

**Systematic metabolic characterization of
hydrocarbon and exo-polysaccharide producing
microalga *Botryococcus braunii***

Dissertation

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Summary

Renewable energy in the form of bio-diesel, bio-methane, bio-hydrogen, and hydrocarbons from microalgal biomass is gaining attention for research in recent years. The use of microalgal biomass as a source of proteins, photosynthetic pigments, antioxidants, peptides, fatty acids and polysaccharides have led to many applications in biofuels, food and feed industry and for its use in the pharmaceutical and cosmetic industries.

The green colony forming microalga *Botryococcus braunii* is well known for its ability to produce large amount of hydrocarbons (~75% of dry biomass) and exo-polysaccharides and is divided into three different races (A, B and L), based on the types of hydrocarbon they produce. The production of metabolically expensive compounds such as hydrocarbons and polysaccharides is one of the main reasons for its slow growth rate and is largely depend on the physiological state of the cells. Apart from slow growth rate, variations across different races and strains in terms of growth behavior, formation of product and biomass yield are some of the main challenges for the commercial applications of this microalga. This leads to the identification and characterization of novel strains of *B. braunii* which can perform better despite of challenges associated with it.

Therefore, the current study focuses on the systematic metabolic characterization and comparison of *B. braunii* race A and race B strains in order to address some of the challenges associated with its slow growth rate and product formation. Furthermore, the result obtained in the current work shows the impact of different physiological state of the cell on product formation and on overall metabolome profile. Additionally, the strains of race A and race B were compared with respect to their growth behavior, metabolome profile and product formation abilities.

The study revealed that the investigated *B. braunii* strains differed greatly in terms of biomass accumulation and hydrocarbon/EPS formation. Race A and race B strains have distinct growth and hydrocarbon/EPS formation phases depending on their physiological state, based on the total chlorophyll content. Comparison between race A and B strains revealed extended linear growth phase for race B strain AC 761. Moreover, the hydrocarbon production in race A strain CCAP 807/2 and race B strain AC 761 differed during the duration of cultivation. For race A strain (CCAP 807/2) the hydrocarbon biosynthesis was promoted during late linear and early stationary phase, whereas race B produces hydrocarbons continuously from the beginning of the cultivation and the hydrocarbon productivity is maximum during the linear phase.

Besides, the primary metabolome analysis of race A and B strains also showed significant differences in the abundance of metabolites related to carbohydrate metabolism especially sucrose. Moreover, race B showed higher intracellular lipid content than race A strains.

Strikingly, even though the race A strains CCALA 778 and CCAP 807/2 showed similar growth behavior, they significantly differed in their ability to produce hydrocarbons. CCALA 778 was majorly carbohydrate producer with presence of only detectable amount of hydrocarbons whereas CCAP 807/2 was able to produce both hydrocarbons and carbohydrates. Furthermore, the gradual increase in the hydrocarbon formation was proportional to the decrease in oleic acid. Notably, intracellular lipid content in CCAP 807/2 was two-fold more than CCALA 778. The very long chain fatty acid (VLCFAs) which are intermediates for the hydrocarbon biosynthesis in race A were detected in both race A and race B strains. In addition, the primary metabolome analysis showed sucrose as the most abundant metabolite in both the strains of race A.

Altogether, this work presents for the first time the distinct and shared characteristics of growth behavior, metabolome and product formation for the *B. braunii* race A strains CCLA778 and CCAP 807/2 with race B strain AC761. In addition, the results in this study also show the potential link and impact of different physiological state of the cell on product formation and on the overall metabolome profile.

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Abbreviations

ACP	acyl carrier protein
ATP	adenosine triphosphate
CAD	charged aerosol detector
CaCl ₂	calcium chloride
CO ₂	carbon dioxide
DAG	diacylglycerol
DMAPP	dimethylallyl diphosphate
DW	dry weight
DXS	1-deoxy-D-xylulose-5-phosphate synthase
Cyt b6f	cytochrome <i>b6f</i>
CuSO ₄	copper sulphate
CoSO ₄	cobalt sulphate
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FAME	fatty acid methyl ester
FAS	fatty acid synthase
Fd	ferredoxin
FeNaEDTA	ethylenediaminetetraacetic Acid, Ferric-Sodium Salt
FID	flame ionization detection
FNR	ferredoxin-NADP ⁺ oxidoreductase
EPS	exo-polysaccharides
ECM	extracellular matrix
g	gram
GC-MS	gas chromatography-mass spectrometry
GMD	GOLM Metadatabase
HPLC	high-performance liquid chromatography
H ₂ O	water
H ₃ BO ₃	boric acid
H ₂ SO ₄	sulphuric acid
IPP	isopentenyl diphosphate
K ₂ HPO ₄	potassium hydrogen phosphate
KNO ₃	potassium nitrate
M	mol
m	meter
max.	maximum
MEP	methyl-D-erythritol 4-phosphate
mg	milligram
MgSO ₄	magnesium sulphate
ml	milliliter
MnSO ₄	manganese sulphate
μl	microliter
μmol	micromole

mM	millimol
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MVA	mavolonate
N / N ₂	nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate
Na ₂ O ₄ Se	sodium selenate
Na ₂ MoO ₄	sodium molybdate
NIST	National Institute of Standards and Technology
nm	nanometer
oDW	organic dry weight
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PQ	plastoquinone
PS I	photosystem I
PS II	photosystem II
ROS	reactive oxygen species
s	second
TAG	triacylglycerol
TCA	cycle citric acid cycle
UV	ultraviolet
VLCFA	very long chain fatty acid
ZnSO ₄	zinc sulphate

1 Introduction

1.1 Global energy demand and sustainable development through Bioenergy

Sustainable development in terms of environmental policies, economic growth and energy supply is an important challenge of modern era. Most of the energy demand for today's modern society is being met from conventional fossil fuels. These conventional fossil fuels are rapidly depleting (Wijffels et al., 2010) and therefore, there is a need to address the ever increasing demand of energy. The use of fossil fuels is attributed to emission of greenhouse gases and subsequently affecting environment, however this impact is not limited to environment only but also economy due to the ever increasing oil prices (Nigam and Singh, 2011a). One of the most promising sustainable source for energy is 'Biofuel' (Hannon et al., 2010). The solid, liquid and gaseous form of fuel that is derived from biomass is referred as biofuel (Nigam and Singh, 2011b). Realizing the necessity for completely or even partly replacing the conventional fossil fuel, most of the developed and developing countries are focusing on the policy establishment to produce biofuel. The recent example of importance of policy establishment resulted into *Renewable Fuels Standard* making it mandatory to produce 36 billion gallons of biofuels by 2022 to displace petroleum in transportation fuel mixture (Somma et al., 2010). Although some serious efforts are continuously invested for policy making, technology upgradation, research on novel sources for energy, renewable sources for liquid fuels are far less developed (Schenk et al., 2008).

At present, sucrose is considered as the most abundant source of biofuel. Bioethanol produced from the plant starch and sugars has emerged as the most successful liquid biofuel till date (Li et al., 2010a). Historically, ethanol production was done using fermentation techniques where simple sugars and starch were used as substrates (Nigam and Singh, 2011b). The sources of these substrates were primarily plants such as sugarcane, corn, maize and thus bioethanol production from such plants was considered as first generation biofuels (Li et al., 2010a; Nigam and Singh, 2011b). However, starch and simple sugars are only a small fraction of total plant biomass. The production of biofuel from these entities is economically challenging apart from the fact that they are food source thus presenting food vs fuel issue (Li et al., 2010a).

