UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

Bio-based solvents for hydrocarbon extraction from *Botryococcus braunii* maintaining cell viability in a two-phase extraction system

Doctoral thesis in fulfillment of the requirements for the degree Doctor of sciences in natural resources

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Esta tesis fue realizada bajo la supervisión del director de tesis Dr. Rodrigo Navia Diez de la Facultad de Ingeniería y Ciencias de la Universidad de La Frontera, y ha sido aprobada por los miembros de la comisión examinadora.

Bio-based solvents for hydrocarbon extraction from *B. braunii* maintaining cell viability in a two-phase extraction system

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Dedicated to my mother Ruth Muñoz Opazo

Acknowledgments

When you start a new chapter of your life you do not know exactly what to expect (at least not in detail) or who will cross your path. In my case, at the beginning of the doctoral program I had an approximate idea about the process itself but, of course, I did not have the faintest idea about who I would spend time with over the next few years. I was very lucky to find wonderful people. I would prefer to not name each person I owe gratitude to individually as I am afraid that my memory will fail me and cause terrible omissions, I will try my best, however, as the acknowledgements are a holy obligation.

First and foremost, I would like to say thank you to Dr. Rodrigo Navia who accepted me as a PhD student even without a wide background on the topic of the study that I would work on, as I was working on economics and data science for nine years before embarking on my PhD trajectory. I remember I started classes a few weeks late due to the 8.8 earthquake on February 28th 2010, in Chile, as I was in the city of Talca at the time. From the very beginning all my classmates were very friendly and helpful. I especially thank Maritza Durán and Gabriela Velásquez for their support.

My classmate Álvaro Torres kindly and patiently familiarized me with with the workings of the laboratory when I first started using the lab in 2011. He did not only explain the devices, instruments, and procedures to me but also the group organization. During this period at the lab many people supported and advised me, among them Dr. Claudio Toro, Dr. Oscar Valerio, Dr. Gustavo Ciudad, Dr. Rodrigo Mella, and in particular Mrs. Noelia Sepúlveda.

In 2012 I had the privilege to realize part of my research in the outstanding laboratory at the Centre for Environmental Research-UFZ, Leipzig, Germany, in charge of Dr. Hermann Heipieper. It was a very challenging time, but many people supported me professionally, emotionally, and even financially. I am especially grateful to Dr. Hermann Heipieper, Dr. Lukas Wick, Dr. Paula Martínez, Dr. Pedro Inostroza, Dr. Vianey Marín, Dr. Karla Locher, Dr. Meliz Muz, Dr. Ricardo Massei, Mr. Florian Parva, Ms. Ioanna Popescu, Dr. Natalia Carrasco, Dr. Jan Bannister, Dr. Julia Chacón, and Dr. Sara Herrero-Martin.

After my return to Chile, I initiated the second phase of my study at the laboratory of the BIOREN-UFRO. During this period I received help and advice from Dr. Laura Azócar, Dr. María Eugenia Gonzalez and I am especially thankful to my colleague Ing. Lena Birkhauer (KIT, Karlsruhe), who helped me to set up and conduct one of the experiments.

Moreover, without the competent administrative help of Gladys Oyarzún and Mariela Gonzalez we would have been completely lost and the English teacher María Eugenia Osses was indispensable and corrected all our texts for presentation at our periodical exams.

Beyond academia we try to have a life and spend it with people that make it worthwhile. During my time at Universidad de La Frontera I enjoyed spending time with the following colleagues, classmates, and friends: Leonardo Almonacid, Erwin Yañez, Evelyn Padilla, Paula Aguilera, Jocelyn Tampe, Ana Luengo, Ana Cerda, Mabel Delgado, Loreto Manosalva, Gabriela Velásquez, Maritza Durán, Álvaro Torres, Robinson Muñoz, Laura Azócar, Leslie Meier, Patricio Neumann, Francisco Cabrera, Juan Díaz, Fabiola Valdebenito, Claudio Toro, Oscar Valerio, Gustavo Ciudad, Rodrigo Mella, Carolina Beltrán, Hernán Díaz, Karina Godoy,

Naiane Sangaletti, Dana Stara, Kristýna Mrkvičková, Gabriel Capson-Tojo, Natalia Anferova, Fredy Valdés, Fernanda Cid, Cristóbal González, Karla Araya, Fernanda Jiménez, Jorge *Musu* and many others.

I would like to express my special gratitude to Dr. Regina Lohndorf who supported me financially and helped me with the English language editing of my doctoral thesis.

I also want to thank all Chilean tax payers, because they funded this research. I hope at some point in the future I can compensate them by inventing something useful and putting it in public domain for free. The study was made possible thanks to three grants awarded to the autor by the *comisión nacional de investigación científica y tecnológica* (project numbers 781211006, 24121442, and 75120043). Additionally, Anillo de Investigación en Ciencia y Tecnología GAMBIO project number ACT172128 and FONDECYT 1150707 contributed with financial resources.

Finally, I would also like acknowledge the many unknown, generous, solidary and highly qualified people, thanks to whose work I could enjoy for free high quality software to search and read papers (Firefox, Okular), to seek and manage bibliographic data (Xapers and Jabref), to make high quality plots and manage huge matrices with millions of data entries (R, Rkward), to write/read e-mails (Thunderbird), to analyze videos and images (image J, mplayer, GIMP), to store and share my files in a cloud (Overleaf, rclone), and to write this thesis (Debian GNU/Linux, LaTeX, texstudio, bibtex).

English Summary

Due to the high energy demand in support of economic growth and the abuse of exhaustible resources such as carbon, petroleum and gas to satisfy this demand, we are facing the unstoppable process of climate change and the dramatic threat of unsatisfied energy demands in the medium-term. These two reasons are sufficient to encourage research on environmental friendly and economically feasible energy sources. If wind, solar, hydroelectric, biomass and some less utilized forms of renewable energy (such as geothermal and wave power) are considered together, just 10.9% of the primary energy production is renewable. Over the past decades, microalgae have been incorporated as a new form of biomass for renewable energy production, used to generate biogas and liquid fuels or for direct burning.

The traditional method to produce liquid fuels from microalgae is to cultivate the alga, harvest, dry, grind and disrupt the cells, to extract the internal lipids and to transesterify them using some petroleum-derived solvent in order to obtain biodiesel. However, this method has three major drawbacks: (1) the stages of drying, grinding, and cell disruption are highly energy demanding, accounting for around 84% of the total energy used in the process to produce biodiesel, (2) the solvents, derived from an exhausting source such as the petroleum, are highly contaminating and hazardous, and (3) microalgae producing highly energetic molecules, normally posses a low growth rate, thus, it is too time-consuming to destroy them to extract lipids and grow new biomass.

This study proposes to use the microalga *B. braunii* to produce hydrocarbons in a two-phase system using a bio-based, renewable and environmental friendly solvent. In this way the three aforementioned issues would be resolved, as in a

two-phase system hydrocarbon production/extraction, and biomass growth occur simultaneously, preventing cell disruption of the microalgal biomass and the highly energy demanding stages of the traditional method.

To examine these relationships two experiments were done: (1) in the first experiment, the biocompatibility and *B. braunii* tolerance mechanism were studied, using n-decane, n-decanol and limonene (a bio-based and renewable terpene) as second-phase solvents, and (2) in the second experiment solvent biocompatibility and hydrocarbon extraction yield were studied, using n-decane and biodiesel (a vegetable oil fatty acid methyl ester solvent) as second-phase solvents.

From the first experiment it was concluded that n-decane was a biocompatible solvent compared to n-decanol and limonene under experimental conditions, and microalga *B. braunii* is able to change cell membrane fatty acid composition to tolerate permanent solvent contact. From the second experiment, it was concluded that biodiesel is a better biocompatible solvent compared to n-decane, and is able to recover hydrocarbons from *B. braunii*, nevertheless n-decane was faster.

The overall findings of the whole set of experiments revealed that it is possible to extract hydrocarbons from microalga *B.braunii* using biodiesel as a biocompatible solvent, and that there is ample room to improve the biocompatibility and extraction yield of the solvent, as the system used in the curret study is quite simple and better ideas have been raise to improve two key system parameters: contact-time and contact-surface of solvent-biomass.

Resumen en Castellano

La gran demanda de energía para apoyar el desarrollo económico (satisfacer nuestro estilo de vida) y el abuso de los recursos agotables como carbón, petróleo y gas para satisfacer esta demanda, nos enfrentan hoy a un cambio climático imparable y a la amenaza latente de que en el mediano plazo podamos tener una escasez energética importante. Sólo estas dos razones hacen urgente buscar alternativas ambientalmente amigables y económicamente factibles para producir energía. Al 2018, apenas el 10.9 % de la demanda de energía mundial fue satisfecha por energías renovables, sumando energía eólica, solar, hidroeléctrica, biomasa y otros aportes de fuentes menores (como la geotérmica y undimotriz). A la energía proveniente de biomasa, recientemente se han incorporado las microalgas, ya sea que se utilicen para producir biogás, combustibles líquidos o se quemen directamente.

La propuesta habitual para producir biocombustibles líquidos desde microalgas es hacer un cultivo batch, cosechar la biomasa, secarla, molerla, romper las células y extraer los lípidos usando un solvente orgánico derivado del petróleo, para luego transesterificarlos y obtener biodiésel. Pero este modelo de producción tiene tres desventajas clave. Primero, las etapas de secado, molienda y disrupción celular son altamente demandantes de energía (ca. 84 % de la energía total usada en el proceso); segundo, utiliza solventes altamente contaminantes, peligrosos de manipular y provenientes de una fuente en agotamiento como el petróleo, y tercero, las microalgas que producen moléculas altamente energéticas normalmente tienen una baja tasa de crecimiento, por lo que romperlas para extraer los lípidos, y esperar a que se forme nueva biomasa es una desventaja.

En esta tesis se propone utilizar la microalga *Botryococcus braunii* para producir hidrocarburos en un sistema de dos fases utilizando un solvente biocompatible y renovable derivado de biomasa. De esta forma se pretende resolver los tres problemas mencionados, pues en un sistema de dos fases el crecimiento de la microalga y la extracción de los hidrocarburos ocurren simultáneamente, evitando las etapas altamente demandantes de energía y romper las células para extraer los lípidos internos. Para ello se desarrollaron dos experimentos principales: (1) en el primero se usaron los solventes n-decano, n-decanol y limoneno (un terpeno renovable obtenido de biomasa) como segunda fase y se estudiaron los mecanismos de tolerancia al solvente de las microalgas y la biocompatibilidad de los solventes, y (2) en el segundo, se usaron los solventes n-decano y biodiesel (biosolvente renovable) y se estudió su biocompatibilidad y su capacidad de extraer hidrocarburos desde la microalga.

Del primer experimento se concluyó que n-decano fue el solvente más biocompatible y que *B. braunii* modifica la tasa de insaturación de su membrana celular para tolerar y sobrevivir al contacto con los solventes. Del segundo experimento se obtuvo que biodiesel es un solvente más biocompatible que n-decano y que es capaz de extraer hidrocarburos desde *B. braunii*, pero n-decano es capaz de extraer más hidrocarburos y más rápido que biodiesel. Del conjunto de experimentos se concluye que una extracción de hidrocarburos *in-situ* desde la microalga *B. braunii* utilizando un solvente renovable y ambientalmente amigable como el biodiesel es conceptualmente posible, y que hay mucho espacio para mejorar el sistema, sus rendimientos de extracción y biocompatibilidad ya que el sistema de dos fases utilizado es muy simple y han aparecido ideas más sofisticadas para mejorar dos puntos claves del sistema: la superficie y el tiempo de contacto solvente-biomasa.

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Chapter 1

General Introduction

1.1 Microalgae

Algae is not a phylogenetic term, but it is a concept refering to a group of highly diverse organisms that perform photosynthesis or possess plastids (Keeling, 2004). Phylogenetically, algae are placed into two domains/superkingdoms: Eukaryota and Bacteria (Ruggiero et al., 2015), the latter including cyanobacteria. Algae play a key role in different global cycles, such as carbon, oxygen and nitrogen cycles (Fowler et al., 2013). Moreover, they account for approximately 50% of the total annual biomass production on earth (Behrenfeld, 2001).

Microalgae are unicellular and phothosynthetic microorganisms that use solar energy and CO_2 from the atmosphere to produce biomass (Demirbas, 2010). Microalgae also play a fundamental role in several global geochemical cycles (Graham et al., 2009). These microorganisms can be found practically in any kind of environment on earth and they can float or be attached to any surface consisting of biological or inorganic material for instance. Microalgae possess a wide variety of shapes and sizes. Shape can be a simple sphere (e.g. *Chlorella vulgaris*) or a complex arrangement (e.g. *Pediastrunm sp.*) (Promdaen et al., 2014). Microalgae can also be flagellated and can live grouped in colonies or forming long chains. Size is in the order of 1 to a few hundred micrometers.

Microalgae are quite flexible microorganisms. They can grow in extreme conditions of salinity, temperature or pH. For instance, *Dunaliella salina* can grow in water ranging from 1.5x to 11x seawater salinity (around 5 M). Concerning temperature, *Chloromonas nivalis* and *Raphidonema nivale* can grow on ice and snow (Leya et al., 2009), meanwhile *Mastigocladus laminosus* is able to withstand 70°C. Genus *Dunalliela* also includes a specie that is able to grow at pH 1: *Dunalliela*

acidophila (Pick, 1999), whereas *Coccomyxa onubensis* can grow between pH 2.5 and 9.

All microalgae possess some kind of covering, which play a role in different aspects of cell life. It is very important in cell-cell adhesion, in defense, or even like a sensor for environmental changes (Domozych, 2016). The cell covering can be a simple gel layer or a strong cell wall, chemically and mechanically resistant as in case of *Botryococcus braunii* (Weiss et al., 2012).

Within the wide variety of microalgae, it is possible to find several different ways of nutrition. At the extremes are obligate photolithotrophs and obligate chemoorganotrophs. Obligate photolithotrophs are photosynthetic microalgae that cannot use organic carbon for growth, but instead use CO_2. On the other side of the spectrum are obligate chemoorganotrophs, which are not photosynthetic and can only use organic carbon as energy and carbon source. In spite of being photosynthetic microorganisms, there are heterotrophic microalgae, that can use organic molecules as carbon source to grow in the dark. If photosynthetically competent microalgae use organic molecules as carbon source to stimulate their growth in light, they are called mixotrophic. According to the cell mechanism of carbon intake, the microalgae can be either osmotrophic, if they take in the carbon as solution, or phagotrophic, if they take in the carbon as a particle (Flynn et al., 2012). For nutrition, microalgae also need vitamins and micronutrients such as Fe, Mn, Zn, Cu, Mo, Co, V, Ni and Cd (Quigg, 2016).

The main microalgae constituents are carbohydrates, lipids and proteins, therefore, they can be used as raw material in several different industrial processes. However, microalgae are also able to produce a wide spectrum of other useful and valuable secondary metabolites such as carotenoids (astaxanthin, β-carotene,

lutein), phycobilins, vitamins, polysaccharides, polyhydroxyalkanoates (bioplastics) and even glycerol (Markou and Nerantzis, 2013). All of these compounds can be extracted under the umbrella concept of biorefinery for maximum efficiency (Vanthoor-Koopmans et al., 2013).

Microalgae could contain up to 77% dry weight (dw) protein (*Spirulina platensis*, Ciferri (1983)), 76% dw lipids (*Limicolaria martensiana*, Nagarkar et al. (2004)) and 38% dw carbohydrates (*Nostoc commune*, Vargas et al. (1998)). Oil content in microalgae is on average 30% (Nautiyal et al., 2014), however, this oil content can be increased under certain environmental or culture conditions such as nitrogen starvation (Widjaja, 2009; Manisali et al., 2019; Soru et al., 2019). Tryacilglycerol is the most common storage lipid in microalgae growing under deprivation conditions (Tornabene et al., 1983), however, under starvation and when microalgae use the energy to produce and store energetic molecules, normally, they grow at lower rates (Sheehan et al., 1998; Vasudevan and Briggs, 2008).

Microalgae are considered as an interesting source of energetic molecules, such as, lipids, hydrocarbons or carbohydrates due to their advantages in therms of high photosynthetic efficiency, fast growth rate and high biomass productivity. Moreover, they can grow on non-cultivable land using brackish water or even sewer water, they use less water than terrestrial crops, and can be harvested anytime of the year compared to once or twice of traditional terrestrial crops (Chisti, 2007; Yeang, 2008; Amaro et al., 2011). Over the past decades, some microalgae species have been widely studied because they possess specific features that make them attractive for lipids or hydrocarbon production, this is the case of *Botryococcus braunii*.

1.2 *Botryococcus braunii*

Botryococcus braunii was first described by Friedrich Traugott Kützing in the book *Species Algarum* (1849). It is able to grow in brackish or fresh water (Banerjee et al., 2002) and form colonies with 50 to 100 cells, which can also be connected forming big aggregates. Cell morphology ranges from ovoid to conical, depending on the location in the colony. Normally, cells near the colony center tend to be more ovoid and cells near the colony border tends to be more conical. However, there exists a high morphological variability and numerous species were proposed in the past based on morphological differences (Komárek and Marvan, 1992), whereas today a chemical classification is preferred. Colonies are enclosed in an extracellular matrix (ECM) which could have a role in several functions like cell-cell adhesion, expansion control, sensing of environmental stressors, defense, and morphogenesis (Domozych, 2016).

