Identification and Transfection of the *Botryococcus braunii* Lipid Exudation Mechanism in Microalgae with High Growth Rate

1.Introduction

Microalgae are single-celled photosynthetic organisms that effectively sequester and transform atmospheric carbon dioxide into biomass and other bioactive compounds that can be used in the cosmetics, biofuels, pharmaceutical and food industries (BOŠNJAKOVIĆ; SINAGA, 2020; KUMAR et al., 2020). In particular, microalgae have attracted substantial attention in recent years due to their high lipid production rate (GOH et al., 2019), significantly surpassing the most used oilseeds to obtain this product, such as soy, corn and rapeseed (BOŠNJAKOVIĆ; SINAGA, 2020; SINGH; SINGH, 2015). In some microalgae species such as *Nitzschia sp.* and *Schizochytrium sp.*, the lipid portion represents more than 50% of the total biomass (ADENIYI; AZIMOV; BURLUKA, 2018), which makes its production on an industrial scale even more advantageous. However, the need for optimized production systems makes such applications more expensive (SLADE; BAUEN, 2013).

The production of products from algae is done in three main steps: Biomass cultivation; harvesting and dehydration; and biomass extraction, fractionation and conversion (FASAEI et al., 2018). In this scenario, microalgae cultivation, harvesting and dehydration are the main contributors to the cost of using algae on an industrial scale (NORSKER et al., 2011; RUIZ et al., 2016a). In particular, the necessary rupture of the cell wall of these organisms for harvesting, cultures with a high volume of water and the small algae proportion, and recultivation make it difficult to scale up industrial production as these processes require the use of costly strategies, with high cost and consumption of energy (MOLINA GRIMA et al., 2003; PAHL et al., 2013; SEO et al., 2016).

One of the alternatives that would circumvent some of these problems would be the use of the microalgae *Botryococcus braunii*. In addition to being able to accumulate high lipid content, this microalga performs an unusual phenomenon of secreting these components into the extracellular environment (PRATHIMA; KARTHIKEYAN, 2017; TASIĆ et al., 2016). This competence is extremely important, as it allows the extraction of hydrocarbons without compromising cell integrity and not requiring constant recultivation, making the production of lipids through microalgae cheaper (JACKSON; BAHRI; MOHEIMANI, 2017). However, algae with high lipid content such as *B. braunii* (up to ~75% lipids in dry mass) grow slowly and can only be harvested a few times a week and its implementation in the industry is still a challenge (CHENG et al., 2019; GENDY; EL-TEMTAMY, 2013). For this reason, most research and development projects and pre-commercial projects still use algae

with lower lipid content, but which grow faster, such as *Dunaliella sp*. and *Nannocholopsis sp.* (~20-40% lipids in dry mass). These algae can be harvested a few times a day or daily, but their cultivation and harvest still have problems, as previously mentioned, of high recultivation cost (ADENIYI; AZIMOV; BURLUKA, 2018; GENDY; EL-TEMTAMY, 2013; RUIZ et al., 2016b).

In this context, genetically engineered algae can help to overcome these limitations in algae culture. It is possible to increase the production of metabolites, lipids or combine characteristics of algae to make an organism more effective in the production of a particular product (KUMAR et al., 2020). Genomic information on one of the pivotal algae in the development of the algae farming industry is available: *B. braunii* (BROWNE et al., 2017). From this information, it is possible to track genes and, consequently, proteins that confer to *B. braunii* the characteristic of secretion of lipids in high amounts, in addition, a possible transfection in algae with a high growth rate as *Dunaliella sp.* or *Nannochloropsis sp.*

2. Hypothesis

Transfecting genes responsible for lipid exudation from the microalgae *Botryococcus braunii* to another, faster growing, microalgae such as *Nannochloropsis oculata, Chlamydomonas reinhardtii, Microchloropsis gaditana* or *Dunaliella salina* is possible and is enough to make these algae able to exude lipids.

3. Justification

High growth rate algae such as *Dunaliella sp.* and *Nannochloropsis sp.* are used in the algae farming industry. Obtaining the lipids on an industrial scale requires the lysis of these microalgae that do not exude their products. This is one of the biggest contributors to the high cost of extracting lipids from microalgae cultures because successive algae recultivation is required.