Apart from ethanol, lipids (oils/TAGs) also serves as the source of bioenergy/liquid biofuel since they are produced as energy storage compound in plants and algae (Li et al., 2010a). Lipids can be readily extracted from plants and algae and can be converted into biodiesel through the process of transesterification. In contrast to ethanol, lipid possess high energy density due to high C/O ratio. Moreover, ethanol cannot be used as aviation fuel or for heavy trucks and thus lipids/TAGs have more advantage over ethanol (Merchant et al., 2007b; Li et al., 2010a; Schenk et al., 2008).

The non-food crops such as *Jatropha*, palm oil tree and switchgrass can be used for biodiesel production however, conversion of forest or agricultural land for fuel has a long-term negative impact on environment (Merchant et al., 2012a; Schenk et al., 2008). Therefore, algae have become now an attractive source for biofuel production because of their capability to grow in waste and seawater. Importantly, they can produce large amounts of TAGs/oils and hydrocarbons which can in turn used as biofuel sources.

1.2 Algae

Algae are photosynthetic eukaryotes belonging to thallophyta with the absence of stems, roots and leaves (Hallmann, 2007; Lee, 2008). Even though the term algae is used for photosynthesizing eukaryotes, few exceptions exist. Cyanobacteria (blue-green algae) though evolutionarily related to bacteria are also considered as algae (Lee, 2008). Therefore, the definition of algae is still debatable considering their several phyla or divisions and evolutionary background.

Algae are most commonly found in aquatic biotopes (water) such as freshwater, brackish or marine water (Hallmann, 2007; Lee, 2008). They are ubiquitous and are found in almost every habitat on earth ranging from snowy mountains to extreme hot deserts (Hoffmann, 1989). Some algae are also extremely tolerant to salts like *Dunaliella salina* (Hallmann, 2007). Algae exhibit different morphologies which ranges from meter to pico scale. For example, the brown alga *Macrocystis pyrifera* commonly known as giant kelp grows up to 60m whereas the smallest living eukaryotic alga known as *Ostreococcus tauri* has its cell diameter in picometer (Courties et al., 1994; Hallmann, 2007). Algaebase, the database for algae currently distinguishes 33,248 eukaryotic algae species out of an estimated 39,000 to 140,000 species (Chapman, 2009; Guiry, 2012). There are two types of algal cells 1) prokaryotic algae that lack organelles such as nuclei, plastid, Golgi bodies and mitochondria e.g. Cyanobacteria (blue - green algae) and 2) eukaryotic algae having all the above mentioned organelles (Lee, 2008). Algae are generally divided into several divisions. These includes Rhodophyta (red algae), Chlorophyta (green algae), Phaeophyta (brown algae), Bacillariophyta (diatoms), dinoflagellates and a prokaryotic phylum called Cyanobacteria (blue-green algae) (Hallmann, 2007; Baweja and Sahoo, 2011; Duong et al., 2012). The complexity and number of existing algal species pose difficulties in studying its phylogeny. However, except few strains of Cyanobacteria and Bacillariophyta (diatoms), biological studies have principally focused on Chlorophyta, the green microalgae.

Green microalgae belongs to the kingdom Viridiplantae, one of the major groups of oxygenic photosynthetic eukaryotes (Leliaert et al., 2012). Green algae and plants exhibit few similar features (Graham, 1996). Green algae, like plants are characterized by chloroplast, enclosed by membrane and thylakoids that are arranged in lamellae along with chlorophyll a and b (Leliaert et al., 2012; Lee, 2008).

90% of green algae are found predominantly in fresh water with 10% of green algae existing in marine water (Lee, 2008).

Several green algae serve as model systems providing valuable insights not only into their molecular evolution and biological mechanisms but also their ecological and economical importance. One such algal model organism is the unicellular flagellate *Chlamydomonas reinhardtii*. This alga has been well characterized for the processes of photosynthesis, cell cycle, chloroplast biogenesis and circadian rhythm (Grossman et al., 2003; Harris, 2001; Breton and Kay, 2006; Leliaert et al., 2012). Apart from *C. reinhardtii*, other green algae such as *Chlorella* and halophilic *Dunaliella salina* have also been well characterized with regard to light-independent reactions of photosynthesis (Calvin cycle), biochemistry and physiology (Calvin, 1948; Tafreshi and Shariati, 2009). Furthermore, the availability of complete nuclear genome sequences of *C. reinhardtii* (Merchant et al., 2007b), *Volvox carteri* (Prochnik et al., 2010) and *Chlorella variabilis* (Blanc et al., 2010) have greatly enhanced the understanding of algal biology in terms of their evolution, photosynthetic machinery and their metabolism.

1.3 Algal photosynthesis as a global energy provider

Photosynthesis is a primary source for energy on the earth. Microalgae are capable of photosynthesis and thus fixing large amount of light and CO₂ which is freely available in the atmosphere. Oxygenic photosynthesis in microalgae is primarily driven by photosynthetic molecules such as chlorophyll wherein light energy and carbon dioxide are converted to glucose and oxygen (Richmond Amos, 2004).

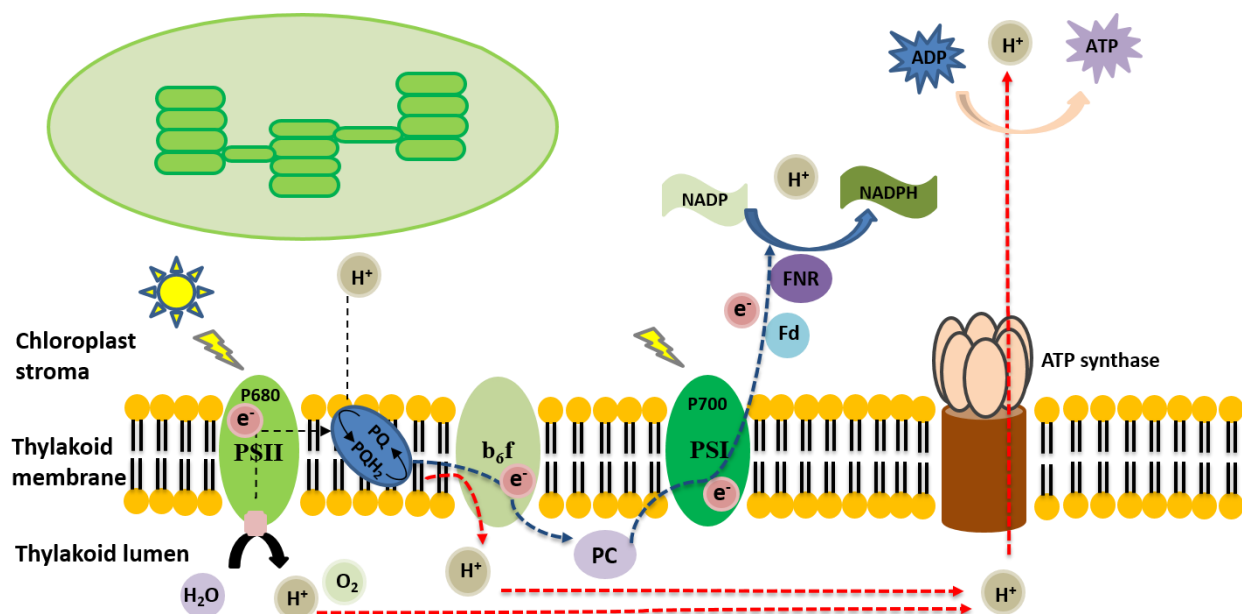


Figure 1 Schematic representation of the light reactions and electro transport chain in microalgae. Not all the components are shown. Abbreviation used PSII-photosystem II, PQ-plastoquinones, PC-plastocyanin, PSI-

photosystem I, Fd- Ferredoxin, FNR- Ferredoxin NADP reductase. Modified from (Johnson, 2013; Meyer et al., 2009).