ECM is composed of three parts: (1) the most interior layer is made of β-1-3 or β-1-4-glucan (cellulose) and is surrounding every cell; (2) the second layer is a cross-linked hydrocarbon network, and (3) the whole colony is enclosed by a wall made with a fibrillar sheath whose main component is arabinose-galactose polysaccharide (Weiss et al., 2012). This ECM plays a key role in the most oustanding *B. braunii* characteristic, which is to store high quantities of hydrocarbons. Every cell is associated with the ECM and contributes to its formation. The whole colony can be divided internally forming drapes as a consequence of retaining-wall folding. The colony size inside the ECM depends mainly on strain, but it is also affected by culture conditions (Khatri et al., 2013; Tanoi et al., 2013). *B. braunii* has the ability to grow under hetero, mixo or phototrophic conditions,

and it has been found that the carbon source, along with light intensity, affects cell morphology (Tanoi et al., 2011).

B. braunii is classified in three races according to the type of hydrocarbons produced, but they also differ in some morphological features. Cells from races A and B are bigger than cells from race L, with a size of 13 µm x 7-9 µm, while race L cells have 8-9 µm x 5 µm (Metzger et al., 1988). Another difference among races is the color they get at stationary growth phase. Normally, race A turns from green to yellow, meanwhile races B and L turn red- or brownish-orange due to the keto-carotenoids accumulation (Grung et al., 1989; Tonegawa et al., 1998).

A third difference among *B. braunii* races is the cell wall composition. On one side, races A and B possess long aliphatic compounds crosslinked by ether bonds. On the other side, race L cell wall is mainly consisting of licopadiene hydrocarbons linked by ether bonds (Banerjee et al., 2002). Races A and B have been collected from quite different climates like continental, alpine or temperate, meanwhile, race L has been identified only in the tropics (Metzger and Largeau, 2005). Regardless of race, all *B. braunii* microalga seem to reproduce using the autosporic method (Senousy et al., 2004).

The relative protein, carbohydrates and lipids/hydrocarbon content in *B. braunii* change along the time with the culture stage. Typical contents can be found in Blifernez-Klassen et al. (2018). The maximum relative protein, carbohydrates and hydrocarbons found were 25.0, 63.3 and 24.0% dw, respectively. The maximum protein content was produced at the beginning of the culture in the lag and linear growth phase. Then, in the stationary phase carbohydrates reached the maximum relative content. In the last decline phase of the culture, hydrocarbons reached the maximum quantity (Blifernez-Klassen et al., 2018). Along with hydrocarbons, *B.*

braunii also synthesizes traditional fatty acids, triglycerides and sterols (Metzger and Largeau, 1999). A new type of ether lipids has been found, which is closely related to hydrocarbons, and in some strains ether lipid production can be the main component (Metzger and Largeau, 2005).

Normally, microalgae have less than 5% dw hydrocarbons (Borowitzka, 2010), however, one of the main characteristic of *B. braunii* is to produce, accumulate and excrete high amounts of liquid hydrocarbons: up to 61% in race A and 86% in race B collected in the nature (Metzger et al., 1985). According to the type of hydrocarbon produced, *B. braunii* is classified in three races: race A produces odd n-alkadienes and alkatrienes (C_{23} to C_{33}), race B produces botryococcene and squalene (C_nH_{2n-10}, n=30 to 37), and race L produces the tetraterpene lycopadiene (Metzger et al., 1997). In 2012, a new race was proposed, it was tentatively identified through phylogenetic technique: S race, which produced saturated n-alkanes (C_{18} and C_{20}) and epoxy-alkanes (Kawachi et al., 2012).

This particular microalga has been proposed as a source of renewable biofuels because of its oil producing capacity. Moreover, it could also help to mitigate climate change by reducing greenhouse gas emissions (Pedroni et al., 2003) as the oil production uses CO_2 from the atmosphere.

The close similarity among some particular tetramethylsqualene and botryococcane found in crude oil from Sumatra, coal samples from China, and petroleum from Australia, make the scientists think that *B. braunii* could have been one of the most important contributors to crude oils and petroleum formation (McKirdy et al., 1986; Summons et al., 2002).

1.3 Hydrocarbons in *Botryococcus braunii*

Hydrocarbons are organic molecules only composed by carbon (C) and hydrogen (H) elements. Considering their properties, they can be used as raw material for the production of fuel, lubricants, solvents, fibers, plastic or rubber, and they have a central role in chemical industry to create many other products. Therefore, they have played a main role in the development of modern societies. Hydrocarbons are classified in (a) saturated, which only possess single bonds (alkanes); (b) unsaturated, which have one or more double or triple bonds (alkenes and alkynes); and (c) aromatic, which have at least one aromatic ring. The main type of hydrocarbons in *B. braunii* are alkenes and alkynes and they are produced in direct correlation with cell growth (Casadevall et al., 1985; Kojima and Zhang, 1999; Khatri et al., 2013), which is an important difference compared to lipid production. Normally, lipid concentration increases in microalgae, when nitrogen starvation is applied to the culture and the excess of carbon is used to produce energetic molecules such as triglycerides, but then the biomass production decreases (Zhila et al., 2005).

B. braunii is classified in three races (A, B and L) according to the type of hydrocarbon they produce. In 2012 the race S was proposed as new, but it is not completely accepted yet.

Hydrocarbons produced by race A are odd-numbered unbranched alkanes, with one to four unsaturations (C_{23} - C_{33}). Expressed as dry weight, they can present values from 0.4 to 61.0% dw (Metzger and Largeau, 1999). More than 50 different types of hydrocarbons have been detected in cells from race A and many of their structures have been elucidated. Hydrocarbons in race A are mostly alkadienes,

with a central unsaturation in *cis* configuration. The second unsaturation is present in almost all hydrocarbons in the terminal bond (Metzger and Largeau, 1999). Trienes possess two conjugated unsaturations in the central part of the chain, they are rarely found in the terminal position. Tetraenes are unusual and only one has been identified and determined, with three conjugated double bonds in the terminal position (Metzger et al., 1993). Templier et al. (1984) showed that the precursor of hydrocarbons in *B. braunii* race A is the oleic acid and that an elongation-decarboxylation is the mechanism by which long-chain alkanes are formed in *B. braunii*. In the eighties, Largeau et al. (1980) proposed that in *B. braunii* race A hydrocarbons are excreted from cytoplasm to ECM. A study by Hirose et al. (2013) supported this hypothesis, suggesting that hydrocarbon synthesis would not be completed in the cytoplasm, but precursors would be excreted to the cell surface, where hydrocarbon synthesis would be completed. Some examples of hydrocarbons synthesized by *B. braunii* race A are shown in Figure 1.1.

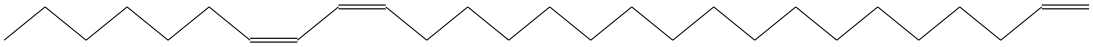
Hydrocarbons from race B are known as botryococcene, they are C_{30} to C_{37} triterpenoids with the general formula C_nH_{n-10} and can or cannot contain cyclic parts (Figure 1.1) (Metzger and Largeau, 2005). Additionally, race B is able to produce a similar type of hydrocarbon called squalene (Figure 1.1). There is a great structure variability in hydrocarbons from race B (Metzger et al., 1988). In this race, hydrocarbons can account for 27 to 86% dw for samples collected in nature (Brown et al., 1969), however, realistically cultures in laboratory settings range between 30 and 40%dw (Metzger et al., 1986; Borowitzka and Borowitzka, 1988).

Okada et al. (2004) showed that botryococcene is synthesized through farnseyl

Race A

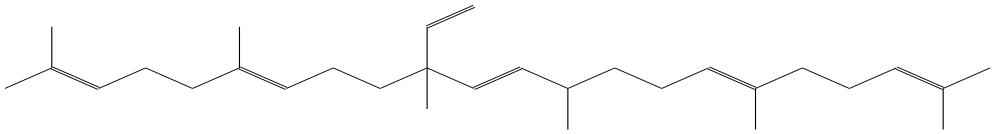
C_{27} Diene

C_{27} Triene

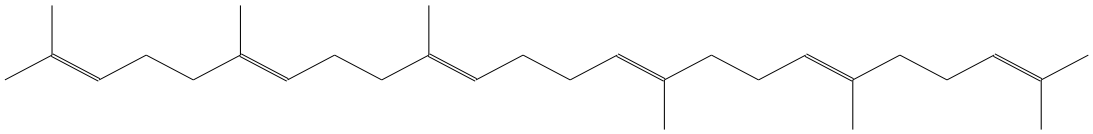


Race B

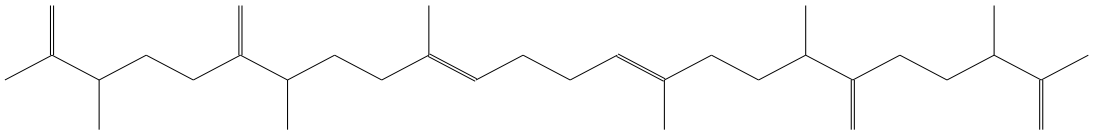
C_{30} Botryococcene



C_{30} Squalene

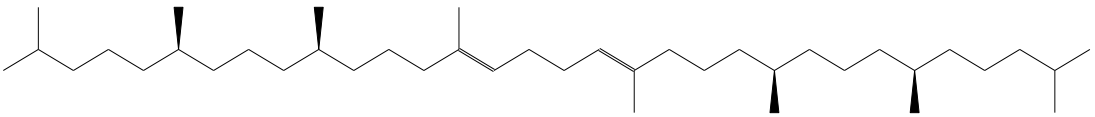


C_{34} Tetramethylsqualene



Race L

C_{40} Lycopadiene



Race S

C_{20} n-eicosane

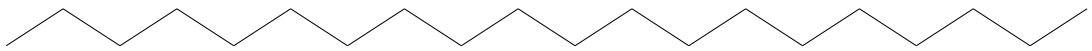


Figure 1.1: Hydrocarbons from microalga *B. braunii* races A, B, L and S. Reproduced from Metzger et al. (1986), Metzger and Largeau (2005) and Griehl et al. (2014).

diphosphate condensation, with presqualene diphosphate as intermediate between farnesyl and botryococcene or squalene (Huang and Poulter, 1989). In turn, farnseyl diphosphate is synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Alike other organisms, in *B. brunii* IPP is obtained through the methylerythritol phosphate (MEP) pathway from glucose (Ishimatsu et al., 2012; Zhao et al., 2013). The resulting C_{30} botryococcene is the precursor for synthesizing all the superior compounds with more than 30 carbons, using S-Adenosyl methionine as methylating agent (Metzger et al., 1986). The procedure to produce methylated squalenes is similar, using methionine as methyl donor to obtain C_{31}−C_{34} homologues (Achitouv et al., 2004). There are different methylation processes, apparently one occurring inside the cell and another one on the ECM (Metzger et al., 1985). Hydrocarbons in *B. braunii* are located for the most part outside the cell membrane (Largeau et al., 1980). Botryococcenes are stored externally on the outer cell wall and also in cytoplasmic inclusions. For this race a hydrocarbon excretory process was found, from cytoplasm towards the ECM (Metzger et al., 1986). The stage of maximum hydrocarbon productivity is the active growth phase (Metzger et al., 1985), i.e., hydrocarbon production is proportional to cell growth (Kojima and Zhang, 1999; Khatri et al., 2013).

Race L was first identified in 1987 by Metzger and Casadevall (1987) in samples obtained in the Ivory-Coast and in Thailand. Race L produces an hydrocarbon known as Lycopadiene (tetraterpene), whose structure is presented in Figure 1.1. It possess 40 C and 78 H, with two central double bonds. Lycopadiene can account for 0.1 to 8% dw (Metzger and Largeau, 2005) and the maximum content is reached at the end of the active growth phase (Metzger et al., 1990). After that hydrocarbon content decreases, possibly, because it is used as raw material to syn-

thesize epoxides and related compounds found in the microalga. Similar to alkenes and botryococcene, lycopadienes seem to be stored in two different locations in the cell: on the ECM and intracellularly (Metzger et al., 1990). The enzyme that catalyzes one of the final steps of lycopadiene synthesis was identified in 2016 (Thapa et al., 2016). This hydrocarbon pathway is started by the enzyme lycopaoctaene synthase, which catalyzes a head-to-head condensation of two C_{20} geranylgeranyl diphosphate (GGPP) molecules to produce C_{40} lycopaoctaene, which in turn is reduced to lycopadiene by a still unknown enzyme (Thapa et al., 2017). This hydrocarbon has also been found in samples of *Botryococcus neglectus* (Metzger et al., 1997) which suggests *B. braunii* and *neglectus* are phylogenetically related. Moreover, it has been reported that lycopadiene and botryococcene are more resistant to bacterial attack compared with alkenes from race A (Metzger et al., 1990). Lycopane, found in Chinese crude oil, can be derived from lycopadiene produced by *B. braunii*.

Race S is a new *B. braunii* strain, first described by Kawachi et al. (2012) who found it in a phylogenetic study of *B. braunii*. This race produces unsaturated C_{18} epoxy-alkanes and saturated C_{20} n-alkanes (Figure 1.1).

Due to the ability of *B. braunii* to accumulate high quantities of hydrocarbons in the ECM, it is possible to think of a system to extract these hydrocarbons in a non-destructive way instead of following the traditional method including harvesting, drying, cell disruption, and extraction. It is possible to cultivate the microalgal cells directly in contact with a biocompatible solvent for a simultaneous growth and an in-situ hydrocarbon extraction. Alternatively, cells can be grown in a separate bioreactor in culture media or biofilms (Wijihastuti et al., 2016) and repetitive hydrocarbon extractions can be performed (Moheimani et al., 2013b;

Griehl et al., 2014). In both cases the highly energy demanding steps like harvesting, drying or cell disruption are avoided, thereby improving the process energy balance and lowering costs.

1.4 Two-phase and milking extraction systems

Microalgae are becoming increasingly interesting to produce bioproducts and biofuels. However, to be economically feasible in biofuel production, costs have to be reduced around one order of magnitude (Kleinegris et al., 2011b). The traditional microalgae downstream consists in cultivating cells, harvesting, drying, grinding and disrupting the biomass to finally perform the extraction and refining of products. However, all of these activities are responsible for a relevant part of the costs and are highly energy demanding (Lardon et al., 2009). New approaches have been proposed to avoid these stages in bioproduct production. Two-phase culture systems, and its variations, allow for a simultaneous cell growth and metabolite extraction without any harvesting or drying. The idea is to grow cells in direct contact with a biocompatible solvent to allow an in-situ metabolite extraction. Two-phase systems have some advantages over traditional systems as they offer a single step process to recover, separate and purify the target product. Moreover, they are easy to manipulate, time-, cost- and energy-efficient, environmentally friendly and reliable to scale-up (Raja et al., 2012; Iqbal et al., 2016). Hydrophobic organic solvents are useful to recover poor-water soluble target molecules. For the implementation of an organic solvent recovery system, several set-ups can be used. The simplest one is a two-phase bioreactor with the aqueous culture media in direct contact with the organic solvent. This system has

been successfully used to extract several different metabolites from bacteria and microalgae (Hejazi et al., 2002; Mojaat et al., 2008; Zhang et al., 2011a; Kleinegris et al., 2011a), particularly lipids/hydrocarbons from *B. braunii* (Sim et al., 2001; Zhang et al., 2013; Moheimani et al., 2013a).

However, two opposite goals compete in these kind of systems. One goal is to obtain the maximum metabolite recovery, and another goal is to achieve minimum cell damage. In a two-phase system, organic solvents recover metabolites as they pass from solvent to aqueous phase and come into contact with cells and metabolites. However, they are toxic to microorganisms for the same reasons. When they come in contact with cells they accumulate in the plasmatic membrane, and if the solvent concentration is sufficiently high, it will produce loss of cell membrane functions, resulting in cell death (Sikkema et al., 1994; de Bont, 1998). Therefore, a balance has to be found to recover the maximum metabolite quantity with the minimum damage to maintain cell viability and growth.

The extraction in two-phase systems occurs in three ways: (1) cells are disrupted and the target molecules are released to the medium, (2) a permeabilization process is induced by the solvent in the two-phase system and, (3) in the ideal case, desired compounds are excreted to the media by the cells (Kleinegris et al., 2011b). For instance, Hejazi et al. (2002) performed a set up of a two-phase system to extract β-carotene from *Dunaliella salina*. In this case cell disruption occurred and target molecules were released to the medium. The same mechanism was used by Fiedler et al. (2007) to recover astaxanthin from *Haematococcus* immobilized in sol–gel silica matrices. The obvious problem of this method is that biomass will not increase if cell disruption is produced by the solvent used.

The second option, permeabilization, can be used when the target molecules

are not or only slightly excreted. The idea is that solvent accumulation in the cell membrane can make it more permeable, allowing leaking of low-molecular weight cellular compounds. However, it is difficult to reach sufficient solvent concentration to allow permeabilization, without producing cell death. Therefore, this option can lead to biomass productivity problems. The ideal situation is that the cell can excrete target molecules (exocytosis). This process can occur spontaneously or can be induced. Two successful cases described in the literature are Dörnenburg and Knorr (1996) and Zare et al. (2009). Normally, when target molecules are released to the medium, direct cell-solvent contact is not necessary, as they are free for recovering in the medium. However, this is not the case for *B. braunii*, one of the most interesting candidates for product excretion, as it stores excreted hydrocarbons in the extracellular matrix, where they are protected and accumulated (Largeau et al., 1980).

