeast extract and 10 g/l peptone) in 2 L STBR, 5% inoculum rate, 600 rpm, 25 °C and 2 vvm at 48 h, however, the biomass concentration and DHA yield dropped to 30.65 g/l and 0.113 g/g CDW, respectively, at the end of fermentation (120 h) (SIRIRAK et al., 2021). They also reported that the high aeration (2 vvm) promoted foaming and the high agitation speed (600 rpm) negatively affected the DHA yield due to mechanical shear stress, which can cause cell damage. Our experiments in STBR also showed foaming even using an aeration between 0.5 and 1 vvm and agitation speed between 400 and 800 rpm, which was controlled by the addition of antifoam. However, foam reduction carries the risk of decreasing mass transfer in the fermentation process, so a chemical-mechanical strategy is more recommended to control the foam (ETOC et al., 2006; JUNKER, 2007; VIESTURS; KRISTAPSONS; LEVITANS, 1982). Foaming can also be explained by the fact that the medium is composed of complex agro-industrial by-products such as corn steep liquor with possible surface-active properties (VECINO et al., 2014).

4. CONCLUSIONS

The thraustochytrid improvement by several strategies such as UV mutagenesis and optimization of the bioprocess through the design of experiments (DOE) methodology allowed to obtain a high production of lipid-rich biomass. Bioprocess modeling allowed the construction of prediction profiles based on desirability. Thus, this multi-step approach allowed to select the best conditions for scale-up in stirred-tank bioreactor by wild-type strain. The highest biomass, lipids and lipid-biomass yield ($Y_{P/X}$) were 39.29 g/l (0.64 g/l.h), 14.98 g/l (0.35 g/l.h) and 0.45

g/g CDW, respectively. Finally, the agro-industry by-products SCM, CSL and RYC were potential substitutes for the commonly used synthetic and defined sources. This process could be improved in the future by optimizing bioreactor conditions such as operating mode (batch, fed-batch, continuous), feeding strategies, aeration, impeller agitation speed, pH and foam control, regulation of accumulation of lipids, temperature shift, among others.

FINAL CONCLUSIONS

The research developed in this doctoral thesis allows to study the sustainable production of high-value lipids using marine thraustochytrids as an alternative source. An integrated analysis of the current challenges and opportunities of this microbial source allowed to clarify key points in the development of biotechnological processes. The exploration of Brazilian regions little studied by next-generation sequencing techniques allowed us to know more about the microbiome of the ecosystem of mangroves and coastal waters. Subsequently, the development of rapid strain selection and screening techniques allowed the bioprospecting of a vast number of LC-PUFA producing thraustochytrids. Additionally, the improvement and optimization of thraustochytrids highlighted the great potential of these protists for large-scale production.

FUTURE PERSPECTIVES

The development of rapid bioprospecting strategies for thraustochytrids is a big bet, as well as the in-depth study of lipid profiles that are widely diverse among species. These lipids could be better defined by techniques such as Gas chromatography–mass spectrometry (GC-MS) that allow the study of these biomolecules clearly. Likewise, improvements in metagenomics techniques in order to cover a greater diversity of species from environmental samples would be important. Also, the improvement of isolated or genetically improved strains that can have maintenance and cell viability through several generations is essential to develop them for commercial purposes. Full genome or transcriptomic sequencing studies would also be relevant in strains improved either by mutation techniques or genetic engineering. Additionally, the analysis of enzymes important in lipid biosynthesis during the fermentation process could help in the regulation of PUFAs. Other scale-up and optimization studies of bioreactor variables such as aeration, agitation speed, dissolved oxygen, impeller configuration, feeding strategies and temperature changes could increase the production of biomass and lipids. Furthermore, improving the separation and purification steps to increase the quality of the final product would be ideal. Finally, obtaining an appropriate bioproduct with all the proximal or bromatological analysis is necessary, as well as the characterization of amino acids, fatty acids, carbohydrates and sugars, and minerals that allow the use of thraustochytrids biomass in formulations for animal and human food.

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ATTACHMENT 1 – PUBLICATION OF CHAPTER 2

https://doi.org/10.1016/j.tifs.2020.03.007

Trends in Food Science & Technology 99 (2020) 244–256

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Omega-3 microbial oils from marine thraustochytrids as a sustainable and technological solution: A review and patent landscape

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ARTICLE INFO

Keywords:
Omega-3
Docosahexaenoic acid
Thraustochytrid
Microalgae oil
Sustainable bioprocess
Functional foods

ABSTRACT

Background: Oils rich in omega-3 have been of industrial and economic importance, mainly for its benefits in human and animal health. Fish is considered the primary source for obtaining omega-3. The resurgence of interest in the use of omega-3 as a functional food has led to the need of alternative sources in this growing market.

Scope and approach: Omega-3 produced by marine thraustochytrids have appeared as an alternative to fish oil and an eco-friendly solution to overfishing. Here, we review key mechanisms that enable sustainable production and technological prospection of omega-3 from thraustochytrids. Sustainability analysis identified strengths, weaknesses, opportunities and threats for omega-3 oils from thraustochytrids. Thus, this study shows the technological prospection based on the worldwide patent landscape.