Light energy/photon is absorbed by light harvesting complex proteins (LHCP) bound to chlorophyll. This excitation energy is further used by photosystem II reaction centers for catalytic oxidation of water forming molecular oxygen, protons and electrons (Anemaet et al., 2010). The low potential electrons are further sequentially transferred through photosynthetic electron transport chain (ETC). This ETC is composed of plastoquinones, cytochrome *b₆f* and plastocyanin. After passing through ETC, the electrons finally reach to the photosystem I. Photosystem I in excitation state reduces ferredoxin to form NADPH from NADP⁺ (Schenk et al., 2008). This reaction is catalyzed by ferredoxin-NADP reductase (FNR). During this process, an electrochemical gradient is formed due to formation of protons (at photosystem II) and their subsequent release into thylakoid lumen. The formation of electrochemical gradient across stroma and thylakoid lumen drives ATP synthase to produce ATP (Anemaet et al., 2010). These NADPH and ATP are utilized as substrates in Calvin cycle that takes place in the stroma of chloroplast. The Calvin cycle mediates CO₂ fixation to form glyceraldehyde-3-phosphate, (3-C) molecules. These 3-C molecules are further assimilated to form sugar, starch, lipids and other molecules (Forti, 2008).

The process of photosynthesis in algae produces ~ 52 billion tons of organic carbon per year (Hallmann, 2007) (Field, 1998). Therefore, the ability of utilizing solar energy (light energy) to produce energy rich carbon compounds makes microalgae a promising source of renewable energy (Hallmann, 2007; Wijffels et al., 2013; Sheehan et al., 1998; Chisti, 2007).

1.4 Microalgae as a feedstock for renewable energy

The global energy demand is rapidly accelerating. Currently, human global energy demand is approximately 15 terawatt (TW) and estimated to be 23.4 TW by 2030 (Barber et al., 2009). Most of this energy comes from the rapidly depleting fossil fuel. This is not only posing a danger to sustainable source of energy but also the use of fossil fuel is detrimental to environment and world economy. In specific, fossil fuel emits greenhouse gases and the high fuel prices is affecting world economy (Singh et al., 2011). It has become necessary to maintain a balance between the ever increasing energy demand and economic feasibility. Therefore, there is a huge requirement for not only to discover and develop new economically sustainable energy sources but also a less polluting one.

Last few decades has seen the evolution of microalgae as a source for renewable energy (Sheehan et al., 1998) (Hallmann, 2007). It is considered as '*third generation biofuel*' (Williams and Laurens, 2010; Hannon et al., 2010; Chisti, 2008). Small size, rapid growth, more productivity, ability to grow on non-arable land, easy to engineer are some of the advantages of microalgae over traditional biofuels sources (Hannon et al., 2010; Behera et al., 2014; Wijffels et al., 2010). Microalgae can produce various types of

high energy density carbon compounds. These includes bioethanol, lipids, hydrocarbons, bio-hydrogen, bio-methane, exo-polysaccharides (Borowitzka and Moheimani, 2013) shown in Fig 2. Some of these compounds are discussed below in brief.

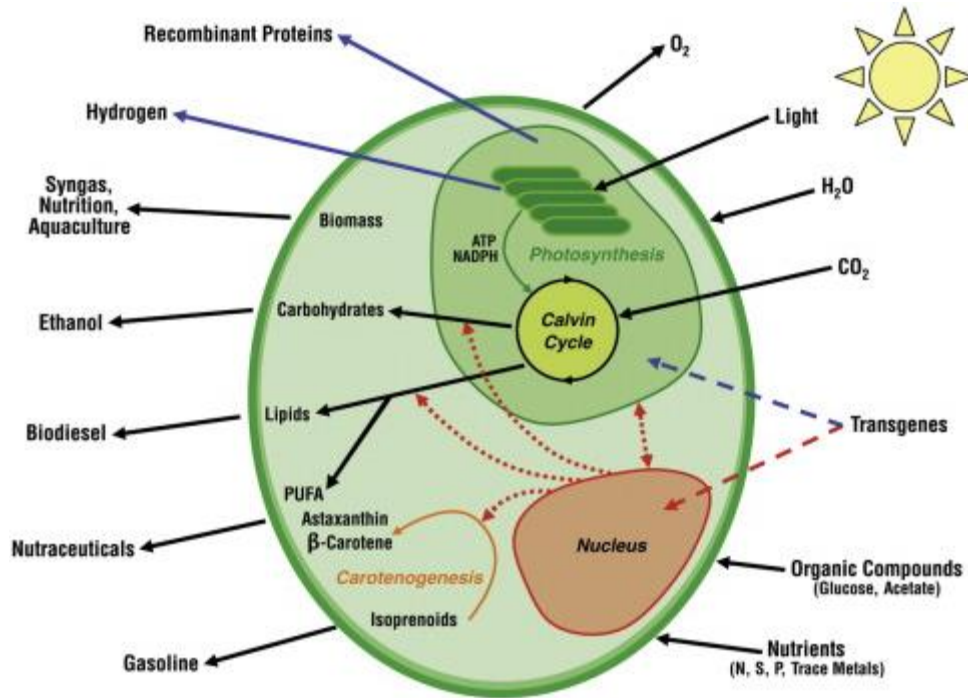


Figure 2 Schematic representation of microalgal cells and intracellular biochemical pathways involved in the production of different carbon compounds (Rosenberg et al. 2008).

Bioethanol

Microalgae are capable of synthesizing ethanol. In *C. reinhardtii*, starch is degraded via glycolytic pathway and pyruvate is broken down into acetyl coA and formate during the dark condition. This acetyl coA is subsequently converted into ethanol and acetate (Gfeller and Gibbs, 1984; Doebbe et al., 2010). This ability of microalgae to produce ethanol render them as an alternative source *per se.*, cyanobacteria *Synechococcus sp.*PCC 7942 (Deng and Coleman, 1999) is most commonly used for this purpose. Unlike plants, the low percentage of lignin and hemicellulose makes algae a potential source for bioethanol (Jones and Mayfield, 2012). Besides ethanol biosynthesis, studies have been conducted with microalgae as feedstock for ethanol production via yeast *Saccharomyces bayanus* (Harun et al., 2010). Lipid extracted biomass of microalgae was used to produce ethanol with ~38% w/w of productivity (Harun et al., 2010).

Bio-hydrogen

From past few years, hydrogen production from microalgae is garnering huge interest for research. Biological hydrogen production occurs in algal chloroplast and depends on electron and proton supply.