Behind solvent features, in a simple two-phase system where the culture is in direct contact with the solvent, metabolite recovering and cell viability depend on contact-surface and contact-time of solvent-biomass, as well as mixing conditions in the system, among others characteristics. If contact-surface increases, normally recovering increases and cell viability decreases. If contact-time of solvent-biomass increases, then recovering increases but cell viability decreases. The same is valid for mixing: if mixing increases recovering increases, but cell viability decreases (Jackson et al., 2019). Consequently, some improvements have been made to simple two-phase systems. For instance, it is possible to use a membrane to separate solvent and biomass in the aqueous phase and still allow product recovery (Valadez-Blanco and Livingston, 2009). Solvent encapsulation is also a solution to prevent direct contact of solvent-biomass. This idea has been successfully used

to protect *Saccharomyces cerevisisae* cells from inhibitory product contact in a fermentation process (Stark et al., 2003). In this study, the solvent was encapsulated into a hydrogel layer and capsules were placed into the bioreactor. Solvent in the capsules was sequestering 2-phenylethanol from the culture media to avoid inhibitory effects on *Saccharomyces*. Another improvement was developed by Griehl et al. (2014), who created a set-up to grow cells in a bioreactor separated from the solvent to reduce the contact-time of solvent-biomass, thereby increasing the biomass growth rate compared to permanent contact. The biomass is circulated through the solvent in short time intervals to recover hydrocarbons from *B. braunii* cells, in between the biomass has time to rest before the process repeats.

Choosing the configuration of a two-phase system, depends basically on the target product, the producing microorganism, the extraction mechanism, and the solvent (Kleinegris et al., 2011b).

1.5 Solvents

In 2013, 30 millions MT (metric tons) of solvents were produced to satisfy the demand of extraction, purification, and cleaning in the chemical industry worldwide (Linak and Bizzari, 2013). Global consumption has been increasing and will continue to increase over the next years. Solvents are present in almost every sector of the economy, because they are essential to chemical synthesis, separation processes, isolation, formation of azeotrope mixtures, favoring contact among reactants or stabilizing transition states. Solvents have demonstrated excellent performance on all of the priorly mentioned functions.

Normally, solvents are the most part of the mass involved in a chemical re-

action, and this is especially true in pharmaceutical or fine chemical industry, where the ratio (mass of waste / mass of product) is very high. In fact, painting, bulk chemical, pharmaceutical and fine chemical products are the most solvent demanding industries (Sheldon, 2007, 2017).

For instance, halogenated solvents and aromatic/aliphatic hydrocarbons are commonly used because of their good properties to dissolve a wide variety of compounds. However, there is an increasing consensus and concern about the problems that can be attributed to petroleum-derived solvents (traditional solvents). The first problem associated to solvent manipulation concerns physical health. Many halogenated and aromatic/aliphatic hydrocarbons display high toxicity by oral or inhalation exposure, or are mutagenic, teratogenic and/or carcinogenic if used improperly (Huang et al., 2014). Currently the use of many of these solvents is regulated, with the recommendation of avoiding them if a replacement is available. A second issue related to the manipulation of traditional solvents is safety. As many of them posses a high vapor pressure, they are highly volatile, and thus a high risk of flammability or explosiveness exist. A third issue is the environmental impact of traditional solvents. Environmental persistence is a negative feature associated with this kind of solvent, as they can stay decades in soil and water. Additionally, in the air they can contribute to global warming, to the formation of ozone in low altitude or to ozone depletion in the high atmosphere. Another important problem in this respect is that most halogenated and aliphatic/aromatic solvents are produced using fossil raw material, which means that even before they are used, their production already contributes to global warming. Moreover, exhaustible raw material will be limited at some point in the near future.

Several efforts have been made to regulate and control the use of traditional

solvents, for instance: (1) in Europe the registration, evaluation, authorization and restriction of chemicals (REACH) proposed a restriction for chemicals such as chlorinated solvents, ethers of ethylene glycol, N-methylpyrrolidinone (NMP) and N,N-dimethyl formamide (DMF)(ECHA, 2019); (2) in 1987, the Montreal Protocol on Substances that Deplete the Ozone Layer release a list of substances with the potential to damage the ozone layer, with the goal of controlling their production and use (ONU, 2018); (3) before that, in 1971, the International Labor Organization (ILO) adopted the Benzene Convention to protect workers against risks arising from benzene use (ILO, 2001).

Beyond environmental, health and safety requirements, solvents also have to meet technical specifications. In the case of an extraction process, the first requirement is affinity, the most fundamental property to be considered because it determines the viability of the process from a technical point of view. There are some data bases with solvent-solute pairs, however, the solvent needs to be tested experimentally as the conditions are specific for every set-up (Grundtvig et al., 2018). Viscosity, vapor pressure and melting point are also important characteristics determining the extraction process success. Highly viscous solvents have liquid-liquid transferring mass problems. The boiling point is important if the solvent-solute separation process will be strictly made by distillation, even though there are other options such as exclusion chromatography or nanofiltration (Marchetti et al., 2014). Chemical stability is also important to avoid secondary reactions of the solvents. The emulsion formation and biocompatibility have to be considered as well, because most of these properties dependent on the characteristics of an specific system. Experimental work is necessary to assess the suitability of the solvent for the process (Grundtvig et al., 2018).

1.6 Biocompatible and bio-based solvents

For the specific case of an extraction in a two-phase system maintaining cell viability, which is of interest to this study, Daugulis (1997) and Frenz et al. (1989a) compiled a list of desirable properties for a biologically compatible (*biocompatible*) solvent: 1) immiscibility with water, 2) an at least 5% different density value compared to water, 3) low boiling point, 4) high selectivity towards the target compound, 5) favorable distribution coefficient for the target product, 6) chemical inertness, 7) thermal stability, 8) inexpensiveness, 9) high availability, 10) low emulsion-forming tendency, and 11) biocompatibility. In particular, biocompatibility plays a crucial role in a two-phase system as the biomass growth has to be maintained to continuously produce the target compound. It has been found that solvents with a high molecular mass, high boiling point and low polarity tend to be biocompatible. In terms of octanol-water partition coefficient (P_{ow}), solvents with a $\log(P_{ow})$ value higher than 5 are considered as biocompatible (Frenz et al., 1989a; León et al., 2001; Zhang et al., 2011b). Additionally to the characteristics already described, Harper et al. (2000), Birch (2000), and Raynie (2000) mention that the desirable solvents should: 12) be easy to handle, 13) possess a low vapor pressure, 14) have the ability to load a high amount of solute per unit of solvent, 15) exhibit a low toxicity, and due to environmental concerns, the solvent should also 16) produce a low environmental impact and 17) be renewable.

In the last decades, due to pollution and global climate change, societies and particularly academia, have payed more attention to the use of highly contaminant and hazardous materials and processes. The declaration of the 12 principles of green chemistry produced an inflection point in the efforts for a reduction of

hazardous materials and processes (Anastas and Eghbali, 2010). These efforts have sparked more interest in the so called green solvents, which are those that show a low environmental impact, less safety and health hazards, and a reduced life cycle impact (Capello et al., 2007).

Bio-based materials are those derived from renewable feedstock, including solvents. Bio-based solvent production is in agreement with principles of green chemistry, consequently, in the last years it has been an active research topic in an effort to replace fossil based solvents. Some important bio-based solvents are: glycerol (and its derivatives), carbohydrates in solution (and its derivatives), lactic acid (and its derivatives), gluconic acid aqueous solution, 2-methyltetrahydrofuran, limonene and p-cymene, γ-valerolactone, lignin-derived solvents, fatty acid methyl esters (FAMEs) and isopropyl ester (Gu and Jérôme, 2013).

Some of these bio-based solvents possess many of the desired properties for a biocompatible two-phase extraction, such as high hydrophobicity, easy availability, low vapor pressure or chemical stability. However, they have not been tested yet for affinity towards hydrophobic compounds or biocompatibility with microalgae. In this study, one member of terpenes (limonene) and one of the FAMEs family (biodiesel), will be tested for their biocompatibilty and hydrocarbon extraction capacity.

Limonene is a terpene found naturally in citrus fruit. It can be obtained as a by-product of the citrus juice production (Negro et al., 2016), but also by bio-conversion of glucose made by *E. coli* or *S. cerevisiae*, thereby avoiding the dependency on citrus fruits (Chemat et al., 2019). It has been used as a carbon source for bacteria (De Carvalho et al., 2005), as a fragrance, a degreasing agent (Toplisek and Gustafson, 1995) and even as a flavor as it is recognized as a

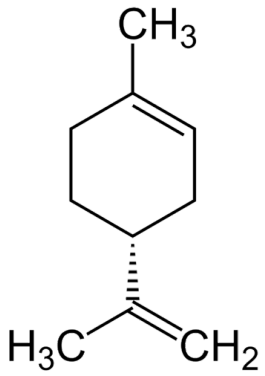


Figure 1.2: d-limonene molecule (1-methyl-4-(1-methylethenyl)-cyclohexene). Limonene can be found naturally in citrus fruit or be obtained by bio-conversion of glucose made by *E. coli* or *S. cerevisiae*.

GRAS material (Generally Recognized As Safe) by the USA FDA (Food and Drug Adminstration) (Mira et al., 1999). As a solvent limonene has been used to replace toluene, n-hexane, and chlorinated organic solvents (Ciriminna et al., 2014) and has been successfully used to extract oil from rice bran (Liu and Mamidipally, 2005; Mamidipally and Liu, 2004) and olives (Virot et al., 2008b,a). In these cases the extraction has taken place using a microwave-assisted Soxhlet method, with a high solvent recycling rate (90%) and a reduced time (only 32 min) compared to a normal Soxhlet extraction system (8 h) (Chemat et al., 2012).

On the other hand, the major component of vegetable oil are tryacylglicerols (TAGs). These are composed by a glycerol backbone with three attached fatty acids. These fatty acids can be differents, typically they possess between 16 and 18 carbon atoms and possess saturated or polyunsaturated bonds. When this vegetable oil is subjected to a chemical thermal reaction (transesterification) with a catalyst and alcohol (normally methanol or ethanol), the corresponding alkyl esters are obtained. The alkyl esters of methanol that are used as fuel in diesel engines are called biodiesel (Knothe, 2010). Biodiesel can be produced

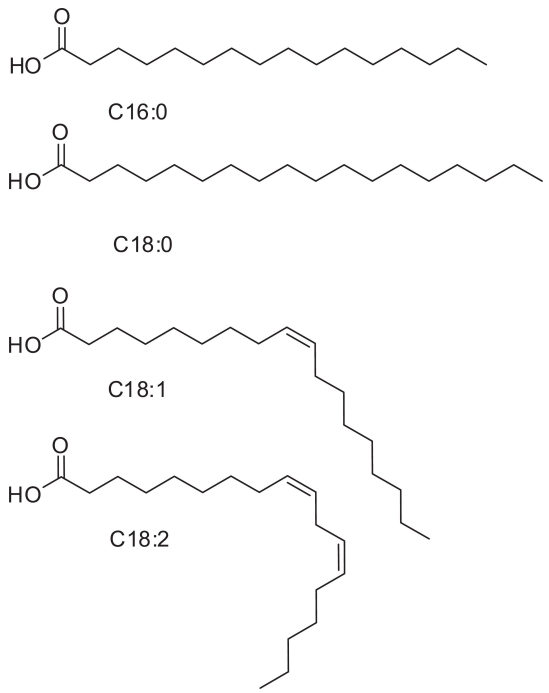


Figure 1.3: Typical fatty acid methyl esters (FAMEs) from vegetable oil. FAMEs can be obtained by vegetable oil transesterification.

using many different feedstock, such as oily vegetable seeds or animal fats. The feedstock quality will determine biodiesel features. Biodiesel is mainly used as a biofuel, but other uses are possible, for instance, it can be used as a plasticizer (Wehlmann, 1999) or as a high-boiling absorbent for gaseous industrial emissions cleaning (Bay et al., 2006) or even as a solvent (Gonzalez et al., 2007; Knothe and Steidley, 2011).

As a solvent, biodiesel is highly hydrophobic with an octanol-water partition coefficient (P_{ow}) value of ca. 6.2 (depending on the oil used as raw material) (Mabanaft, 2018). It has a density of around 0.875 $kg\,L^{-1}$ (Bajpai and Tyagi, 2006) which makes it easy to separate from water. It has a low vapor pressure at room temperature (Yuan et al., 2005; Castellanos et al., 2012) compared to other solvents such as hexane or decane, that are unsafer for manipulation. It is

chemically stable (Mabanaft, 2018), has a low toxicity (Khan et al., 2007; Pereira et al., 2011; Müller et al., 2019), is biodegradable (Zhang et al., 1998), and is one hundred percent renewable when produced by using bioetanol. All of these features make biodiesel a good candidate for a biocompatible solvent. In this study limonene and biodiesel will be tested in a two-phase hydrocarbon extraction system in permanent contact with microalgal biomass to test their biocompatibility and hydrocarbon extraction capacity. Their performance will be compared to the standard organic solvents n-decane and n-decanol.

1.7 Hypothesis

It is possible to develop a petroleum independent methodology to extract external hydrocarbons from the microalga *Botryococcus braunii*, while simultaneously maintaining cell viability by using bio-based solvents.

1.8 General objective

To test two bio-based solvents in a two-phase extraction system in terms of bio-compatibility, cell viability and external hydrocarbon recovery yields from the microalga *Botryococcus braunii*.

1.9 Specific objectives

- To test the bio-based solvent limonene (1-methyl-4-(1-methylethenyl)-cyclo hexene) in a two-phase system in terms of biocompatibility and to examine changes on cell membrane as microalga cell tolerance mechanism.
- To test fatty acid methyl esters derived from canola oil as a bio-based solvent in a two-phase extraction system in terms of biocompatibility and hydrocarbon extraction yield.

Chapter 2

Effects of limonene, n-decane and n-decanol on growth and membrane fatty acid composition of the microalga *Botryococcus braunii*

Botryococcus braunii is a promising microalga for the production of biofuels and other chemicals because of its high content of internal lipids and external hydrocarbons. However, due to the very thick cell wall of *B. braunii*, traditional chemical/physical downstream processing very often is not as effective as expected and requires high amounts of energy. In this cases, the application of two-phase aqueous-organic solvent systems could be an alternative to cultivate microalgae allowing for a simultaneous extraction of the valuable compounds without significant negative effects on cell growth. Two-phase systems have been applied before, however, there are no studies so far on the mechanisms used by microalgae to survive in contact with solvents present as a second-phase. In this study, the effects of the solvents limonene, n-decane and n-decanol on growth of the microalga *Boytococcus braunii* as well as the adaptive cell response in terms of their phospholipid fatty acid contents were analized. A concentration-dependent negative effect of all three solvents on cell growth was observed. Effects were accompanied by changes of the membrane fatty acid composition of the alga as manifested by a decrease of the unsaturation . In addition, an association was found between the solvent hydrophobicity (given as log octanol-water partition coefficient (P_{ow}) values) and their toxic effects, whereby n-decanol and n-decane emerged as the most and least toxic solvent respectively. Among the tested solvents, the latter promises to be the most suitable for a two-phase extraction system.

2.1 Introduction

Botryococcus braunii is a microalga that can be found in fresh, brackish, and saline water all around the world (Aaronson et al., 1983). This microalga is

considered to be a source of lipids and hydrocarbons and can thus possibly serve as a base for renewable fuel production (Ashokkumar and Rengasamy, 2012). It is known that lipid productivity in *B. braunii* is higher when it is cultivated under nitrogen-depletion or other stress conditions (Cheng et al., 2013). However, the total amount of lipids available for biotechnological applications depends on the biomass productivity, that is normally reduced under stress conditions. Unlike lipids, hydrocarbon production is proportional to cell growth. Accordingly, more hydrocarbons are obtained when more biomass is produced (Kojima and Zhang, 1999).

For extracting biotechnologically valuable products from microorganisms generally two different methods are used: (i) intensive extraction from harvested biomass (Cooney et al., 2009; Kumar et al., 2015) and (ii) continuous extraction in a two-phase aqueous-organic solvent system, during ongoing microbial growth (Kleinegris et al., 2011a). This second approach has been used to extract valuable compounds such as carotenoids, lipids, and hydrocarbons from microalgae maintaining, whereby cell growth is, at least partially, maintained (Sim et al., 2001; Hejazi and Wijffels, 2004; Zhang et al., 2011c).

Two-phase systems may also be advantageous for the extraction of hydrocarbons from *B. braunii* in a biofuel production context as: (i) most hydrocarbons of *B. braunii* are located outside of the cell wall (approx. 95% according to Largeau et al. (1980)), and are therefore more easily extractable than internal lipids; (ii) two-phase systems potentially allow for both, the ongoing cultivation of cells and the harvest of external hydrocarbonswhich move from aqueous to solvent phase.

Maintaining microbial growth in a two-phase system depends on the tolerance and adaptive properties of microorganisms to the conditions and solvents applied.

Responses of bacteria in contact with solvents have been widely studied (Manefield et al., 2017) and data show that solvent effects on cell membrane include alterations in order, packing, and interaction of lipids-lipids and lipids-proteins, or impairments on membrane functions such as the selective permeability and enzymatic activity (Weber and de Bont, 1996; Isken and de Bont, 1998; Mattos, 2001). Adaptive bacterial responses to counteract solvent effects include alterations of the content of their membrane phospholipid fatty acids, morphological changes, active solvent transport out of cell membrane, and modification of surface charge and hydrophobicity (Heipieper et al., 2007; Segura et al., 2012; Guan et al., 2017; Kusumawardhani et al., 2018).

A convenient proxy for the adaptation of the membrane of eukaryotic cells (including fungi and algae) is the fatty acid unsaturation index (UI) (Heipieper et al., 2000). This index is the average number of double bonds present in every lipid unit in the sample. In this experiment UI is the unsaturation level index of membrane fatty acids. Therefore, a decrease in the UI is related to a decrease in membrane fluidity and an increase in the rigidity of the cell membrane (Weber and de Bont, 1996), as a response, for instance, to membrane fluidizing solvents.