Key findings and conclusions: Thraustochytrids have also been known as microalgae on the international market, which has been well received by consumers as a natural or vegan source of omega-3 supplementation. Thus, omega-3 oils can be developed in controlled bioprocess systems preventing the production of toxins and pollutants. Hence, the technological prospection showed a growing trend with 731 patents published between 1999 and 2018. Most patents were classified within the chemical and human areas, which highlighted omega-3 oils involving biosynthesis by microorganisms such as *Aurantiochytrium* in food applications. These technologies have been mainly developed by countries, such as the United States, China, Netherlands and Japan, which refer to bioreactor cultivation, strains improvement and food formulations. Thus, global patent search allowed to analyze technological advances, trends and market gaps of omega-3 oils from thraustochytrids as a renewable technological solution.

ATTACHMENT 2 – PUBLICATION OF CHAPTER 3

https://doi.org/10.1016/j.biortech.2021.125176

Bioresource Technology 333 (2021) 125176

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Integrating metagenetics and high-throughput screening for bioprospecting marine thraustochytrids producers of long-chain polyunsaturated fatty acids

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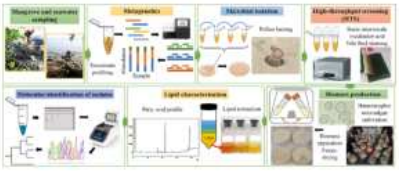
HIGHLIGHTS

- Eukaryote metagenetics as a bioprospecting tool for thraustochytrids.
- High abundance and diversity of thraustochytrids in mangrove sediments.
- Microscale cultivation was suitable for high-throughput screening of isolates.
- Brazilian thraustochytrids highly producers of LC-PUFAs.
- *Aurantiochytrium* sp. isolate B36 had a DHA yield of 0.16 g/g_{CDW}.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Thraustochytrid
Metagenetics
Bioprospecting
LC-PUFAs
Omega-3

ABSTRACT

Omega-3 produced by marine thraustochytrids has appeared as an alternative to fish oil and an eco-friendly solution to overfishing. Herein, an integrative analysis of metagenetics and high-throughput screening was used for bioprospecting marine thraustochytrids from southern Brazil mangrove and coastal seawater. All sampled environments showed biodiversity and abundance of SAR clade. Environmental samples detected with potential lipid-accumulating labyrinthulomycetes were further processed for direct plating and pollen baiting isolation. Microtiter plate system and fluorescence spectroscopy were combined for high-throughput screening of 319 isolates to accumulate lipids. Twenty isolates were selected for submerged cultivation and lipid characterization. Among them, B36 isolate, identified as *Aurantiochytrium* sp. by 18s rRNA sequencing, achieved the highest biomass (25.60 g/l CDW) and lipids (17.12 g/l CDW). This lipid content had a high biological value with 44.37% LC-PUFAs and 34.6% DHA, which can be used as a sustainable source in vegan, seafood-free and animal feed diets.

ATTACHMENT 3 – PATENT FROM CHAPTER 4

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2020 026212 2

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): BIOPROCESSO DE PRODUÇÃO DE BIOMASSA DE TRAUSTOQUITRÍDEO RICA EM ÁCIDO DOCOSAHEXAENÓICO UTILIZANDO RESÍDUOS AGROINDUSTRIAIS E BIOPRODUTO COMPOSTO DE BIOMASSA DE TRAUSTOQUITRÍDEO

Resumo: A presente invenção trata de um bioprocesso tecnológico para produção de biomassa de traustoquitrídeo rica em ácido docosahexaenóico (DHA) do tipo ômega-3 em biorreator de tanque agitado por cultivo submerso, e também em um bioproduto composto de biomassa de traustoquitrídeo. Este bioprocesso aproveita resíduos agroindustriais gerados pelas indústrias agroalimentares como o melaço de cana-de-açúcar, o creme de levedura e a milhocina como fontes de carbono e nitrogênio no meio de cultura. O bioprocesso é caracterizado pela produção sustentável, controlada e de baixo custo de biomassa microbiana, um bioproduto que pode ser utilizado na alimentação animal.

Figura a publicar: 2

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ATTACHMENT 4 – PUBLICATION AS CO-AUTHORSHIP

https://doi.org/10.1016/B978-0-12-821879-2.00009-0

CHAPTER

9

Valorization of solid and liquid wastes from palm oil industry

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9.1 Introduction

Palm oil annual production reaches 19.72 million tonnes worldwide, which is 80% higher than the annual worldwide production of soybean oil, and with 6 times higher productivity [1]. Since it does not require frequent replants, it is suitable for small producers and favors the recovery of degraded lands [2].

Palm oil production has been doubling every decade since 1965 and, despite slowing down in Malaysia and Indonesia (the world's largest producers), it is increasing in other regions, such as Colombia, the northern region of Brazil, and in some African countries. Furthermore, other products are entering the market, driven mainly by the potential of palm oil as raw material for biodiesel production [3]. This expansion of palm cultivation may have severe consequences for the environment in the near future, but it will undoubtedly bring countless economic and social benefits for the regions if the bioprocessing of this biomass is done using clean and sustainable technologies.