These electrons and protons are derived either from the process of water splitting in photosystem II or from degradation of organic molecules such as starch (Kruse and Hankamer, 2010; Doebbe et al., 2010; Toepel et al., 2013). The process of hydrogen production occurs in anaerobic condition in *C. reinhardtii* and can produce up to 540 ml of hydrogen/L of culture with 98% of purity (Kruse et al., 2005). However Fe-Fe hydrogenase, the enzyme involved in this process is very sensitive to oxygen and thus demands strict anaerobic condition (Melis et al., 2000). Currently *C.reinhardtii* is the best photosynthetic hydrogen producer green microalgae besides cyanobacteria *Nostoc*, *Arthrospira (Spirulina) maxima* and *Synechocystis* PCC6803 (Ananyev et al., 2008; Lindberg et al., 2004).

Owing to the complexities of the processes underlying hydrogen production it might be still a long way until hydrogen production from microalgae will be economically efficient. Thus, optimization of hydrogen production process, studying the bottlenecks of hydrogen synthesis and strain development is an active area of research.

Bio-methane

In recent times, the ability of microalgae to produce methane has been exploited for commercial use. Microalgae produce biogas through anaerobic fermentation. Microalgae such as *Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Scenedesmus obliquus* are studied for biogas production (Klassen et al., 2015). Their varying levels of natural biodegradability renders them suitable for biogas production (Mussnug et al., 2010). In model organism *C.reinhardtii*, different growth conditions such as mixotrophic growth and low nitrogen resulted in 587 ± 8.8 mL and 698 ± 23 mL per gram of volatile solids of biogas (Klassen et al., 2015). Interestingly, removal of harmful algal blooms which produce eco-system damaging secondary metabolites can be used for biogas production (Yuan et al., 2011). Although a lot of research has been done on using micro or macro algae for production of biogas and thereby methane, there are still some challenges hindering the application of micro-algal biomass for fermentative biogas production. These challenges include production cost for biomass, resistance of microalgal cell wall towards degradation and high protein content in the cell (Slade and Bauen, 2013; Passos et al., 2014; Yen and Brune, 2007). To overcome these challenges, use of combined production and bioremediation strategies for multi-purpose bio-refinery is an efficient way (Murphy et al., 2015).

Pigments

Photosynthetic organisms such as algae not only synthesize chlorophyll for light harvesting but also photosynthetic pigments, which are widely known as carotenoids. These pigments are anti-oxidants and are involved in photo protective mechanism, excess heat dissipation, electron transport and increasing the efficiency of light harvesting (Takaichi, 2011). Algal pigments are commercially valuable as they are used

in food industry for food odor, as colorant, in cosmetic and health industry as anti-oxidant and photo-protective chemicals. Astaxanthin, strong anti-oxidant produced by green microalga *Haematococcus pluvialis* is an abundant carotenoid pigment used as colorant in aquaculture industry (Pulz and Gross, 2004; Olaizola, 2000). β -carotene is another carotenoid which is commercially produced from halotolerant green microalga *Dunaliella Salina* (Pulz and Gross, 2004; Raja et al., 2007). Besides, hydrophobic photosynthetic pigments from green microalgae, phycobiliproteins produced by cyanobacteria, rhodophytes, cryptomonads, and glaucocystophytes are also commercially important. Phycobiliproteins such as phycoerythrin and phycocyanin are used in food, pharmaceuticals and cosmetics industry (Pulz and Gross, 2004; Hallmann, 2007).

Proteins

From last few years, intense efforts have made to explore microalgae as a source of proteins for the ever growing world population. Despite being a high nutrient protein, algal biomass has not gained much attention and importance as a food substitute (Becker, 2007). Green microalgae such as *Dunaliella*, *Scenedesmus obliquus*, *Chlorella sp.* are used for large scale production of proteins (Becker, 2007). Studies have been conducted for the utilization of algae as a protein source for humans (Dam et al., 1965). *C.reinhardtii* is used for recombinant protein production from past decade and thus it has been attributed for understanding the feasibility for protein production in green alga (Lauersen et al., 2013).

Bio-fertilizers

Microalgae as soil conditioners are important for the ecosystem. Algae have water and soil mineral binding abilities which can be used for agriculture purposes (Pulz and Gross, 2004). In addition to their polymers producing ability for particle adherence and water storing feature in soils or nitrogen-fixing, algal derived bio-active compounds have been successfully demonstrated to increase, carotenoids and sugar contents in tomatoes (Coppens et al., 2015). Furthermore, microalgae have the ability to recover nitrogen and phosphorous from wastewater and soils. This can be utilized for removal of pollutants and consequently as soil conditioning fertilizers (Wuang et al., 2016).

Polysaccharides

Algal polysaccharides can be used as gelatinous agar, gelling agent, stabilizers, texturants, thickeners and viscosifiers. These polysaccharides are produced by different classes of algae that include microalga such as *Gelidium spp.* and *Gracilaria spp.* producing hydrocolloid agar. Red macroalgae *Kappaphycus spp.*, *Eucheuma spp.*, *Betaphycus gelatinum* are well known for their ability to produce carrageenan. Brown macroalgae like *Laminaria spp.*, *Macrocystis pyrifera*, *Ecklonia spp.*, *Lessonia spp.*, *Durvillaea spp.*, and

Ascophyllum nodosum are alginate producers, a carboxylated polysaccharides which is used as stabilizer and emulsifier in food industries (Hallmann, 2007; Pulz and Gross, 2004).

Polysaccharides obtained from green microalgae such as *Chlorella sp.* was used in dietary supplements (Walker et al., 2005). Apart from chlorella, *Botryococcus sp.* is capable of synthesizing polysaccharides (Casadevall et al., 1985; Banerjee et al., 2002). Polysaccharide compounds from cyanobacteria have been used as effective immune modulators in pharmaceuticals and nutrition management. (Hallmann, 2007) (Pulz and Gross, 2004). Exo-polysaccharides produced by green alga *Chlamydomonas maxicana* can be used as soil conditioner (Kroen and Rayburn, 1984).

Although, the enormous potential of microalgal products is yet to achieve its full capacity, in recent years, many algal species and their associated new products were discovered. Currently, a great amount of research is being carried out to use microalgae for their application in pharmaceuticals and for biofuels. The robust and abundant biomass, high amount of lipids especially TAG content, ease of handling for genetic manipulation and downstream processing are some of the qualities which are desired for economical and sustainable growth of algal industry (Mata et al., 2010; Mutanda et al., 2011; Hannon et al., 2010). Unlike other conventional biofuel sources such as soya beans, Jatropha, sunflower, the diverse habitat of algal species and their adaptiveness to survive in different environmental conditions makes them highly suitable.

Oil and Hydrocarbons

One of the key features of microalgae is their ability to produce oil in the form of non-polar triacylglycerols (TAGs), hydrocarbons and other lipids. Many microalgae accumulate TAGs as their storage compounds under various stress conditions which includes photo-oxidative stress and nitrogen starvation (Merchant et al., 2012b; Hu et al., 2008). These TAGs were then converted into the 'biodiesel' via a process of transesterification in the presence of acid and methanol (Sheehan et al., 1998). Although, most of the microalgae that have been studied earlier for their ability to produce high amount of TAGs, the economic viability of the whole process still remains unfeasible.