Previous studies examining the effect of stress on the fatty acid profile of microalgae include effects of NaCl, irradiation, CO_2, temperature and heavy metals (Vazquez and Arredondo, 1991; McLarnon-Riches et al., 1998; Sushchik et al., 2003; Rao et al., 2007; Chen et al., 2008; Kalacheva et al., 2002; Zhila et al., 2011; Yoshimura et al., 2013; Dawaliby et al., 2016). However, to our knowledge so far no study has addressed the solvent stress on changes of the UI of microalgae membrane fatty acids.

Depending on goals, solvent selection should be a balance among different

solvent characteristics (Daugulis, 1988). In this study, on the one hand hydrocarbon extraction capabilities and biocompatibility are necessary, but on the other hand a sustainable solvent production and an easy solvent-hydrocarbon separation (low energy cost) are desirable from a renewable fuel production perspective. These last two conditions, are reasons to consider limonene and decane candidates. The former is a non-petroleum derived, renewable solvent (Njoroge et al., 2004), whereas the latter is one of the lowest molecular weight highly biocompatible alkanes (León, 2003). Decanol, the alcohol derived from decane, is less hydrophobic and, therefore, more water soluble, what could provide a higher hydrocarbon extraction although a lower biocompatibility. In this study, only biocompatibility will be tested.

Limonene has been used before to extract hydrophobic compounds such as oils and carotenoids from diverse types of matrices with good results (Mamidipally and Liu, 2004; Virot et al., 2008b,a; Chemat-Djenni et al., 2010; Tanzi et al., 2012). Oil extraction yields, based on dry weight, have oscillated between 13.1% (Chemat-Djenni et al., 2010) and 48.6% (Virot et al., 2008a), although these values depend on oil content in their respective matrices. Limonene used to extract lipids from the microalga *Chlorella vulgaris* recovered 38.4% of its respective total lipids (Tanzi et al., 2012). Nevertheless, in our knowledge, limonene has never been used as solvent in a two-phase system to extract hydrophobic compounds.

Decane has been used as solvent to extract hydrophobic compounds from two-phase systems with alive microalga. Results have varied in biocompatibility and extraction capacity, oscillating from high (León et al., 2001; León, 2003; Zhang et al., 2011b) to low (León et al., 2001; Hejazi et al., 2002) biocompatibility and from acceptable (León, 2003; Zhang et al., 2011b) to poor (Mojaat et al., 2008)

compound extraction capabilities. These results, however, depend on extraction system conditions and microalga species and should therefore be taken with caution.

In this study, we tested the effects of both mineral solvents, n-decane and its derived alcohol n-decanol, as well as the effects of the renewable solvent limonene, on the growth and membrane fatty acid profile of the microalga *B. braunii* in a two-phase aqueous-organic solvent system.

2.2 Materials and methods

2.2.1 Preculture conditions

A 6 L preculture was established to supply biomass in an exponential growth phase for two-phase cultures. The microalga strain used in this experiment was *Botryococcus braunii* race A (UTEX LB572) provided by the Universidad de Antofagasta, Chile. The preculture was carried out in a 10 L glass bottle (Cat.No.11 602 00, Duran Group) using the medium described by Bazaes et al. (2012), but replacing HPO_3 for NaH_2PO_4. The pH was set at 6.5 using HCl (5 M) and the medium was autoclaved at 121°C for 21 min. The microalga grew under continuous (24:0 h light:dark cycle) cool fluorescent illumination at ca. 1500 lx, and 25±1°C with neither aeration nor CO_2 source. To prevent microalga precipitation the flask was shaken twice a day manually.

2.2.2 Two-phase cultures

The experiment was set up as a two-factor factorial design, with solvent and solvent concentration as factors. When the biomass in precultures reached the exponential growth phase, 48 parts of the culture were taken (100 mL volume) and either limonene, n-decane and n-decanol were added in the necessary amount to obtain the following solvent concentrations (mM): (1) limonene: 123.3, 12.3, 1.2, 0.6, 0.3; (2) n-decane: 513.0, 282.2, 51.3, 28.2, 5.1; (3) n-decanol: 157.3, 15.7, 1.6, 0.8, 0.4. Concentrations were determined based upon literature (Liu and Mamidipally, 2005; Frenz et al., 1989a; Mojaat et al., 2008; Zhang et al., 2011b) and toxicity assays (OECD, 2002). According to the authors reports and pilot studies this range of concentrations produce quick cell death (higher rates among higher concentrations) but also fully functional cells to observe changes in membrane fatty acid composition. Three replicates, placed in 240 mL flasks with rubber caps, were used for each treatment, totalling 48 runs including three control samples (cultures without solvents). After 24 h, two aliquots were taken from every flask, one to measure biomass concentration changes (growth) and the other one to determine membrane fatty acid profile.

All conditions for two-phase cultures were the same as in preculture, including culture media and continuous illumination.

2.2.3 Cell growth measurement

Cell growth in culture and preculture was determined using a Coulter counter device (isoton II solution as diluent, 100 µm electrode, 1:500 dilution) (Ríos et al., 2012; Nguyen et al., 2013). Samples were taken in the morning, and after that

Table 2.1: Physico-chemical properties of solvents used in the two-phase aqueous-organic system. P_{mw} and [M]/[M_{dec}] were included as references.

Solvents	Molar mass (g/mol)	Max. water solub. (mM)	log $P_{ow}^{(a)}$	log $P_{mw}^{(b,c)}$	MMC$^{(d)}$	$M/M_{dec}^{(e)}$
n-decane	142.29	0.000366	5.01$^{(f)}$	4.22	6	1
Limonene	136.23	0.101299	4.23$^{(g)}$	3.46	294	≈ 49
n-decanol	158.28	0.230000	3.97$^{(h)}$	3.21	374	≈ 62

(a) Logarithm of octanol-water partition coefficient.

(b) Logarithm of water-membrane partition coefficient.

(c) Calculated according to Sikkema et al. (1994).

(d) MMC: Maximum membrane concentration of solvent. Calculated according to Neumann et al. (2005).

(e) Solvent concentration in membrane (M) divided by n-decane concentration in membrane (M_{dec}).

(f) Data from Mojaat et al. (2008).

(g) Data from Falk et al. (1998).

(h) Data from Frenz et al. (1989a).

two-phase cultures were shaken manually twice a day (12.00 and 20.00 h), to avoid that solvent droplets in samples modify microalga cell concentrations.

2.2.4 Solvent concentration in cell membrane

According to Sikkema et al. (1994) there is a direct correlation between the hydrophobicity given as log P_{ow} values of a solvent and their partitioning in biological membranes. The following empirical relation was estimated: $log(P_{mw}) = 0.97 * log(P_{ow}) - 0.64$, where P_{mw} and P_{ow} are membrane/water and octanol/water partition coefficients, respectively. This equation allows to calculate solvent concentration in a membrane for a resting-system case, which will be helpful for result interpretation (Neumann et al., 2005).

2.2.5 Characterization of membrane fatty acid profile

Membrane fatty acid profile was characterized for the control samples and biomass in contact with solvents 24 h after the first solvent-microalga contact. Membrane lipids were extracted according to Bligh and Dyer (1959) and transformed into Fatty Acid Methyl Ester (FAME) as described by Morrison and Smith (1964). FAME identification was performed using a GC-FID Agilent 6890N, equipped with a capillary chromatographic column (CP-Sil 88 capillary column, Chrompack, ID: 0.25 mm, longitude: 50 m, film: 0.2 µm). A proxy for the relevant presence of double bonds in the membrane fatty acid profile was calculated as follows:

$$UI = \frac{(\%C16:1 + \%C18:1) + (\%C18:2 * 2) + (\%C18:3 * 3)}{100} \tag{2.1}$$

, where UI is the unsaturation index (Heipieper et al., 2000; Kaszycki et al., 2013).

2.2.6 Data processing

The experiment was set as a two-factor factorial design, with solvent and solvent concentration as factors. All experiments were carried out in triplicates. The obtained data were analyzed using analysis of variance (ANOVA) to detect significant differences between solvents or solvent concentration effects. The probability of α (type I error) was set at 5%. All data processing and plots were made using the statistical computing software R (version 3.3.3) (Team, 2017).

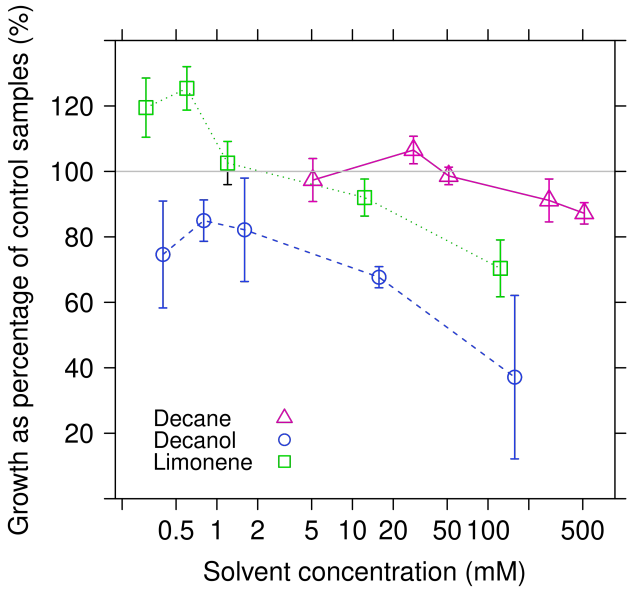


Figure 2.1: Effect of different concentrations of limonene (□), n-decanol (◯) and n-decane (△) on *Botryococcus braunii* UTEX LB572 growth, after 24 h solvent-biomass contact. Growth is expressed as a percentage of control samples. Every point is the average of three independent samples. Error bars represent standard error of the mean of the same three samples.

2.3 Results

2.3.1 Cell growth

The effect of three solvents of different log P_{ow} on *B. braunii* growth was measured (Figure 2.1). n-decanol (log P_{ow} = 3.97) was found to be the most toxic solvent tested, resulting in a lower cell concentration compared to n-decane and limonene at quasi identical solvent concentrations (p-value < 0.01). In the case of limonene (log P_{ow} = 4.23), cultures with concentrations lower to 1.2 mM showed higher growth than control samples (p-value = 0.03), i.e., values greater than 100% as illustrated in Figure 2.1.

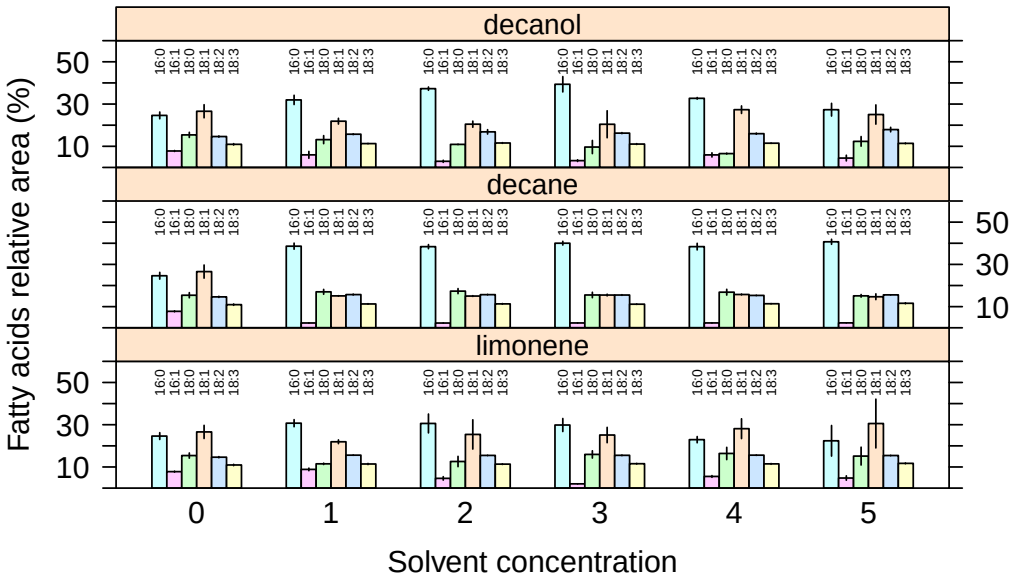


Figure 2.2: Effect of n-decanol, n-decane and limonene on membrane fatty acid profile of *Botryococcus braunii* UTEX LB572 after 24 h biomass-solvent contact for 5 different concentrations (1 to 5) and control samples (0). The number 1 correspond to the lowest concentration for every solvent, meanwhile number 5 correspond to the highest one. All data represent the average and standard error of the mean of three independent samples.

Cultures using n-decane as second-phase (log P_{ow}=5.01) grew similarly to the control samples up to 51.3 mM of solvent concentration (p-value = 0.89), and then slowly started to decrease to 70%[1] of control sample growth. As expected, the general trend for all the solvents was a lower relative growth when solvent concentration increased and when log P_{ow} decreased (see Figure 2.1).

[1] It should say 87%

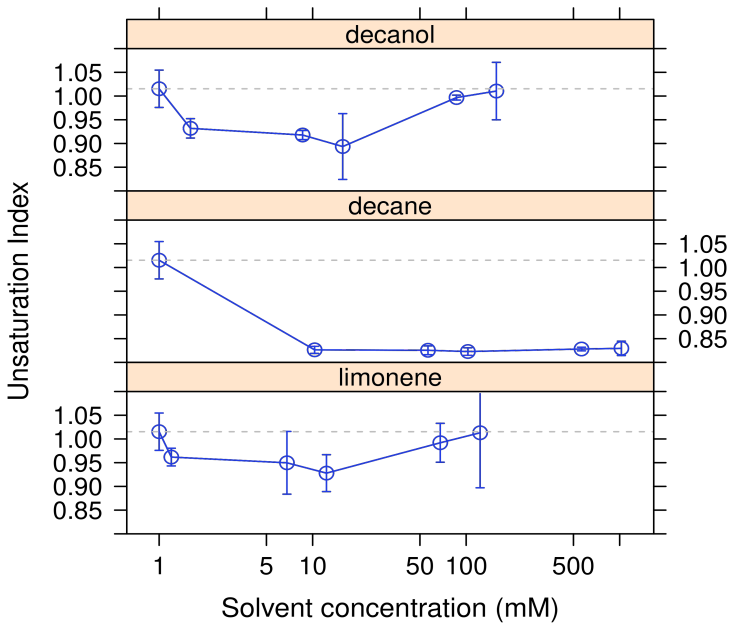


Figure 2.3: Effect of solvents on membrane fatty acid UI of *B. braunii* UTEX LB572, after 24 h solvent-biomass contact. Slashed horizontal line show control samples UI in every panel. Every point is the average of three independent samples. Bars show standard error of the mean.

2.3.2 Characterization of fatty acid profile from cells in contact with solvents

The fatty acid profile of cells from control samples, revealed that cell membranes of *B. braunii* contain mainly oleic acid (C18:1cis9, 28.0%) and palmitic acid (C16:0, 25.9%). The main effects of solvents on membrane fatty acid profile were observed on C16:0 and C18:1, and to a minor degree on C16:1. C18:2 and C18:3 showed no significant changes (Figure 2.2).

On the one hand, cells in contact with n-decanol and n-decane synthesized higher amounts of C16:0 (p-value < 0.01) on average, followed by a decrease in the content of C18:1, especially in decane (p-value<0.01). On the other hand, limonene presents a monotonic ascending trend for C16:0 and the opposite for C18:1, for increasing solvent concentrations. These changes in fatty acid profile were reflected by different UIs, showing differences in response to both the solvent type and solvent concentration, suggesting predominance of saturated fatty acids and those fatty acids with one double bond.

Addition of n-decane resulted in a decreased UI remaining at $UI \approx 0.82$, regardless of the solvent concentration. The presence of limonene and n-decanol at the three lowest concentrations levels likewise lowered the UI to the following range: $UI \approx 0.90$-0.96. For these solvents, two higher concentrations did not result in important changes on the UI compared to the control samples (Figure 2.3).

2.4 Discussion

The aim of this study was to test the effects of n-decane, n-decanol, and limonene on growth and membrane fatty acid composition, in particular the UI of *B. braunii* UTEX LB572 cells. Addition of solvents to *B. braunii* led to differing extents of growth inhibition. At quasi equimolar concentration n-decanol was found to be the most toxic solvent followed by limonene and n-decane, which showed the least inhibitory effects (Figure 2.1). Such data provide valuable information for a better evaluation of the relative physiological status of the cells and associated changes of their fatty acid profile and UIs as will be discussed below.

In 1994, Sikkema et al. (1994) hypothesized that solvent toxicity is primarily governed by the amount of solvent dissolved into the membrane rather than its chemical structure. Thus, the accumulation of molecules in the cell membrane of microorganisms would be the cause of negative effects on bilayer stability, packing of acyl chains and ion leakage problems, resulting in stress, arrest of growth, or even cell death in the extreme case (Weber and de Bont, 1996). This hypothesis was supported by results of Heipieper et al. (1995), who, working with different types of solvents on Pseudomonas putida S12, found that the concentration in membrane that produces a 50% loss in growth is similar for all of them: between 60 and 200 mM (solvents used were: methanol, ethanol, 1-butanol, phenol, 1-hexanol, p-cresol, 4-chlorophenol, toluene, 1-octanol, and 2,4-dichlorophenol).

In this study, the membrane solvent concentration was calculated for the maximum water solubility for every solvent, according to the works by Sikkema et al. (1994) and Neumann et al. (2005). Results in Table 2.1 show that, in a resting system, n-decane reached a maximum membrane concentration (MMC) around 6 mM. A low value compared with the range 60-200 mM. In contrast, MMC for limonene and n-decanol were higher than two hundred mM, 294 and 374 mM respectively, suggesting that this is the reason for the low toxicity of n-decane and high toxic effects of n-decanol on the microalga *B. braunii*. Although this is an approximate estimation of the actual solvent concentration in the cell membrane, it was consistent with results of the growth curves in Figure 2.1. These curves show that, on average, solvents with higher log P_{ow} are more biocompatible. This finding is in line with previous research on *B. braunii* and other microalgae (Frenz et al., 1989a,b; León et al., 2001; Zhang et al., 2011c).