In contrast, *B. braunii* is known for its high production and exudation of lipids but also for its slow growth. Combining the high growth rate plus the ability to exude lipids would bring advantages to the industry by reducing the costs of lipid production, bringing

potential implications and impacts on the efficiency and economic viability of the process, in order to achieve a more sustainable and competitive production model.

4. Objectives

Identify and transfect the *B. braunii* component(s) that composes the lipid exudation system into microalgae with high growth rate, so these algae can adopt the lipid exudation mechanism in a functional way.

Catalog microalgal genetic elements for the purpose of creating biological parts that will be used in the future in genetic engineering, biotechnology and synthetic biology applications.

Test and validate a laboratory-scale photobioreactor to be used both in wild microalgae cultures and in genetically modified cultures, constituting a hardware that allows control and monitoring of the cultivation conditions and the growth rate of the species of interest.

5. Methodology

5.1 Search for the exudation mechanism candidate

The sequenced genome of *B. braunii* with predicted proteins, deposited at Phytozome (https://phytozome-next.jgi.doe.gov/info/Bbraunii_v2_1) (GOODSTEIN et al., 2012) will be used as our reference for this species. Although there is already protein prediction, protein annotation is not available. To resolve this situation we will perform a protein search by protein similarity against all entries of the Viridiplantae kingdom (12,287,201 proteins) deposited at Uniprot (https://www.uniprot.org/) (APWEILER et al., 2004) Search will be performed using the MMseqs2 software (STEINEGGER et al., 2017). The best hit related to the predicted *B. braunii* protein will be chosen using a multiplication between the percentage of identity, if greater than 30%, and the extension of the alignment.

We will also search for ATP binding cassette transporters (ABC transporters) deposited at Uniprot and manually revised (6,259 sequences), regardless of taxonomic proximity to *B. braunii*. The best hits will be chosen considering the identity percentage, if it is greater than 30%, and if the alignment length is greater than 100 amino acids.

From then onwards, manual curation of the results of the search for ABC transporters will be carried out, in accordance with the described function. The predicted proteins that most resemble proteins that are related to lipid transport, after curation, will have their annotation against proteins from the kingdom Viridiplantae verified. Also, homologs of the chosen candidate protein from *B. braunii* will be searched in the proteomes of *N. oculata*, *M. gaditana*, *D. salina* and *Chlamydomonas reinhardtii*, to ensure that it is a protein unique to the gene donor.

5.2 Cell culture

Cells will be cultivated in 3.5% m/v saline solution enriched with solutions of nitrate, phosphate, trace metals and vitamins (thiamine, biotin and cyanocobalamin). The pH of the solution will be kept between 7.5-8.0 using HCl and NaOH. We will use the Guillard culture Medium without silicate enrichment due to the absence of a silica-rich wall algae species employed. Initially, the algae will be under cultivation in a makeshift greenhouse and receive constant light and aeration, being under white LED lighting.

As a second step, we will cultivate the algae in a low-cost photobioreactor, made of 3D printed parts, LED strip lights, and glassware. We have established a partnership with Vitor Marchesan, Master in Biotechnology Engineering with experience in Bioreactors and Photobioreactors.

The algae culture will be homogenized using a magnetic bar stir and culture conditions will be monitored using a thermometer and a pHmeter. In addition, we are developing a method of using a coupled spectrophotometer to check algae growth and a nutrient injection system together with a flow gas system. The purpose of this hardware is to promote a low cost and easy to replicate construction using the DIY philosophy. We engage with a whole open science project, every aspect, from the files to the final construction is available on https://github.com/VitorFrost/photobioreactor.

5.3 Growth control and algae maintaining

Growth control will be measured by absorbance in a spectrophotometer. We will use serial dilution to calibrate the curve. The predicted maximum absorption peak in the visible spectrum will be at 680 nm and will be the wavelength used to measure the absorbance of the solution, relating it to the density of algae in the medium.

6. Project schedule

2021

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Brainstorm	x											
Choice of the project		x										
Theoretical research	x	x	x	x	x	x	x	x				
Algae receivement (*D. salina, M. gaditana* e *N. oculata*)								x				

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