Hydrocarbons are another type of non-polar lipids synthesized by some microalgae. Although, the amount of hydrocarbons produced by microalgae is less than 5% (Lee and and Loeblich, 1971). However, *Botryococcus braunii* is the only green microalga that produces large amount of hydrocarbons i.e. ~ 75% of its dry biomass. Interestingly, some of these hydrocarbons are similar to those found in petroleum (Cane, 1977). These hydrocarbons can easily be converted into gasoline, and other forms of fuel which are suitable for engines (Kitazato et al., 1989).

Therefore, given the variety of uses of microalgae and the need of improving and developing these sources, a great deal of efforts is underway to optimize the microalgal cultivation as a feedstock for biofuels and

biomass. Some of them are identification of novel strains, metabolic engineering for strain improvement and development, optimization of cultivation process. The use of new sequencing and analytical technologies have contributed immensely for the better understanding of system biology of microalgae.

1.5 Algal OMICs for bio-product diversity

The term “omics” includes the study of genomics, transcriptomics, proteomics and metabolomics. This technology based analytical field have provided deeper insights into the potential of alga for its use in a variety of applications in biofuel, pharmaceuticals and nutraceutical industries.

The availability of genome of more than 30 organelles and whole algal genome have provided the basic and essential understanding of native biosynthetic and metabolic capacity of algal cell as a potential cell factory for valuable products (Guarnieri and Pienkos, 2014). For example, the genome of *C.reinhardtii* (Merchant et al., 2007a) has been instrumental in providing insights into microalgal fatty acid biosynthesis and TAGs accumulation under nutrient deletion (Merchant et al., 2012b).

The development in the field of next-generation sequencing has enabled researchers to study algal transcriptome. The study of transcriptome has been instrumental for examining the differential expression of algal genes involved in the biosynthesis of different products. A prime example is the study of TAGs and fatty acid biosynthesis under nitrogen starvation in *C.reinhardtii* (Miller et al., 2010). Besides *C.reinhardtii*, the other microalgae such as *Chlorella vulgaris* (Guarnieri et al., 2011) *Neochloris oleoabundans* (Rismani-Yazdi et al., 2012) were also studied in detail using transcriptome as a tool for understanding TAG accumulations. All these components of OMICs play a crucial role not only in establishing the conceptual knowledge but also provides important information about the potential applications of microalgae in the field of biofuel industry, food and feed industry, nutraceutical and pharmaceutical industries. Metabolome serves as an important component of OMICs based studies and the current study focuses on metabolomics as an effective tool for studying the bio-product diversity of microalga.

1.5.1 Metabolomics: an important tool of system biology

Systems biology is the quantitative study of a complex network of interacting and interchanging molecular participants and their environment. These includes DNA, mRNA, proteins and metabolites (Nielsen and Jewett, 2007). Given the highly complex nature of cellular networks, the study of system biology became challenging. This is mainly because of three reasons, 1) There is no equal relation between gene and the metabolites, for example many metabolites can be formed from a single enzyme and in turn many proteins from a single mRNA. 2) mechanisms such as the post transcriptional modifications, translational

regulations directly or indirectly affect the metabolites and 3) finally, small changes in the overall environmental conditions and within the organism sometimes can have large effect because of the complex network of these biochemical pathways and their interrelation (Nielsen and Jewett, 2007).

In order to understand the complex biological behavior of an organism, study of their response to the different environmental and physiochemical conditions is important. The comprehensive analysis of expression characteristic of any biological system is comprised of transcriptome, proteome and metabolome. These three expression profiles provide a complete view of the RNAs, proteins and metabolites enabling researchers 1) to derive relation between macromolecules. 2) identify functional linkages between phenotype and genotype expressions and 3) construct the models that quantitatively summarize the dynamics of biological system. However, it is important to study the phenotypes to validate the relation between gene and its predicted functions (Stitt and Fernie, 2003). The metabolites, the end products of any intracellular biochemical pathways are the ultimate factors to assess the response of all the genetic and environmental changes that takes place in the cell (Fiehn, 2002). Therefore, attributing to their role in the expression of the information from different expression features (RNAs, proteins) (Nielsen, 2003) including the effect of the external factors, quantitative study of these metabolites is of prime importance for system biology.

Metabolomic profiling has been used for many different applications. These includes drug discovery (Horning and Horning, 1971; Cascante et al., 2002), strain classification (Frisvad and Filtenborg, 1983) and functional genomics (Bino et al., 2004). Metabolomics as a tool for understanding the biological response to different physiological conditions and its effect on the product formation in microalgae have been studied effectively and extensively (Bölling and Fiehn, 2005; Doebbe et al., 2010; Lee et al., 2012).. For example, Bölling and Fiehn reported the response of *C.reinhardtii* to nutrient depletion (Bölling and Fiehn, 2005). The elucidation of metabolome profile under sulphur depleted condition for the production of hydrogen from *C.reinhardtii* was also described(Matthew et al., 2009). Sometimes, these studies include the targeted approach to study the particular set of metabolites such as fatty acids, carotenoids for selective physiochemical conditions such as nutrient depletion, pH and temperature for biotechnological purposes. For example, the metabolic changes of starch and lipids in *Chlorella zofingiensis* following nitrogen starvation (Zhu et al., 2013) and the for enhancement of lutein in *Chlorella sorokiniana* (Cordero et al., 2011). These studies have contributed enormously for the better understanding of the system biology and cellular response of the microalgae.

Taken together, the identification and quantification of the metabolites is essential in order to study the kinetics and dynamic changes in the metabolome of an organism. This in turn will aid to understand the intracellular biochemical pathway and their response to various stimuli (Bino et al., 2004). However, the

bigger challenge is to identify the role of each metabolites and to correlate the changes in the metabolites and their network to physiological and developmental phenotypes of the cell or organism (Bino et al., 2004). The current study is an attempt to provide the overall metabolic profile of one of the very interesting microalga *Botryococcus braunii*.

1.6 *Botryococcus braunii*

Botryococcus braunii, a unicellular photosynthetic green microalga was first identified by Kützing in 1849 (Kützing, 1849). The morphology and classification of *B.braunii* was studied during the first quarter of 19th century by Poulton (Poulton, 1930; Blackburn, 1936). In 1938, Fremy and Dangeard reported a detailed study on *Botryococcus braunii* from a Swedish lake and Tertiary black clay of France (Fremy and Dangeard, 1938). It took more than two decades to further investigate this alga when Belcher, Maxwell, Brown and Knight gave a comprehensive overview of physiology, biochemical characterization of *Botryococcus braunii* and its products which are mainly the hydrocarbons (Maxwell et al., 1968; Brown et al., 1969; Knights et al., 1970). However, even after long periods of intensive research, *B. braunii* has remained ambiguous in terms of its cell biology and its relation to product formation. Geochemical significance of *B. braunii* made this alga widely studied in the past. Cane in 1977 reported that the oil-rich deposits dating from the Ordovician period to the present (Cane, 1977) are composed of hydrocarbons from *B. braunii*. Later Moldwan and Seifert described that two Sumatran crude oils has nearly 0.9 and 1.4% of botryococcane, C₃₄H₇₀, the hydrogenated derivative of botryococcene, (Moldwan and Seifert, 1980). Thereafter, numerous reports have suggested the presence of *B.braunii* derived hydrocarbons in coastal kerosene formations, sediments and oil shales (McKirdy et al., 1986; Lichtfouse et al., 1994; Behrens et al., 2000). Together, these above mentioned studies have provided valuable insights into our understanding of the morphology and physiology of *B. braunii*. Collectively, the biofuel producing characteristics promote *B. braunii* as an attractive source for biofuel.