Notably, for some concentrations of limonene and n-decane, growth reached

values greater than 100%. A possible explanation is that within a certain range of concentration, solvents produced cell membrane instability, which in turn favours mass transfer between cells and culture medium. Consequently, nutrients and oxygen permeate more easily through cell membrane, accelerating growth (León et al., 2001). This high growth associated with limonene and n-decane is also in agreement with previous studies on *Aerobacter aerogenes* and *Saccharomyces cerevisae* (Rols et al., 1990; Jia et al., 1997), which reported that oxygen dissolves more easily in organic solvents compared with water, working as improved oxygen-vectors, thus incrementing the oxygen transfer rate and growth in *B. braunii* cultures. Another reason for the high growth could be that solvents are actually working, simultaneously, as carbon sources (De Carvalho and Da Fonseca, 2004; De Carvalho et al., 2005), which is possible as *B. braunii* has been reported as a mixotrophic microalga (Zhang et al., 2011b; Tanoi et al., 2011).

The adaptive response of *B. braunii* to solvent contact was similar for all solvents tested in our studies. The greatest changes in fatty acid profile were produced by n-decane, where C16:0 abundance was remarkably increased while C18:1 decreased. A reduction of C16:1 also occurred (Figure 2.2). As a result of these changes, an UI drop from 1.02 (control samples) to around 0.82 for all n-decane concentrations was produced (Figure 2.3). As Figure 2.1 illustrates, cells in contact with n-decane seem to have a growth comparable to control samples for all concentrations. These outcomes suggest that n-decane, dissolved in culture media and cell membrane, was enough to stimulate cells to produce *de novo* synthesis of saturated and/or less unsaturated lipids to counteract increased fluidity, but not enough to stop cell growth (Figures 2.1 and 2.3).

According to Piper (1995), solvent accumulation in cell membrane produce changes in membrane fatty acids similar to those produced by an increase in temperature, due to both stressors induce an increment in fluidity and loss of selective permeability. *B. braunii* exposed to rising temperatures showed a reduction in its UI (Kalacheva et al., 2002; Sushchik et al., 2003), as was also found in this study. Sushchik et al. (2003) observed that an increment from 30°C up to 40°C increased C16:0 from 56.3% up to 73.0% of total fatty acids, while simultaneously C18:2 and C18:3 were reduced from 14.9% to 8.8% and from 19.4% to 10.3%, respectively. In this study, however, there were no significant changes in linoleic (C18:2) or linolenic (C18:3) acid abundance, probably because the increase in membrane rigidity due to the reduction from 3 to 2, or 2 to 1 double bond is not as large as when the change is from 1 to 0 double bond, since the structure of a saturated fatty acid is linear.

The underlying logic in a reduction of membrane fatty acid unsaturation is that saturated fatty acids counteract increasing membrane fluidity and permeability, due to rising temperature or solvent contact with cells, as they can be packed more tightly due to their straightness (Sikkema et al., 1995). Other microalgae exposed to a rise in temperature, such as *Nannochloropsis sp.* (Hu and Gao, 2016) and *Chlorella vulgaris* (Sushchik et al., 2003) also showed a similar behaviour, reducing unsaturation. Meanwhile a reduction in temperatures, produce the opposite effect, i.e., increased fatty acid unsaturation to maintain membrane fluidity (Thompson et al., 1992; McLarnon-Riches et al., 1998; Mikami and Murata, 2003; Chen et al., 2008).

Microalgae can also change fatty acid unsaturation levels to regulate membrane fluidity altered by modifications in environmental or anthropogenic factors

such as light, heavy metals, CO_2 or NaCl. However, the direction of changes are not always clear (Tsuzuki et al., 1990; McLarnon-Riches et al., 1998; Zhila et al., 2011; Hu and Gao, 2016) since eukaryotes use others complementary mechanisms to regulate membrane stability, such as production of sterols or synthesis of metabolites to counteract osmotic pressure produced by salts (Vazquez and Arredondo, 1991; Rao et al., 2007).

With regard to limonene and n-decanol an UI reduction was found (compared to control samples) at the three lower solvent concentrations, meaning cells were still able to perform changes at fatty acids synthesis level, despite of the stress produced by solvents. At the two higher solvent concentrations the UI remained comparable to the control samples for both solvents. Coincidently, higher concentrations of limonene and n-decanol produced lower growth rates compared to control samples suggesting that stress reduces synthesis of fatty acids, which is a requisite for a change in the saturated/unsaturated ratio (Segura et al., 2004).

In conclusion, this study confirms for *B. braunii*, what has been known for bacteria: *B. braunii* performs changes in lipid profile and unsaturation of membrane lipids in contact with solvents as a strategy to maintain membrane fluidity, tolerate stress and keep its growth. Additionally, as predicted by log P_{ow}, n-decanol was identified as the most aggressive solvent as second-phase; limonene had an intermediate effect, whereas n-decane seems to be able to maintain high growth rates even at high concentrations, being the most suitable solvent to extract valuable lipophilic compounds like hydrocarbons in a two-phase culture, under conditions used in this study.

2.5 List of abbreviations

- FAME: Fatty Acid Methyl Ester
- lx: lux
- mM: milimolar
- MMC: Maximum membrane concentration
- P_{ow}: Partition coefficient octanol-water
- P_{wm}: Partition coefficient water-membrane
- UI: Unsaturation index

2.6 Funding

This study was funded by CONICYT award numbers 781211006, 24121442, 75120043 and FONDECYT 1150707

2.7 Author's contributions

EC and HJH designed experiment, analyzed data, and wrote the paper. EC performed the experiment. LYW, GAC and RN wrote the paper. All authors read and approved the final manuscript.

Chapter 3

Hydrocarbon extraction from microalga *Botryococcus braunii* in a two-phase system using biodiesel as a biocompatible solvent

Obtaining valuable compounds from microalgae presents two main problems: it is energetically expensive to concentrate biomass for further compound extraction and the solvents traditionally used are hazardous for humans and environment. In-situ extraction methods such as two-phase systems, avoid energetically expensive stages of compound extraction from microalgae, although, solvents used as second-phase (mainly alkanes) have several drawbacks: they could be toxic, mutagenic, teratogenic, flammable, explosive, carcinogenic, environmentally persistent, among other issues. Biobased solvents can help to avoid some of these issues. Biodiesel has normally been used as a biofuel, but it could also be used as a solvent. In this study, it was used as a second-phase solvent in a two-phase hydrocarbon extraction system from microalgae *Botryococcus braunii*, to test extraction capacity and biocompatibility. After nine days of solvent-biomass contact it was found that for 50% solvent concentration, biodiesel performance was similar to decane in hydrocarbon extraction yield (20.6% with biodiesel; 22.3% with decane) and was able to maintain a higher biomass relative growth (39.0% biodiesel; 16.3% decane). However, decane was faster than biodiesel in recovering hydrocarbons from the biomass. Contact angle measurements support the hypothesis that hydrocarbons have been removed by solvents, producing lower contact angles for decane compared to biodiesel, indicating that more hydrocarbons have been removed by decane. In addition, lower contact angle values for higher solvent concentrations were obtained, indicating more hydrocarbons have been removed by higher solvent concentrations.

3.1 Introduction

Microalga *Botryococcus braunii* possess two unusual features that make it a candidate for renewable liquid biofuel production. In fact, it can produce a high amount of hydrocarbons, as much as up to 61% expressed as a percentage of its dry weight (Metzger et al., 1985), and in addition, about 95% of these hydrocarbons are stored outside the cell membrane on the outer part of its wall (Largeau et al., 1980). Two possible ways can be applied to extract these hydrocarbons with liquid solvents. The classic solid-liquid extraction process, in which an aqueous culture with around 2 $g\,L^{-1}$ solid concentration has to be converted into a solid mass by removing the water, which is a highly energy-consuming process (Lardon et al., 2009), or by using a two-phase aqueous-hydrophobic solvent system, which allows for hydrocarbon extraction without removing the water (Hejazi et al., 2002). In this type of system, biomass growth and hydrocarbon extraction occurs simultaneously, being more efficient in terms of energy balance (Saga et al., 2015). However, microalgae growth in the presence of solvents is not an easy task, mainly due to the possible toxic effects of several solvents on the growth of microorganisms (Sikkema et al., 1995; de Bont, 1998). Solvents that allow microorganisms growth are called biocompatible and in a two-phase system, these solvents are requested to meet some specific features to perform a feasible and efficient process. Daugulis (1997) has defined desirable solvent features for metabolites extraction from microorganisms while maintaining cell viability. Previously, Frenz et al. (1989a) defined a similar list of solvent features to extract hydrocarbons from *Botryoccocus braunii*. After analyzing both research works it is possible to state that the desirable solvent characteristics are: 1) immiscibility

with water, 2) an at least 5% different density value compared to water, 3) low boiling point, 4) high selectivity towards the interest compound, 5) favorable distribution coefficient for product and substrate, 6) chemical inertness, 7) thermal stability, 8) inexpensiveness, 9) high availability, 10) low emulsion-forming tendency, and 11) biocompatibility. In particular, biocompatibility plays a crucial role in a two-phase system as biomass growth has to be maintained to continuously produce hydrocarbons. In this regard, it has been found that solvents with a high molecular mass, high boiling point and low polarity tend to be biocompatible. Measuring polarity in terms of octanol-water partition coefficient (P_{ow}), solvents with a $\log(P_{ow})$ value higher than 5 are considered as biocompatible (Frenz et al., 1989a; León et al., 2001; Zhang et al., 2011c). Additionally to the characteristics already described, Harper et al. (2000), Birch (2000), and Raynie (2000) mention that the desirable solvents should: 12) be easy to handle, 13) possess a low vapor pressure, 14) have the ability to load a high amount of solute per unit of solvent, 15) exhibit a low toxicity, and due to environmental concerns, the solvent should also 16) produce a low environmental impact and 17) be renewable.

Aromatic and aliphatic hydrocarbons have normally been used as solvents, as they have poor water solubility, which makes them easily separable from water systems. However, these solvents can present several drawbacks such as high oral and inhalation toxicity, mutagenicity, teratogenicity, carcinogenicity, environmental persistence, high volatility, risk of acute and chronic problems to users, flammability, explosiveness, and their derivation mainly from fossil feedstocks (Huang et al., 2014; Pena-Pereira and Tobiszewski, 2017). These characteristics make them hard to choose if a safe and environmentally friendly process is desired.

Regarding biofuel production from microalgae, solvents screening in experi-

mental processes using two-phase systems to extract hydrocarbons or lipids have been focused on organic aliphatic solvents, mainly hexane (Griehl et al., 2014), heptane (Moheimani et al., 2013a; Jackson et al., 2019), octane (Choi et al., 2013), decane (Mojaat et al., 2008), dodecane (Mehta et al., 2019), tetradecane and hexadecane (Sim et al., 2001; Zhang et al., 2011c). However, these solvents possess several non-desirable properties and some of them are non-biocompatible (Frenz et al., 1989a). Moreover, organic solvents are derived from non-renewable sources such as petroleum, which biofuels will supposedly replace due to scarcity. Thus, it would be inconsistent to use organic solvents as a raw material to produce biofuels to replace petroleum. Additionally, due to human health and environmental concern, many efforts have been made to reduce the use of organic solvents (ILO, 2001; ONU, 2018) and more green processes have been developed in different areas of chemistry to avoid them (Anastas and Eghbali, 2010; Pena-Pereira and Tobiszewski, 2017; Gu and Jérôme, 2013).

Biosolvents such as terpenes, vegetable oils or biodiesel possess several of the desirable properties to be used as a solvent in a two-phase hydrocarbon extraction system. In particular, biodiesel is highly hydrophobic with a $\log(P_{ow})$ value ca. 6.2 (depending on the oil used as raw material) (Mabanaft, 2018), which makes biodiesel a biocompatible solvent (Frenz et al., 1989a; Mojaat et al., 2008; Zhang et al., 2011c). In addition, it has a density of around 0.875 $\mathrm{kg\,L^{-1}}$ (Bajpai and Tyagi, 2006) which makes it easily separable from water forming a second-phase. Moreover, it is obtained from vegetable oils by transesterification using an alcohol and a catalyst, which makes it easily available and it has a low vapor pressure at room temperature (Yuan et al., 2005; Castellanos et al., 2012) compared with solvents such as hexane or decane, which makes it safe for manipulation. Biodiesel

is also easy to handle as it does not emit toxic vapors, it is chemically stable (Mabanaft, 2018), it possesses a low toxicity (Khan et al., 2007; Pereira et al., 2011; Müller et al., 2019), it is readily biodegradable (Zhang et al., 1998), and it is hundred percent renewable when produced using bioetanol. However, some properties of biodiesel as a solvent have not been tested yet, such as biocompatibility with microalgae in a two-phase system, or selectivity and capacity for hydrocarbon extraction. In this study, the use of biodiesel as a biocompatible solvent to extract hydrocarbons from *B. braunii* while simultaneously maintaining cell viability in a two-phase extraction system will be explored, and compared with decane as a standard organic solvent.

3.2 Materials and methods

3.2.1 Preculture conditions

A *B. braunii* preculture was established to provide biomass. The green microalga *B. braunii* UTEX LB572 was obtained from the University of Antofagasta, Chile. It was cultivated in the medium described by Bazaes et al. (2012), but instead of HPO_3, double the amount of NaH_2PO_4 was used. The preculture (6 L) was performed in a 10 L Schott-bottle under constant air-CO_2 mixture (2% CO_2) aeration (500 mL min^{-1}). The preculture medium was shaken with a magnetic stirrer at 200 rpm. The cultivation temperature ranged between 24 and 26°C, with an average illumination intensity of 100 µmol m^{-2} s^{-1} and a 24:0 h light-dark cycle.

3.2.2 Two-phase culture conditions

When the microalgal preculture reached its exponential growth phase, different volumes were taken to establish two-phase cultures. Culture media and solvents were added in the proportions presented in Table 3.1. Solvent concentrations were defined based on previous assays in our laboratory. Every combination of culture medium and solvent was established in triplicate. In addition, a control without a solvent was used. The resulting 21 flasks, with 100 mL of liquid volume, were shaken with a magnetic stirrer at 100 rpm. Temperature, light intensity and light:dark cycle were the same as in preculture. Air-CO_2 mixture (2% CO_2) was bubbled into every flask at a rate of 30 mL min^{-1} throughout the culture period.

Table 3.1: Solvent concentration used in solvent-microalgal biomass two-phase cultures.

Solvent concentration (%)	Culture medium (mL)	Solvent (mL) (Decane or Biodiesel)	Total volume (mL)
5	95	5	100
30	70	30	100
50	50	50	100

The experiment lasted nine days. During this time, five samples from culture medium and solvents were taken from every flask, the first after 24 h solvent-biomass contact, and after that, four additional times every 48 h. Solvent samples were constant in volume: (100 µL for decane and 20 µL for biodiesel). To maintain the correct proportion of solvent:culture medium, the appropriate amount of culture medium was taken every time. Solvent samples were used to measure

the hydrocarbon mass transferred to the solvent, and biomass samples were used to measure biomass concentration. As described before, solvent physicochemical characteristics are important for both cell growth and hydrocarbon extraction, therefore, four relevant solvent features are presented in Table 3.2.

Table 3.2: Physico-chemical properties of solvents used in the two-phase system.

Solvents	Molar mass (g/mol)	Max. water solubility (mM)	log $P_{ow}^{(a)}$	log $P_{wm}^{(b,c)}$
Decane	142.29	0.000366	5.01	4.22
Biodiesel	$273.13^{(d)}$	$0.000051^{(e)}$	6.30	$5.47^{(e)}$

(a) Logarithm of octanol-water partition coefficient.

(b) Calculated according to Sikkema et al. (1994).

(c) Logarithm of water-membrane partition coefficient.

(d) Calculated with data from Hidalgo et al. (2015).

(e) Calculated with data from Von Wedel (1999).

3.2.3 Culture growth

Microalgal cell concentration in cultures was measured using a Flow Cytometer FACS Canto II (Becton Dickinson, USA). This device counts the cell number in a known culture medium volume. The cell number was correlated to dry weight in order to obtain a calibration curve. Two-phase culture growth was expressed as a percentage of control growth.

3.2.4 Hydrocarbon extraction

To quantify the total amount of hydrocarbons in *B. braunii* cultures at the beginning of the experiment, three microalgal biomass samples were taken from the preculture, just after mounting the set-up, filtered with filter paper (glass microfiber filters GF/C 47 mm, Whitman) and used in a two-step extraction process with a Soxhlet system. In the first extraction step, 30 mL chloroform-methanol solution (1:2) was used for 7 h. The second extraction step was performed with 30 mL hexane for an additional 7 h on the same biomass. The solvents were evaporated and the extracts were re-suspended in acetone with fluoranthene as an internal standard and were measured in a chromatograph as described in section 3.2.5. The same two-step extraction was repeated at the end of the experiment with all the biomass in the flasks, to obtain the total amount of hydrocarbons in the biomass-solvent system. To determine the hydrocarbon amount extracted by solvents as a second-phase, 100 µL of decane from the two-phase cultures were evaporated at 60 °C and vacuum of 90 kPa. The remaining extract was re-suspended in 100 µL of an analytic acetone-fluoranthene mixture. In the case of biodiesel, 20 µL of biodiesel samples from the two-phase cultures were diluted with 80 µL of an analytic acetone-fluoranthene mixture and injected in the chromatograph. Additionally, a sample of pure biodiesel with the same dilution was injected in the chromatograph and the result subtracted from chromatograms with hydrocarbons.

3.2.5 Hydrocarbon quantification

Hydrocarbon quantification was performed in a gas chromatograph Clarus 600 Series coupled to a mass spectrometer Clarus 500T (Perkin Elmer, USA). The chromatograph was equipped with a VF-1701 column (length: 60 m, internal diameter: 0.25 mm and film thickness 0.25 µm, Agilent Technologies, USA). Helium was used as carrier gas. The chromatograph was programmed as follows: (a) initial temperature was set at 45°C and was maintained for 4 min, (b) then the first temperature ramp was 1.0 $^{\circ}\mathrm{C\,min^{-1}}$ up to 60 °C, (c) this was followed by a second temperature ramp of 3.0 $^{\circ}\mathrm{C\,min^{-1}}$ up to 280 °C. The injection volume was 1 µL. To quantify hydrocarbons in the samples, fluoranthene dissolved in acetone, as internal standard, was used. TurboMass 5.4.2 GC-MS software by Perkin Elmer in combination with NIST 05 Mass Spectral Library was used for peaks identification. The hydrocarbon content in solvent samples was expressed in two different ways: (1) as hydrocarbon concentration, i.e., the total hydrocarbon quantity divided by the total solvent volume ($\mu\mathrm{g\,mL^{-1}}$), and (2) as a percentage (%) of hydrocarbon quantity in the biomass at the beginning of the experiment (day 0).

3.2.6 Contact angle (CA)

CA can be considered a measurement of surface hydrophobicity. If hydrocarbons are removed from the biomass, then a layer of biomass after contact with solvents should have a lower CA compared with the same biomass before the contact with solvents. In this study, CA measurements were performed at the beginning and at the end of the experiment to confirm that hydrocarbons were

removed. For measuring the CA, the cells were concentrated by centrifugation and then washed three times with a 10 mM KNO_3 solution to eliminate solvent residues and culture medium. Subsequently, the cell suspension was filtered with a cellulose acetate filter (0.2 µm pore size) by applying negative pressure. The wet filter membrane was fixed with a double sided adhesive tape to a microscope slide. After drying at room temperature for 2 h, the CA of the algal lawn was measured. A droplet of 3 µL distilled water was dripped off using a 10 µL microsyringe onto the algal lawn. The process was captured by real-time image shooting (Camera Lumix DMC FZ60). The droplet was enlarged by a magnifying lens of 30 x 21.0 mm and illuminated from behind to produce a defined border. The first frame after droplet and syringe were separated was used to calculate the CA using the computer program ImageJ with the plugin LB-ADSA as proposed by Williams et al. (2010). Every algal lawn was measured three times.

3.2.7 Data analysis

All the experiments were done by triplicate. Error bars in plots are standard errors of the mean. The Kruskal-Wallis test was used to compare medians ($\alpha = 0.05$ for significant results). To evaluate the hydrocarbon mass transfer kinetic from biomass to solvents, the linear fit slope of hydrocarbon concentration-time was calculated (β_1), then the hypothesis $\beta_1 = 0$ was tested. Plots, data processing and comparisons were made using R (version 3.5), a language and environment for statistical computing (Team, 2017).

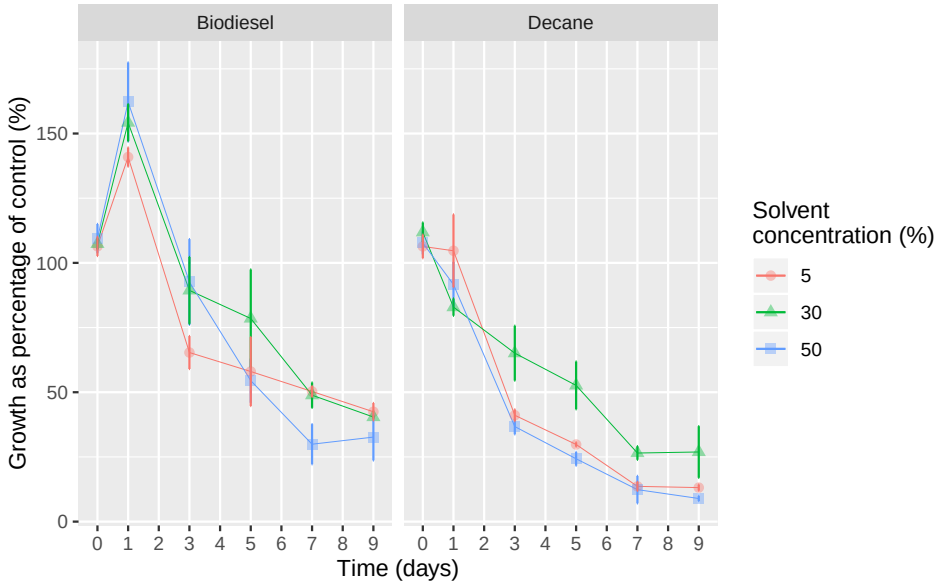


Figure 3.1: Two-phase culture growth as a percentage of control culture. Data are separated into two panels by solvent. Inside every panel data are grouped by concentration: 5, 30 and 50% v/v.

3.3 Results

3.3.1 Cell growth

Figure 3.1 presents the two-phase culture growth as a percentage of control culture for every solvent and concentration from day one to nine. Values from the inoculation day (day 0) are also provided as a reference. After 24 h solvent-biomass contact (day 1), culture growth with biodiesel was higher than controls (up to ≈ 175%). However, after this initial jump, microalgae growth tended to decrease for both solvents, reaching the minimum average relative growth at day nine for both solvents: 39.0% and 16.3% respectively, compared to control culture growth.

At the end of the experiment (day 9), each solvent presented a significant

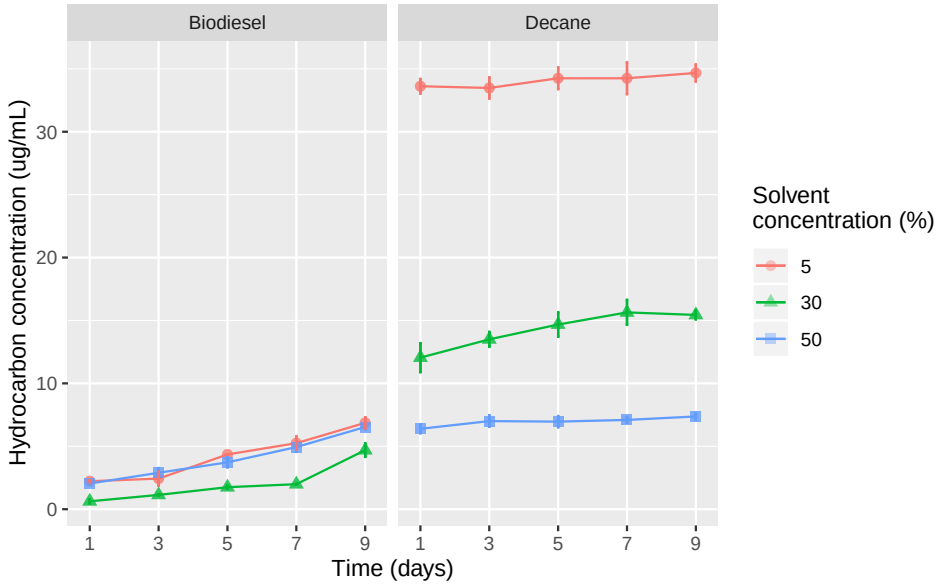


Figure 3.2: Hydrocarbon concentration ($mg\,L^{-1}$) found in biodiesel and decane along 9 days of contact solvent-biomass for 3 solvent concentrations (%v/v): 5, 30 and 50% v/v.

difference in biomass concentration (p-value = 0.00708) with higher values in cultures with biodiesel as second phase. However, solvent concentration showed no clear effects on growth. In the case of biodiesel, biomass concentration showed no significant differences for different solvent concentrations (p-value = 0.31401). For decane, 30% solvent concentration showed a higher dry weight biomass value compared to 5 and 50% (p-value=0.01794).

3.3.2 Hydrocarbon extraction and quantification

In the two-phase systems, the solvent used to extract hydrocarbons is in direct contact with growing cells. In this way, extraction is made constantly in-situ and hydrocarbon concentration in the second-phase will increase with time.

Hydrocarbon extraction results are shown in two different ways. The first is

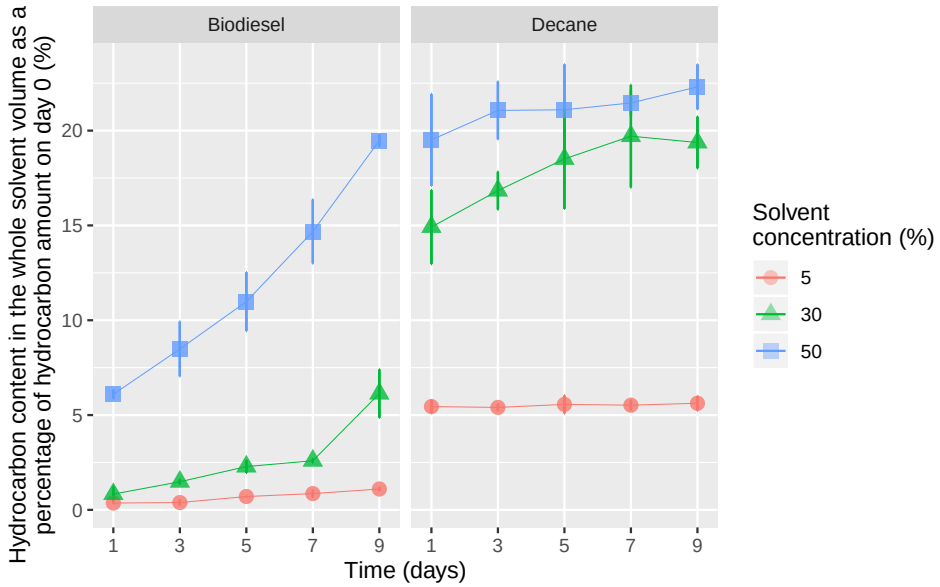


Figure 3.3: Hydrocarbon content expressed as a percentage of hydrocarbon at the beginning of the experiment (day 0), for the three solvent concentrations (%v/v): 5, 30 and 50% v/v decane and biodiesel.

as concentration, i.e., hydrocarbon mass per solvent volume unit (Figure 3.2) and secondly, as a percentage of total hydrocarbons in the biomass at the beginning of the experiment (day 0, Figure 3.3). Additionally, Table 3.3 shows a summary of total hydrocarbon content in the solvent at day 9 compared with total hydrocarbon content in the solvent-biomass system.

Biodiesel showed an increase in hydrocarbon concentration for all solvent concentrations (all p-values $<$ 0.001 for the null hypothesis slope=0, Figure 3.2), unlike decane (all p-values $>$ 0.05). For decane and biodiesel, the higher hydrocarbon concentration was found in cultures with 5% solvent: 34.7 and 6.9 mg L^{-1}, respectively.

Most notable from Figure 3.3 is that the highest hydrocarbon extractions for biodiesel and decane are similar at the end of the experiment (19.5 and 22.3%,

Table 3.3: Hydrocarbon amount at the end of the experiment (day 9) in the whole biomass-solvent system and extracted by the solvent, expressed as a percentage (recovery yield).

Solvent & concentration (%)	Hydrocarbon amount (ug) on day 9			Recovery Yield (%)	Relative growth
	In biomass (1)	In the whole solvent volume (2)	Total (3)=1+2	(4)=2/3	(%)
Biodiesel & 5	3274	34	3308	1.0	42
Biodiesel & 30	2287	141	2428	5.8	41
Biodiesel & 50	1398	362	1760	20.6	33
n-decane & 5	2938	173	3111	5.6	13
n-decane & 30	1947	463	2410	19.2	27
n-decane & 50	1287	369	1656	22.3	9

respectively), both are expressed as a percentage of hydrocarbons in biomass at the beginning of the experiment (day 0). If results are shown as a percentage of total hydrocarbons in the biomass-solvent system at the end of the experiment (day 9, Table 3.3), the highest values for biodiesel and decane were also comparable (20.6 and 22.3%).

3.3.3 Contact angle (CA)

Figure 3.4 shows the CA for lawns of cells which were in contact with biodiesel and decane for 9 days, respectively. Additionally, two controls were included. The first one measured at the beginning of the experiment (ControlAB) and the second one measured at the end (ControlAE). CA is plotted against solvent concentration and data were grouped by solvent. Values are the average of three replicates and every replicate was measured three times, using three drops on different places on the glass slide, meaning that every point in Figure 3.4 is the average of nine measurements.

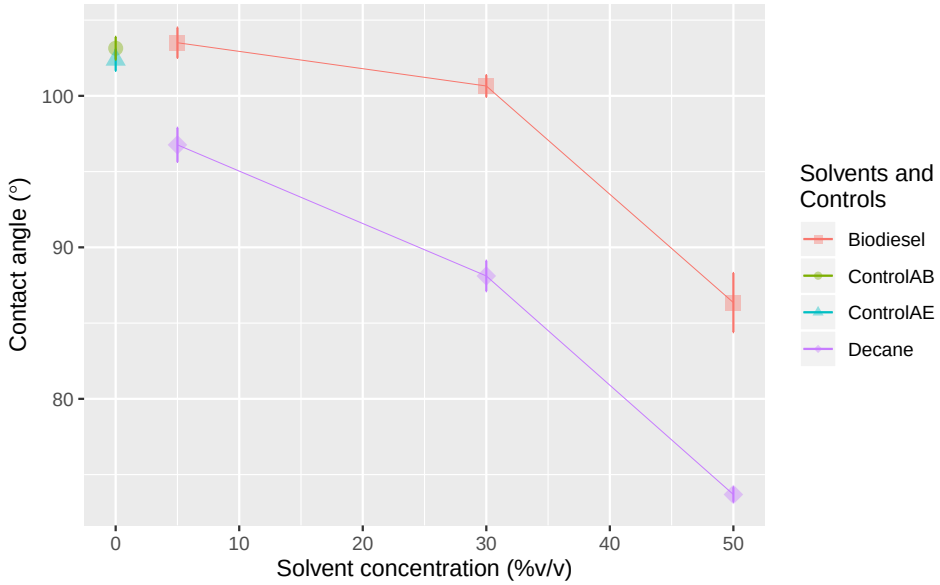


Figure 3.4: Contact angle (°) for control cultures (0% solvent concentration) at the beginning (ControlAB) and the end of the experiment (ControlAE), and for decane and biodiesel at the end of the experiment, for 3 different solvent concentrations: 5, 30 and 50% v/v.

There was no difference between CA for controls at the beginning (mean = 102.6°) and at the end (mean = 101.7°) of the experiment (p-value = 0.37675). It means that CA is constant in the experiment range time for cells not being in contact with solvents. In addition, it was found that both factors, type of solvent and solvent concentration, were highly significant for CA (p-value < 0.0001 for both cases). Considering solvents, CA from samples with biodiesel as a second-phase showed higher values compared to samples from decane. Additionally, for both solvents a negative relationship among CA and solvent concentration was found.

3.4 Discussion

Microalgae responses to solvents in growth media are different, depending on solvent nature, concentration and microalgae species (Tadros et al., 1994). It has been reported that solvents may even promote microalgae growth, depending on their concentration (León et al., 2001; Zhang et al., 2011c). In our study the microalga *B. braunii* was in continuous contact with two solvents, biodiesel or decane, for nine days. Cultures with biodiesel as a second-phase, showed a higher growth rate than control culture during the first 24 hours, unlike decane (Figure 3.1). This result is in line with the expected effects of solvents, according to their physicochemical properties (Table 3.2). Possible explanation for this behavior includes that solvents may act like oxygen vectors (Jia et al., 1996, 1997), or solvent accumulation into cell membrane can permeabilize it, thereby favoring mass transfer from nutrients and oxygen to cells, stimulating growth (Flores et al., 1994). As biodiesel has a higher octanol-water partition coefficient (log P_{ow}) than decane (Table 3.2), a lower amount of biodiesel was dissolved into the culture media, producing less disturbance in cell membrane compared to decane, leading to this beneficial effect of biodiesel in the first hours of the experiment. On the contrary, in the case of decane with a lower log (P_{ow}), the high amount of solvent molecules dispersed in the culture medium produced an excessive amount of molecules in the cell membrane, loosing its structural and functional properties, such as selectivity, resulting in cell death (Sikkema et al., 1994, 1995; de Bont, 1998). In our study, after 9 days of biomass-solvent contact, cells in biodiesel presence reached an average growth rate of 39%, expressed as a percentage compared to control culture growth, whereas decane reached only 16%. A comparable

two-phase system study, obtained 73, 82 and 79% relative growth of cultures with n-dodecane, n-tetradecane and n-hexadecane after 6 days biomass-solvent contact time (Sim et al., 2001). A shorter contact-time, a higher solvent log(P_{ow}) value, the absence of magnetic stirring and lower solvent concentration could explain the higher relative growth values. With a slightly different system, where the solvent was spouted into the broth, a relative growth rate of 85, 62, 76 and 69% was obtained for n-octane, n-dodecane, n-tetradecane and n-hexadecane, respectively, after three days of solvent-biomass contact, with a 50% solvent concentration and magnetic stirring (Choi et al., 2013).