1.6.1 Cell Morphology and biodiversity of *Botryococcus braunii*

Botryococcus braunii is a colony forming green alga found in fresh and brackish water, distributed to temperate and tropical regions all over the world (Belcher, 1968; Banerjee et al., 2002; Metzger and Largeau, 2005). The *B.braunii* cells are pyriform shaped and cell size typically ranges from 4µM wide and 10 µM long (Blackburn, 1936). These pyriform cells cluster together to form a colony and are held together by complex extracellular matrix (ECM) within a colony unit (Belcher, 1968; Weiss et al., 2012). Each of this colony unit contains 50-100 oval shaped cells that are confined in retaining wall. This retaining wall is attached to fibrillar sheath made up of arabinose and galactose polysaccharides (Weiss et al., 2012). The retaining wall is in direct contact with a much thicker cell wall. The inner surface of the retaining wall and

the outer surface of cell wall consists of hydrocarbons except the cell apex (Wolf, 1983; Weiss et al., 2012). The cell wall of *Botryococcus braunii* is composed of polysaccharides (Wolf, 1983) especially fibrous β -1, 4- and/or β -1, 3-glucan components (Weiss et al., 2012). The space between colonies is filled up with hydrocarbon network. Thus, the ECM of *B.braunii* secretes three classes of materials 1) polysaccharides of cell wall 2) proteinaceous granules associated with retaining wall and 3) hydrocarbons (Weiss et al., 2012). The protoplast contains a cup shaped chloroplast with a basal pyrenoid, centrally located nucleus and a large Golgi body at apical cytoplasm (Wolf, 1983; Weiss et al., 2012). The Golgi apparatus is associated with non-apical endoplasmic reticulum (ER), mitochondria appears in close association with chloroplast and cell contains variable number of lipid bodies (Wolf and Cox, 1981; Wolf, 1983; Weiss et al., 2012).

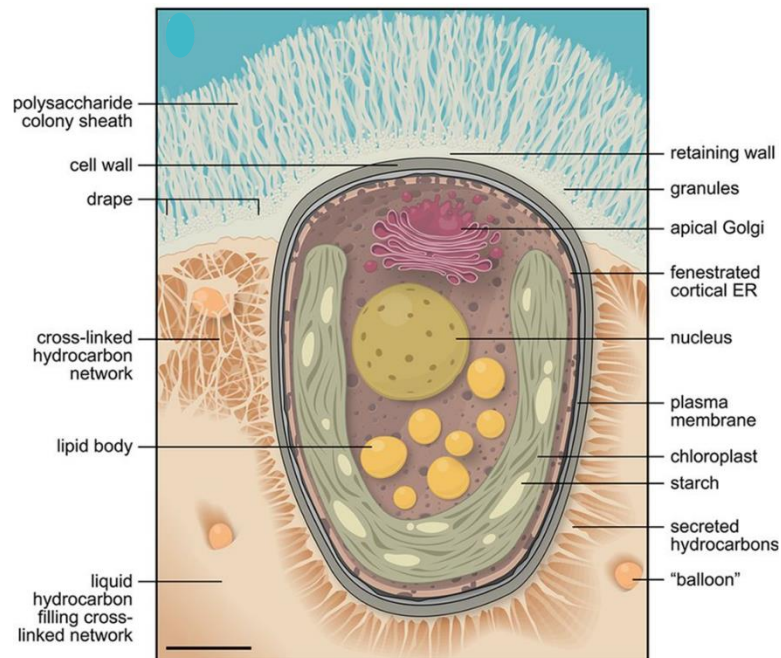


Figure 3 Schematic representation of single cell of *Botryococcus braunii*. Schematic representation of single *Botryococcus braunii* cell showing main cellular organelles and surrounding extra cellular matrices. Not all the cellular organelles shown. Adapted from (Weiss et al., 2012).

Although the above mentioned morphological and physiological characteristics of *B.braunii* cells are from recent studies, initially *Botryococcus* was divided into different number of species based on their morphology (Metzger and Largeau, 2005). For example, Kamarek and Marven (1992) divided *Botryococcus* into 13 species based on their cell size, shape and colony organization (Komárek and Marvan, 1992). Based on 18s RNA sequencing of four different strains of *B.braunii*, it was shown that they formed a monophyletic group (Senousy et al., 2004). Phylogenetic study by Weiss et al. suggested that evolutionary relationship between different races of *B. braunii* are related to the types of hydrocarbons they produce.

The same study based on flow cytometry analysis also reported the genome size of race A and race L (Weiss et al., 2010). The relation between hydrocarbon and molecular phylogeny was recently demonstrated (Kawachi et al., 2012).

1.6.2 Chemical variability

Botryococcus braunii is well known for its ability to produce large quantity of hydrocarbons. 75% of dry biomass can be hydrocarbons (Banerjee et al., 2002; Ranga Rao et al., 2014). 86% of total hydrocarbon (botryococcene) produced in *B. braunii* was described to be produced during the 'resting phase' of the growth (Brown et al., 1969). Depending on the types of hydrocarbons they produce, *B. braunii* strains are divided into three different races namely race A, race B and race L. Recent study on the relationship between the hydrocarbon and molecular phylogeny led to the discovery of a new race, race S and is reported to produce epoxy-n-alkane and saturated n-alkane chains with carbon numbers 18 and 20, respectively (Kawachi et al., 2012). However, not much is known about the new race S yet.

1.6.2.1 Race A

Botryococcus braunii race A strains are known to produce fatty acid derived hydrocarbons. These hydrocarbons include C23 to C31 odd chain alkadienes, alkatrienes (Metzger et al., 1985, 1986b, 1989). Race A strains have the largest range of hydrocarbon content from 0.4% to 61.0% of their dry weight (Metzger and Largeau, 1999; Metzger et al., 1985). There are about 30 odd chain hydrocarbons structure determined from race A strains, all these compounds exhibit terminal unsaturation (Metzger and Largeau, 1999).

Race A derived hydrocarbons are of non-isoprenoid origin, alkadienes and trines and are predominately alkadienes (Metzger and Largeau, 2005). This non-isoprenoid mechanism of hydrocarbon biosynthesis occurs via elongation-decarboxylation pathway. Here, two carbon atoms to oleic acid are added successively from malonyl Co-A until the appropriate chain lengths are attained (Templier et al., 1984, 1987). Decarboxylation of very long chain fatty acid derivatives is the final step to form hydrocarbons. The removal of carbon atoms requires a lot of energy which is provided by β -OH group (ChanYong et al., 1986). Structural identity of these hydrocarbons revealed the presence of terminal double bond leading to the conclusion that Oleic acid (C18:1 cis- ω 9) is a direct precursor for the hydrocarbon biosynthesis in race A strain of *Botryococcus braunii*. However, this elongation-decarboxylation pathways system is non-specific and can accept both oleic (C18:1 cis- ω 9) and elaidic acid (C18:1;trans- ω 9) (Templier et al., 1984). In 1984, Templier et al showed that the extent of hydrocarbon synthesis in race A was strongly dependent on dithioerythritol concentration, as it is well known that thiols inhibit the hydrocarbon biosynthesis by

blocking decarboxylation reaction of very long chain fatty acid derivatives (Templier et al., 1984). Thus, this confirms that hydrocarbon biosynthesis in race A of *Botryococcus braunii* indeed occurs via elongation-decarboxylation pathway.