Stirring or agitation into the broth plays a crucial role in several aspects of hydrocarbon extraction and biomass growth in a two-phase system. In the case of *B. braunii*, as cells are grouped in colonies into a matrix, stirring helps to separate or disperse cells producing shear forces, making hydrocarbons accessible to solvents. It also helps to prevent microalgae precipitation and accumulation on the bottom of the flasks, thereby reducing the contact with solvents. Insufficient mixing between two phases can lead to poor hydrocarbon recovery (Sim et al., 2001; An et al., 2004; Choi et al., 2013). In this study, stirring was established following literature values and was constant along the study. It produced an incipient emulsion after the first day, which entrapped cells into prolonged contact with solvents, favoring cell death. However, stirring was maintained at the same velocity to keep constant conditions along the experiment. As this parameter is critical in a two-phase system, it has to be studied to establish an optimal value for every singular system, considering the flask volume, type of solvent and microalga specie, for instance (Hejazi et al., 2003).

Most studies on biofuels obtained from microalgae have concentrated their ef-

forts on lipid generation and extraction under stress conditions (Yoo et al., 2010; Ashokkumar and Rengasamy, 2012; Cheng et al., 2013; Ruangsomboon, 2012; Kaewkannetra et al., 2012; Kim et al., 2014; Manchanda et al., 2016). However, this approach presents the disadvantage that high lipid concentration in every cell is obtained sacrificing culture growth, i.e., the total biomass. On the contrary, in the case of *B. braunii*, hydrocarbon production is proportional to cell growth (Kojima and Zhang, 1999), giving *B. braunii* an interesting advantage for hydrocarbon production. A second advantage for *B. braunii* is that hydrocarbons are stored outside the cell (Largeau et al., 1980), unlike lipids, which are stored inside the cytoplasm. Therefore, it should be energetically less demanding to obtain hydrocarbons compared to lipids.

In this study, hydrocarbon extraction was performed by a two-phase system using biodiesel or decane as a second-phase. The total hydrocarbon production reached 30.7% dry weight at the end of the study (control), which is around the average for *B. braunii* reported in the literature (Furuhashi et al., 2013; Saga et al., 2015; Tsutsumi et al., 2018). Hydrocarbon concentration in decane was constant from day 1 to 9 for every concentration (Figure 3.2), meaning that the extraction was performed in the first hours, showing that decane has a high affinity for hydrocarbons produced by *B. braunii* compared to biodiesel. However, at the same time, biomass growth was negatively affected by the solvent, reducing it to as low as 8.9% (50% decane concentration) compared to the control culture growth rate. On the contrary, hydrocarbon concentration in biodiesel started at low values (0.6 $\mu g\,mL^{-1}$, 30% solvent concentration), increasing every day for all solvent concentrations, up to the maximum of 6.9 $\mu g\,mL^{-1}$ on day nine (5% solvent concentration). Cell growth was also affected by biodiesel, but at day 9

cell growth in the culture reached an average of 39% compared to the control culture. In terms of absolute biomass quantity, it is comparable to the biomass in the culture at the beginning of the experiment (data not shown). This suggests that biodiesel is affecting culture growth, but cells are reproducing sufficiently to maintain the biomass amount approximately constant.

Recovery yield, expressed as the percentage of hydrocarbons extracted at the beginning of the experiment (day 0) shows that a higher solvent concentration produces a higher extraction yield (Figure 3.3). At the end of the experimental trials (day 9) recovery yield for biodiesel and decane were comparable (20.6 and 22.3%), and were in line with previous findings by Sim et al. (2001) (21, 18 and 21% for n-dodecane, n-tetradecane and n-hexadecane) and An et al. (2004) (20.0% for n-octane) for two-phase systems. In this study however, biodiesel may present environmental, health, safety and economic advantages, as it is renewable, biodegradable, non-toxic, easy to handle, and even cheaper than organic solvents.

Other special systems are those which spout solvent into the broth or the broth into the solvent to increase the contact surface of the solvent and biomass; or biomass cultivation and hydrocarbon extraction in two separate bioreactors (An et al., 2004; Choi et al., 2013; Griehl et al., 2014; Mehta et al., 2019). These systems are able to recover higher hydrocarbon amounts and produce less damage to the biomass. However, these results are most probably related to a better system design and not to solvent characteristics.

Hydrocarbon recovery was also verified by measuring the CA of a biomass film disposed on a cellulose acetate filter, fixed on a piece of glass. As a measurement of cell surface hydrophobicity, CA is the result of a complex interaction of several factors, such as, membrane lipid or membrane protein composition, and in this

case, hydrocarbon content. Lipid or protein composition can change as a result of the physiological state or environmental stimulation. However, in the case of *B. braunii* in this study, the high quantity and distribution of hydrocarbons on the cell surface (Largeau et al., 1980) possibly determine most part of the CA. A higher hydrocarbon presence in the biomass should produce a higher CA because of high hydrophobicity. In general terms, CA results in this study confirm hydrocarbon extraction (Figure 3.4). Decane removed a higher hydrocarbon proportion compared to biodiesel showing lower CA values. Additionally, higher solvent concentrations, which should extract a higher proportion of hydrocarbons, showed lower CA values.

In summary, decane presented a negative effect on biomass growth and showed more affinity for hydrocarbons than biodiesel. Due to these characteristics, decane seems to be more appropriate for systems such as the one proposed by An et al. (2004) or Griehl et al. (2014), where the biomass is in contact with the solvent for a short time. In this case, a short time is sufficient to transfer hydrocarbons from the biomass to the solvent, avoiding toxic effects on cells. On the other hand, biodiesel works well with two-phase systems where the needed solvent-biomass contact time is longer, being sufficient to produce the hydrocarbon transferring from the biomass to the solvent, not showing severe negative effect on cells growth, due to its higher biocompatibility.

3.5 Conclusion

The findings show that both biodiesel and decane are able to recover hydrocarbons from a *Botryococcus braunii* culture, and after nine days the hydrocarbon recovery yield is comparable for both solvents, as well as previous works with similar conditions. Mass transfer kinetics is faster in the system using decane as a second-phase, showing a higher affinity for hydrocarbons produced by *Botryococcus braunii*. Nonetheless, biodiesel is capable of maintaining a higher growth rate in the culture compared to decane, showing higher biocompatibility under the tested experimental conditions. System design for hydrocarbon extraction has evolved to increase contact surface between solvent and biomass, avoiding damage to cells. Hence, future research should consider testing biodiesel with an optimized system for obtaining higher hydrocarbon recovery yields as well as sufficient biomass growth rate.

Chapter 4

General discussion, concluding remarks and future directions

4.1 General discussion

A good predictor of solvent toxicity is the octanol-water partition coefficient P_{ow} (Frenz et al., 1989b; Zhang et al., 2011b). In this study the solvent toxicity on *B. braunii* measured as growth inhibition, from the most to the least toxic solvent was: decanol, limonene, decane and biodiesel (Figures 3.1 and A.1). This result is in agreement with toxicity as predicted by $\log(P_{ow})$ (Tables 2.1 and 3.2) and in line with previous findings as reported by Sim et al. (2001) and Zhang et al. (2011b), confirming the hypothesis that solvent toxicity is mainly produced by solvent molecules accumulation in cell membrane (Sikkema et al., 1994).

Molecule accumulation in cell membrane is proportional to molecule number in solution (Sikkema et al., 1994), which in turn depends on solvent hydrophobicity (Frenz et al., 1989a). The more hydrophobic the solvent, the less molecules will be in solution and less perturbation will occur in cell membrane. It means that solvents with a higher $\log(P_{ow})$ are more biocompatible, as confirmed in this study.

A remarkable finding of this study is that for some solvent concentrations, biomass growth in two-phase cultures was higher than in control cultures (Figures 3.1 and A.1). It is conceivable that solvent molecule accumulation in the cell membrane produced instability which allowed for a higher nutrient and oxygen intake (Flores et al., 1994; León et al., 1998, 2001; Mojaat et al., 2008; Zhang et al., 2011b). However, the flow through the cell membrane is not only from the medium to the cytoplasm, but also in the opposite direction and the cell could lose intracellular content. As this quasi-free substances interchange is not under cell control, the cell will die, if a certain trigger point level is exceeded. In the case of limonene, the higher growth could also possibly occur because limonene

itself can serve as carbon source (De Carvalho et al., 2005).

When microorganisms become in contact with threatening agents such as heavy metals, salts or solvents, they trigger a set of responses to counteract negative effects of the contact. These tolerance mechanisms have been studied mainly in gram positive bacteria (Ramos et al., 2002; Segura et al., 2004), but microalgae are so far under-researched. The mechanisms can include active solvent transport from cytoplasm to the medium, morphological changes, alterations in membrane lipid or protein type or production of secondary metabolites (Heipieper et al., 2007; Guan et al., 2017). The main changes at lipid membrane level are (1) *cis* to *trans* bond isomerization (Heipieper et al., 1994), (2) to elongate fatty acid acyl-chain, and (3) to change the unsaturation level (Weber and de Bont, 1996). As saturated lipids are straighter than unsaturated ones, a higher quantity can be pack in a volume unit compared to unsaturated lipids. This means that a cell membrane with saturated lipids will be less fluid and can thus deal in a better way with fluidizing agents as solvents (Sikkema et al., 1995; Ramos et al., 2002).

In this study, changes in membrane lipid composition were explored as a *B. braunii* response to contact with solvents. The main lipids in *B. braunii* cell membrane were oleic (C18:1*cis*9, 28.0%) and palmitic acid (C16:0, 25.9%), when the culture medium was solvent-free. However, it was found that solvent-biomass contact induced an increase in palmitic acid and a decrease in oleic acid (Figure 2.2). This means that a reduction in unsaturation occurred (Figure 2.3). As a consequence, a more rigid cell membrane was obtained, better-equipped to prevent fluidizing solvent effects. However, this was not found for all solvent concentrations as it depends on the solvent. In the case of decanol, excessively high concentrations prevent this change in unsaturation (Figure 2.3). A possi-

ble explanation could be that this change in cell membrane lipid composition is energy-dependant, i.e., an input of energy is needed to *de novo* synthesis of saturated lipids (Taylor et al., 2012). If the cell is under high stress because of a high quantity of solvent molecules in the cell membrane, producing an uncontrolled flow of cytoplasmic content, then the cell loses physiological functions and is unable to synthesize new lipids. This mechanism prevents the occurrence of changes in the cell membrane.

Solvent-biomass contact produces negative effects on microalgae growth, but it is necessary for hydrocarbon extraction as these molecules are deposited on the extracellular matrix (Largeau et al., 1988). This disposition of hydrocarbons represents an unusual location for target molecules in a microorganism, as they are normally intracellular compounds, demanding cell disruption or aggressive methods for recovering them. In this case hydrocarbon location seems to be an opportunity for an easy recovery. However, it is not too easy, as described by Furuhashi et al. (2016), because the colony structure of *B. braunii* hinders hydrocarbon extraction.

In this study n-decane and biodiesel were tested for its abilities for hydrocarbon extraction while maintaining cell viability. Results showed that extraction kinetics for decane were faster than biodiesel (Figure 3.3), suggesting a higher decane-hydrocarbons affinity. However, after nine days, hydrocarbon extraction yield for both solvents is comparable (around 20%) for 50% solvent concentration, with the advantage that biodiesel showed a higher biocompatibility, maintaining microalgal biomass concentration nearly constant in the culture (Figure A.3).

Another interesting outcome revealed that solvent concentration is important for hydrocarbon extraction yield. A higher solvent concentration always showed

a higher hydrocarbon extraction yield (Figure 3.3), which means that higher hydrocarbon quantities were removed when higher solvent amounts were in contact with the culture. This idea is supported by contact angle results, that showed lower values for higher solvent concentration (Figures 3.4 and A.2). Figure 3.4 also illustrates that the curve associated to biodiesel has a higher CA, suggesting that biodiesel removed less hydrocarbons compared to decane, supporting results in Figure 3.3.

To enhance extraction yield, Furuhashi et al. (2016) proposed to apply a treatment prior to performing hydrocarbon extraction, making them more available. They proposed two treatments: to heat the microalga up to 90°C or to cultivate the microalga in brackish water. Both treatments improved the hydrocarbon recovery, unfortunately, Furuhashi et al. (2016) did not evaluate cell viability after treatments, because the first treatment was destructive. Nonetheless, the treatment with brackish water seems to be interesting because it did not use extra energy.

Tsutsumi et al. (2018) made a second proposal for extracellular matrix dispersion enhancing hydrocarbon extractability: (1) using a jet paster and a (2) bed mill. They obtained a dramatic yield improvement from 2.7 to 82.8% in extraction yield using the jet paster, and were also able to maintain cell viability practically at the same level as before the treatment. They did not use a two-phase system for hydrocarbon extraction, however, it seems to be possible to use the jet paster coupled to a two-phase system.

Several other systems have been proposed to improve hydrocarbon recovery and cell viability in a two-phase or milking system (Hejazi and Wijffels, 2004). For instance, Choi et al. (2013) proposed to spout tiny solvent droplets into the *B.*

braunii culture, to increase solvent-biomass contact surface, recycling the solvent and to stop the system after 9 h to regenerate the biomass. Once this process is finished, it is repeated. In this way the same biomass can be used several times and the hydrocarbon recovery is improved compared to a simple two-phase system with a lower contact-surface. Griehl et al. (2014) introduced another system for hydrocarbon extraction from *B. braunii*, however, just as in two-phase systems microalga growth and hydrocarbon extraction stages are connected but separated. It helps, maintaining a cell viability similar to control culture even when the aggressive solvent hexane was used.

Once hydrocarbons are transferred from the biomass to the solvent, two steps are still necessary before they can be used: hydrocarbons has to be separated from the solvent and, according to necessities, they could be cracked. Several alternatives for separation are available: distillation, evaporation, adsorption, centrifugation (Tsori and Leibler, 2007), chromatography or filtration (Marchetti et al., 2014). Each method has advantages/disadvantages according to the nature of the solvent/solute. In the case of two miscible liquids, as solvent/hydrocarbons for biofuel production, separation might be challenging as it is only justifiable if the energy used in the separation process is (significantly) less than the energy contained in the resulting hydrocarbons. Traditional technologies, such as evaporation or distillation used in the petroleum industry, likely have the disadvantage that they are highly energy demanding. On the contrary, nanofiltration seems to be an excellent alternative as it allows for miscible-liquids separation in mild operating conditions, with an energy consumption of $\approx 2\%$ of the distillation consumption, a capital cost of $\approx 25\%$ for distillation, and an operational cost of less than 50% of the required amount for distillation (Spratt et al., 2019).

4.2 Concluding remarks

This research demonstrates that it is possible to use biodiesel to extract hydrocarbons while maintaining cell growth in a two-phase system using the microalga *B. braunii* as hydrocarbon source. Biodiesel biocompatibility and extraction yield results were not as high compared to other studies and solvents (under different experimental conditions), hence, there is ample space for improving these results considering the simple two-phase system used in this research. New ideas have been raised about how to put solvents and biomass in contact in an extraction process, which can be implemented with *B. braunii* and biodiesel to improve two key parameters of these type of systems: contact time and contact surface of solvent-biomass.

Biodiesel demonstrated a good biocompatibility at as high as 50% v/v solvent concentration. On the contrary, results of experiments with limonene as second-phase are poor in terms of biocompatibility for concentrations higher than or equal to 12.3mM ($\approx$ 0.2% v/v), where the number of viable cells in cultures quickly declined. However, at concentration levels lower than or equal to 1.2mM limonene boosts cell growth. In terms of biocompatibility the order of solvents from the highest to the lowest, compared to similar concentrations is: biodiesel, n-decane, limonene and n-decanol, which is in agreement with the biocompatibility predicted by the octanol-water partition coefficient (P_{ow}).

Another important conclusion is that the microalga *B. braunii* is able to modify its membrane lipid composition by raising the saturation lipid rate to increase membrane viscosity and consequently decreasing solvent leakage into cell membrane, thereby improving contact tolerance with solvents.

Regarding the extraction yield, the highest hydrocarbon extraction (22.3%) occurs at 50% n-decane concentration. This yield is comparable to the highest reached by biodiesel at the same solvent concentration (20.6%). Concerning the extraction kinetics, n-decane recovered the hydrocarbons from the biomass faster than biodiesel, which means that n-decane possesses a higher affinity than biodiesel for hydrocarbons produced by *B. braunii* race A.

Finally, solvent concentrations also make a difference in the extraction yield of the process: higher solvent concentrations consistently produced a higher extraction yield of hydrocarbons, using either biodiesel or n-decane.

4.3 Future directions

Recommendations for future research derived from this study are:

1. Regarding the very simple two-phase system used in this study, improvements can be made easily to increase both hydrocarbon extraction yield and cell viability. In particular, the ideas proposed by Choi et al. (2013), Griehl et al. (2014), Furuhashi et al. (2016), Tsutsumi et al. (2018) and those collected by Jackson et al. (2019) should be implemented in a new set-up to test biodiesel as solvent under better conditions, in order to make it more competitive with fossil-based traditional solvents.

2. To search for other bio-based solvents which promote the ability to survive of *B. braunii* in contact with them, and are simultaneously able to recover high value metabolites from microalgal biomass.

3. Applying the idea of natural selection as follows: under a high environmental pressure on a microorganism population some of them will be able to adapt to the new conditions, and survive. This ability to survive, could be related to intrinsic characteristics, as survival is genetically mediated. Hence, it should be possible to isolate and to reproduce surviving microorganisms in order to produce a population, which is better adapted to resist high environmental pressure. If the environmental pressure consists of the solvent in the culture media, it should be possible to produce an improved *B. braunii* population, better equipped to growth along the solvent. After several cycles of selection pressure and recultivation a higher or faster production of valuable target molecules can be obtained from microalgae populations living in a two-phase or milking system.

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Appendices

Appendix A

Complementary plots

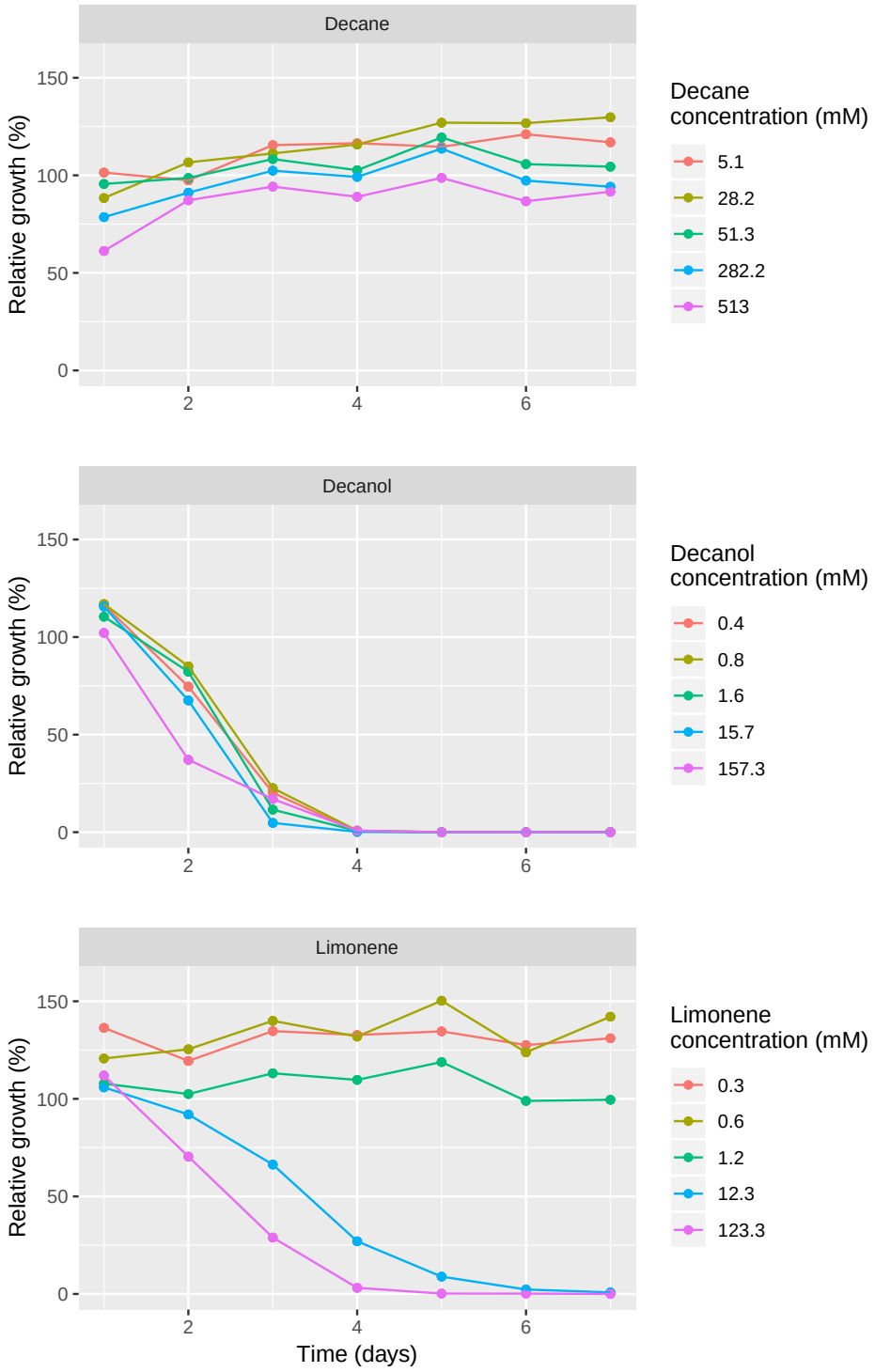

Figure A.1: Effect of different concentrations of n-decane, n-decanol and limonene on *Botryococcus braunii* UTEX LB572 growth, from day 1 to 7. Growth is expressed as a percentage of control samples. Every point is the average of three independent samples.

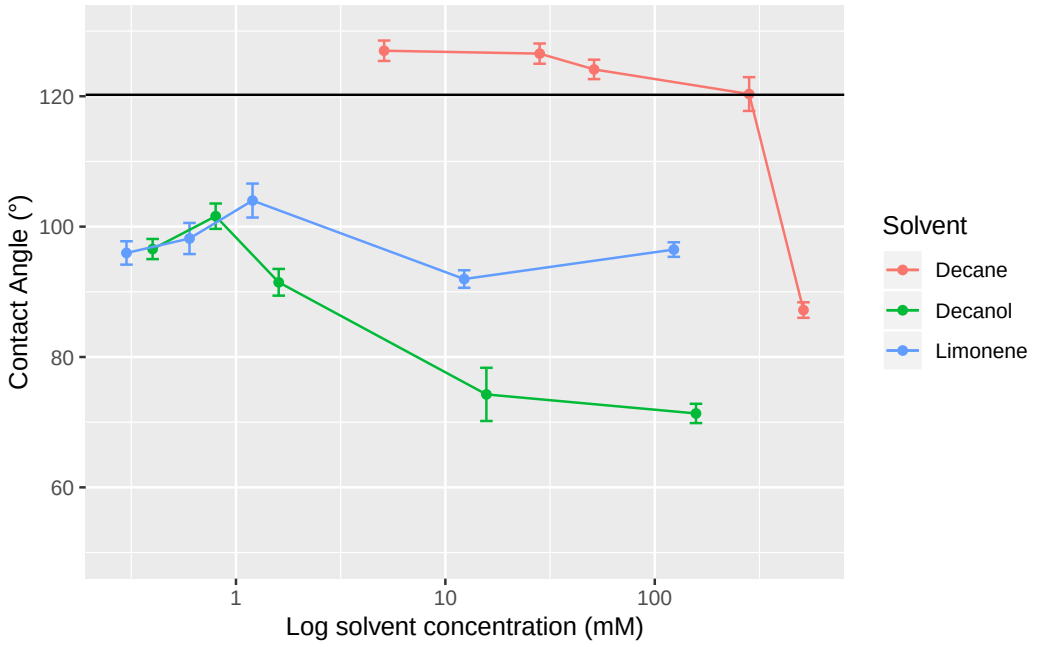

Figure A.2: Contact angle (^{o}) for three solvents (decane, decanol and limonene) and five concentrations. The black continuous line is the contact angle for control samples at the end of the experiment (seven days).

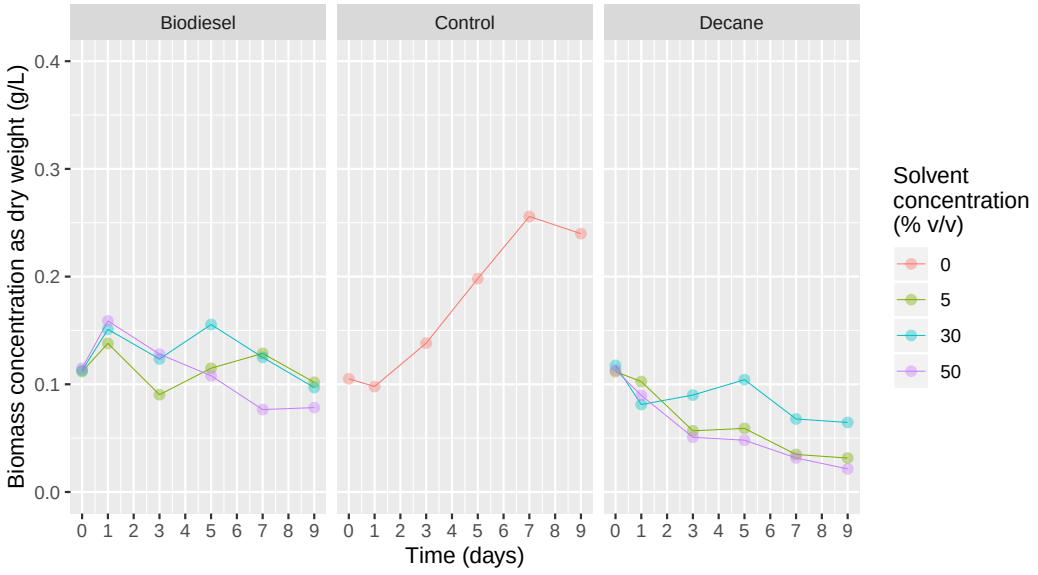

Figure A.3: Absolute growth ($g\,L^{-1}$) for control, and biodiesel and decane for three solvent concentrations: 5, 30 and 50 (%v/v).

Appendix B

Cell viability and bacteria in *B. braunii* cultures

B.1 Cell viability

Cell viability is the ability of a treated sample to exhibit a specific function or functions, expressed as a proportion of the same function exhibited by the same sample before treatment or an identical fresh sample (Pegg, 1989). Defined in this way, it should be considered that a viable cell is not a synonym for a living cell, as the meaning of *viability* will depend on the function or functions defined as relevant. In the case of this study, the relevant function is the ability to grow or produce biomass, because hydrocarbon production depends on growth.

Cell viability can be assessed in different ways, however, modern techniques are based on use of fluorochromes. The idea is to take advantage of the fact that cell membrane of dead or damaged cells lose their permeability control, allowing fluorochromes accumulation inside cells. Then these fluorochromes can be de-

tected by an instrument such as a flow cytometer or a laser confocal microscope. It should be noted that these type of tests are not saying cells are alive or viable, they are only evaluating the integrity of the plasmatic membrane (Pegg, 1989; Pouneva, 1997), whose integrity is a necessary (but not sufficient) condition for having a viable cell.

In this study, *B. braunii* was grown in permanent contact with solvents for nine days with the goal to transfer hydrocarbons from biomass to the solvents and to maintain culture growth. Therefore it was important to measure the number of viable cells in the culture. Several trials were conducted in our laboratory using fluorochrome dye techniques to evaluate *B. braunii* cell viability. Propidium iodine was used as stain, but staining was irregular and incomplete even in dead cell samples. This can possibly be explained by the fact that this microalga is protected by a three layer chemically resistant cover, which makes it difficult for fluorochromes to penetrate into cells even if the cell membrane is not normally working or is not active at all.

Favorably, unlike bacteria or animal cells, microalgae possess a photosynthetic system known for its autofluorescing properties when stimulated with the appropriate light wave length. It is also known that the correct photosystem functioning is correlated with cell viability (Takahashi, 2019). With a broken photosystem microalgal cells are doomed to death. Therefore, this internal autofluorescence in microalgae is an ideal way to evaluate cell viability in this study as it is not necessary to introduce any fluorochromes into cells. In fact, Pouneva (1997) and Koç et al. (2018) both recommend chlorophyll autofluorescence as the best method for microalgae cell viability evaluation. In this study, measurements were made as described in section 3.2.3.

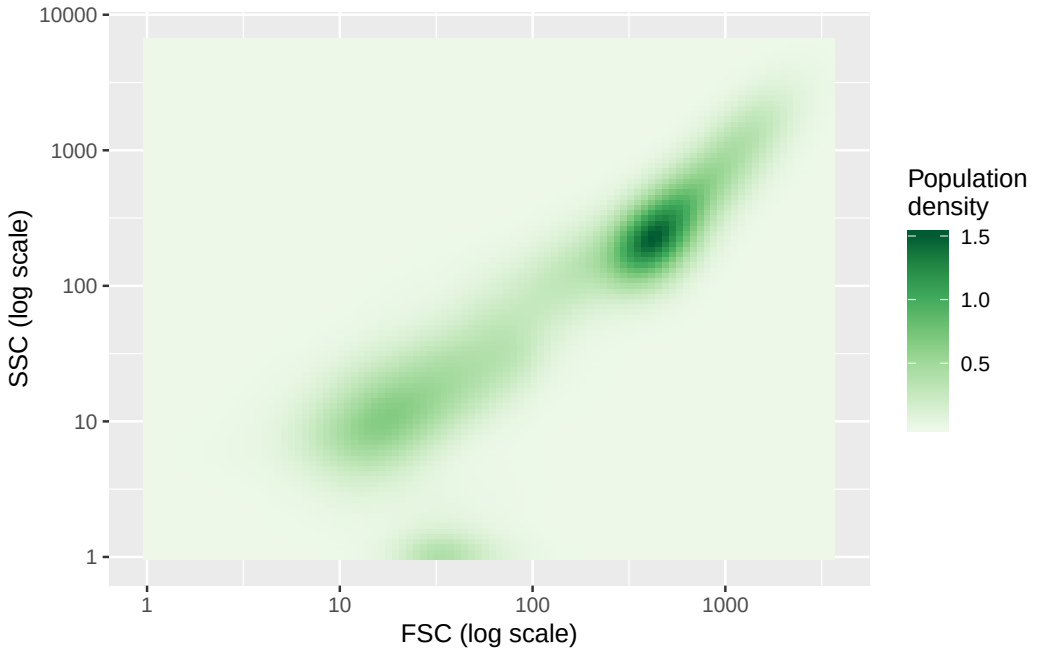

Figure B.1: Forward-scattered (FSC) - side-scattered light (SSC) plot showing three different microorganism populations, *B. braunii* microalga is the set with higher FSC and SSC values in the upper-right corner of the plot. Population density represents a relative scale.

The Figure B.1 presents the results for a sample from the cultures in contact with a solvent, measured in the flow cytometer (a flow of 10 µl min^{-1} passed through the detector by 30 s). Three different populations are displayed: in the left-bottom section are two bacteria populations, whereas the top-right section shows *B. braunii* cells. Bacteria and dead microalgal cells can be easily removed from this measurement using a filter on a channel that has saved values for autofluorescence from the microorganisms, for instance, the channel for Allophycocyanin (APC). The result is shown in Figure B.2.

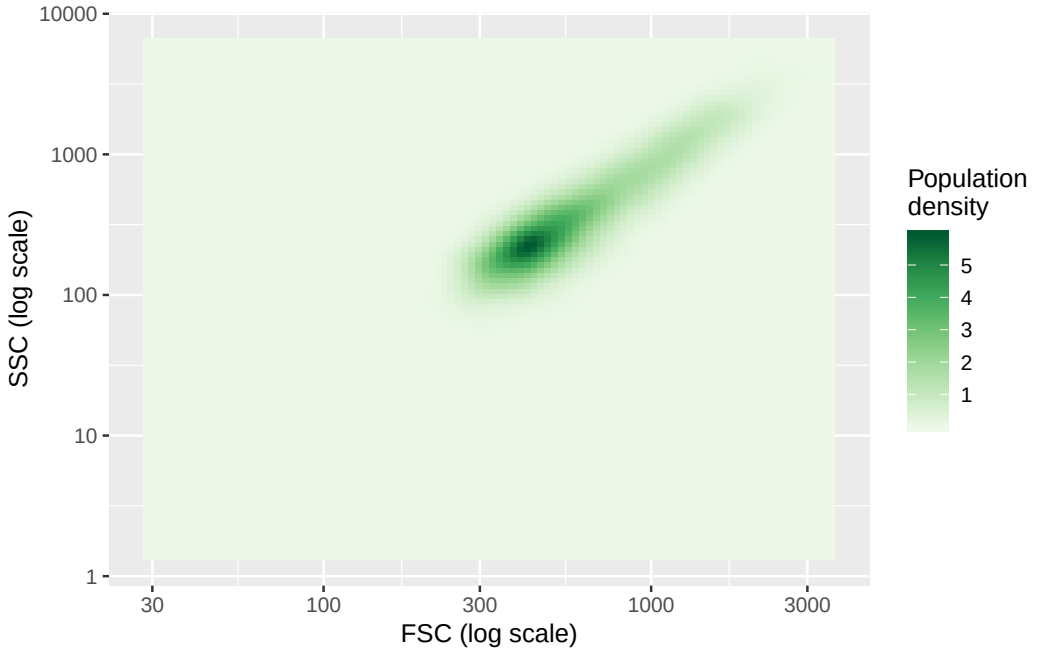

Figure B.2: Forward-scattered (FSC) - side-scattered light (SSC) plot when bacteria or dead microalgal cells are removed using a filter on autofluorescence channel for Allophycocyanin (APC). Population density represent a relative scale.

B.2 Bacteria in *B. braunii* cultures

Bacteria are everywhere and have evolved over time in close proximity to microalgae, establishing mutually beneficial or antagonistic interactions (Ramanan et al., 2015; Segev et al., 2016). On the one hand, bacterial CO_2 production, nutrient exchange, and community resilience to invasion by other bacteria are examples of a synergistic relationship among bacteria and microalgae (Ramanan et al., 2015). On the other hand, algal growth inhibition by competing for nutrients or direct microalgae cell lysis are possible antagonistic interactions (Segev et al., 2016). In *B. braunii* cultures several different bacteria have been found such as *Pseudomona sp., Flaviobacterium sp., Rhizobium sp., Hyphomonadaceae*

spp., Bradyrhizobium or *Bacteroidetes sp.* (Chirac et al., 1982; Rivas et al., 2010; Tanabe et al., 2015; Sambles et al., 2017).

It is important to consider the presence of bacteria in the culture media along *B. braunii*, when measuring biomass growth or extracting/identifying fatty acid methyl ester. Using flow cytometry allows us easily recognizing/eliminating bacteria populations in biomass samples when growth measurements were made in the experiment with biodiesel as a second-phase. For membrane lipid change assessment in the experiment with limonene as a second-phase, optical microscopy allowed us to verify that bacteria were not attached to *B. braunii* colonies. In this case, according to Chirac et al. (1985), most part of the bacteria are eliminated by the filtration used to concentrate the microalgal biomass. As biomass was concentrated by filtration previous to lipid extraction and transesterification to evaluate changes in membrane lipid composition, it is likely that bacteria do not contribute with lipids in an important amount to the measurements made in this evaluation.