Besides hydrocarbons, some of the strains especially race A strains of *Botryococcus braunii*, are also known for their ability to produce (exo) polysaccharides (Casadevall et al., 1985). The polysaccharides are high value products as they are used in textiles, laundry products, adhesives, paper, paint, and foods (Banerjee et al., 2002).

As earlier mentioned, the cell wall of *B. braunii* is made-up of polysaccharides. The internal fibrillary sheath attached to retaining wall consists of arabinose and galactose polysaccharides (Weiss et al., 2012). These polysaccharides slowly dissolves in the culture thereby increasing the viscosity of medium (Casadevall et al., 1985). The production of polysaccharides occurs during growth and stationary phase (Casadevall et al., 1985).

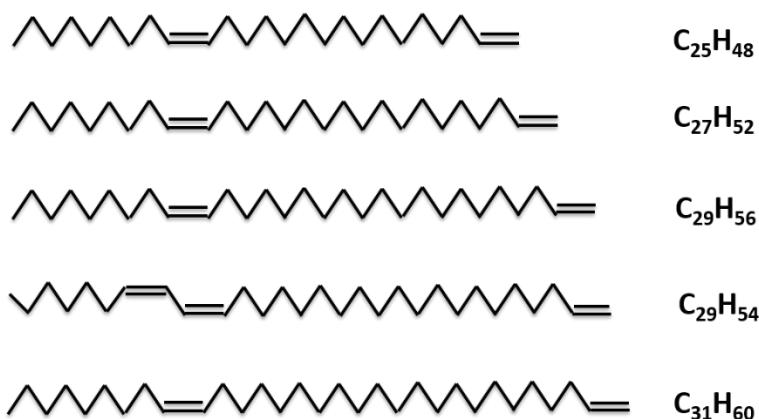


Figure 4 Hydrocarbons produced by *B. braunii* race A strains. The structure of some hydrocarbons produced by race A strains of *B. braunii*. Modified from (Banerjee et al., 2002).

1.6.2.2 Race B

Because of its ancient origin dating back to 500 MYA and its contribution to the existing oil shales and coal deposits, last few years have seen *Botryococcus braunii* race B strains gaining much attention in the few years. (Niehaus et al. 2011). This race of *B. braunii* produces isoprenoid-derived hydrocarbon via the well studied MEP pathway and is among the highest *B. braunii* hydrocarbon producer strain known until now. Race B hydrocarbons are specific cyclic and acyclic triterpenoid compounds known as Botryococcenes (Metzger and Largeau, 2005). Around 50 botryococcene compounds have been identified, however, only few of them are structurally known. The distribution of these isoprenoid-derived hydrocarbons among race

B strains vary depending on culture conditions, origin of strain, temperature and other physio-chemical parameters. In addition to botryococenes, some of the race B strains also produce squalene and methylated squalene from C31-C34 carbon chain (Huang and Dale Poulter, 1989; Metzger and Largeau, 1999).

Hydrocarbon biosynthesis in race B

In eukaryotic organisms such as plants and phototrophic algae, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the fundamental precursors for terpenoid biosynthesis (Davies et al., 2014). For decades, mevalonate (MVA) pathway was thought to be the only pathway through which terpenoid biosynthesis occurs. However, another pathway was discovered in the plants and was named methyl-D-erythritol 4-phosphate (MEP) pathway (Lichtenthaler, 1999; Zhao et al., 2013). Both MVA and MEP pathways yield IPP and DMAPP as their final product. Initially, it was thought that MVA is of eukaryotic/archael origin and MEP pathway is of prokaryotic, although, there are plenty of evidences suggesting the shuffling of these two pathways across kingdoms. Eukaryotic photosynthetic unicellular algae have MEP pathway and are solely dependent on it to produce carotenoids, sterols and other isoprenoid derived products (Davies et al., 2014).

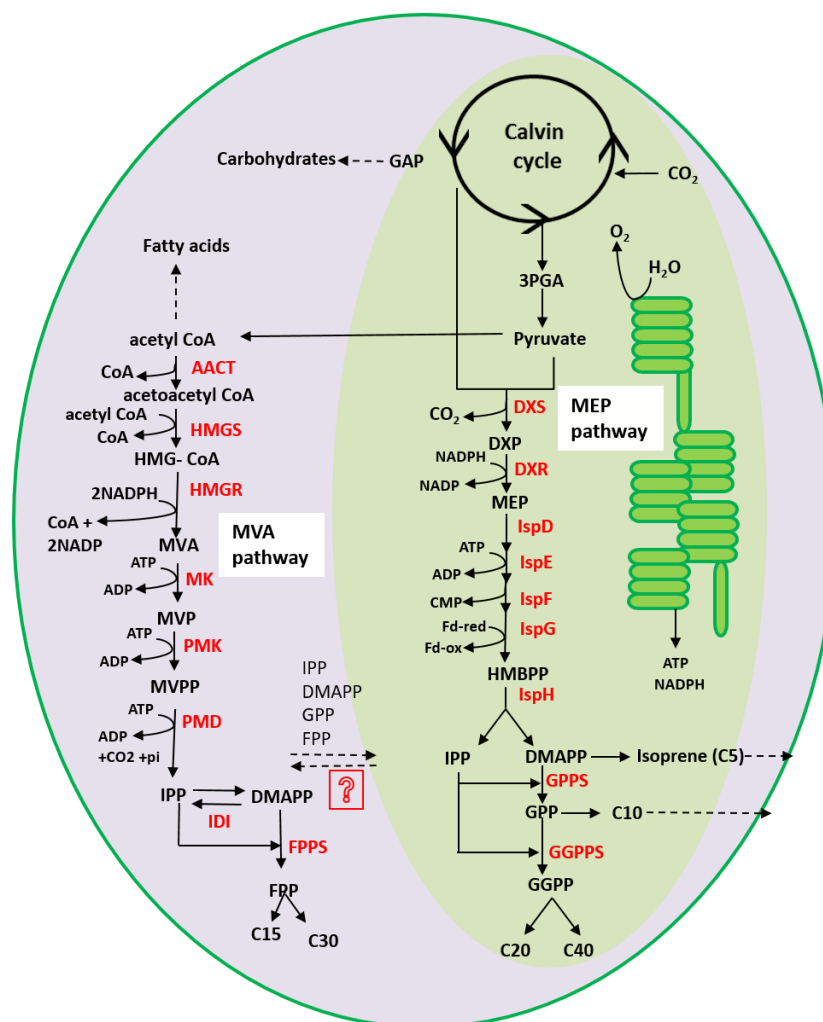


Figure 5 Schematic representation of compartmentalization of MVA and MEP pathway in the plant cell. Cofactors such as ATP, NADPH and reduced ferredoxin (Fdr) that are used by the MEP pathway are produced by the process of photosynthesis in the thylakoid membrane. Abbreviated MVA pathway enzymes are: AACT acetoacetyl-CoA thiolase, HMGS HMG- CoA synthase, HMGR HMG-CoA reductase, MK mevalonate kinase, PMK mevalonate 5-phosphate kinase, PMD mevalonate 5-pyrophosphate decarboxylase, and IDI IPP isomerase. Abbreviated MEP pathway enzymes are: DXS 1-deoxy-D-xylulose 5-phosphate synthase, and DXR 1-deoxy-D-xylulose 5-phosphate reductase. Modified from (Davies et al., 2014).

Unlike the race A strains, hydrocarbons from race B are derived from isoprenoid biosynthesis i.e. MEP pathway. Earlier reports on hydrocarbon biosynthesis in race B using labelling experiment suggested that incorporation of radiolabelled mevalonate (2- ^{14}C) in botryococcene was very low (0.2%) (Casadevall et al., 1984). In year 2003, Sato et al observed that the hydrocarbons produced namely, botryococcenes and methylated squalenes were derived from non-mevalonate pathway after feeding algae with radiolabelled glucose (1- ^{13}C) (Sato et al., 2003). Furthermore, the detailed study on transcriptome of *B. braunii* race B strain Showa, identified genes and enzymes responsible for botryococcene formation and that race B synthesizes hydrocarbons via MEP pathway (Molnár et al., 2012; Niehaus et al., 2011).

The MEP pathways use glyceraldehyde-3-phosphate(GAP) and pyruvate as their primary feedstock molecules to form 1-deoxy-D-xylulose 5-phosphate (DXP) as the first reaction product. DXP then undergoes a series of reactions including reductive isomerization, phosphorylation, cyclization and reductive dehydration steps to generate 4-hydroxy 3-methyl-butenyl 1-phosphate (HMBPP). HMBPP can be further converted to either IPP or DMAPP by reductive dehydration step (Fig. 5) (Lichtenthaler, 1999; Lohr et al., 2012).

Hydrocarbon biosynthesis in *Botryococcus braunii* race B strain for the production of botryococcene and methylated squalene is different from that of other phototrophic eukaryotic algae. In phototrophic unicellular algae, squalene synthesis is a two-step reaction carried out by a single enzyme called squalene synthase. In the first step, two FPP molecules through head-to-head condensation reaction form presqualene diphosphate (PSPP), a stable cyclopropyl intermediate. In the second step, PSPP in the presence of NADPH undergoes reductive rearrangement to yield squalene (Poulter, 1990; Rilling, 1966). In *B.braunii*, Okada et al. using modified experimental procedure discovered that Farnesyl di-phosphate (FPP) is the main precursor for botryococcenes (Okada et al., 2004). Based on proposed similarities (Huang et al., 1988), prediction was that botryococcene synthase and squalene synthase would be similar. However, Niehaus co-workers have shown that there are three genes which encodes for three different squalene synthase like (SSL) enzyme involved in the synthesis of squalene and botryococcene in *B.braunii* (Niehaus et al., 2011). These three enzymes were SSL-1, SSL-2 and SSL-3. SSL-1 catalyzes the first step of squalene synthesis, is the formation of PSPP. While SSL-2 catalyzes the reaction to form squalene, SSL-3 required to give rise to botryococcene from PSPP. These findings showed that the synthesis of botryococcene and squalene in *B.braunii* requires the combination of two separate squalene synthase like enzymes, SSL-1+SSL-2 for squalene and SSL-1+SSL-3 for botryococcene, unlike other unicellular phototrophic alga (Niehaus et al. 2011).

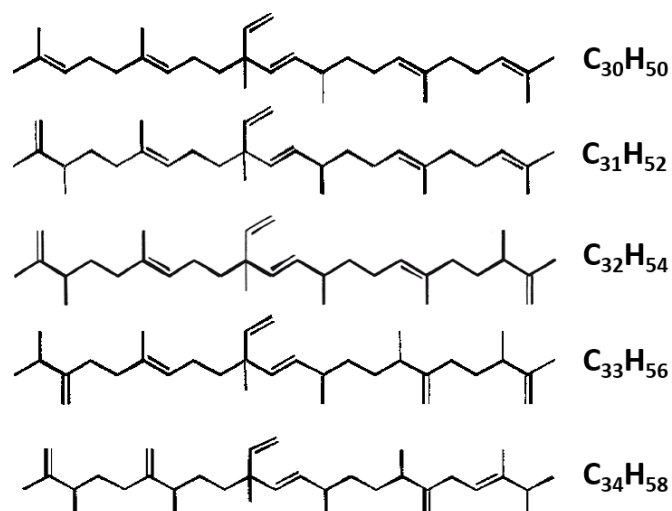


Figure 6 Hydrocarbons produced by *B. braunii* race B strains. The structure of some hydrocarbons produced by race B strains of *B. braunii*. These hydrocarbons include squalene and methylated squalene along with botryococenes from C31-C37 carbon chain. Modified from (Banerjee et al., 2002).

1.6.2.3 Race L

Botryococcus braunii race L strains are usually smaller compared to race A and race B strains (Metzger and Casadevall, 1987). They produce longer chain hydrocarbons such as lycopadienes (Metzger and Casadevall, 1987). Lycopadiene biosynthesis is hypothesized to occur via two pathways. First entails the tail-to-tail combination of two-phytyl diphosphate (PPP) units and in as a second possibility is the head-to-head condensation of two-geranylgeranyl diphosphate (GGPP) molecules to produce lycopersene which is further reduced to lycopadiene (Metzger and Casadevall, 1987). Recently, Devarenne group elucidated the mechanism of lycopadienes biosynthesis. They have successfully showed that lycopadiene biosynthesis takes place via a second route where two molecules of GGPP forms lycopaoctaene and is further reduced to form lycopadiene (Thapa et al., 2016a).

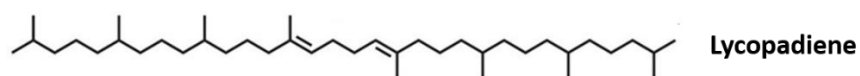


Figure 7 Hydrocarbon produced by *B. braunii* race L. Race L produces hydrocarbons with carbon chain length C40 and more. Structure of lycopadiene was modified from (Thapa et al., 2016b).

Besides producing lycopadienes, number of ether lipids such as lycopanerols were also isolated from race L strain originated from a lake in Yamoussoukro, Ivory Coast (Rager and Metzger, 2000; Metzger et al., 2003). Lycopanerols are comprised of a transtetrahydrofuran (THF)-containing lycopane connected by an ether bridge to another lycopane comprising a tetrahydropyran ring (THP) thus forming a very long n-alkenyl chain by another ether bridge (Metzger et al., 2003). Lycopanerols were found to be 10% of dry

algal biomass in *B.braunii* (Rager and Metzger, 2000). Metzger and co-worker also reported that *B.braunii* race L and *Botryococcus neglectus* are from the similar taxa.

1.7 Why *Botryococcus braunii*?

As previously mentioned, *B. braunii* is a green microalga widely grown in fresh and brackish water microalgae which can produce large quantity of hydrocarbons and polysaccharides. These algae are also known for their unique and outstanding capacity to produce and excrete high quantities of long-chain hydrocarbons as well as an interesting group of polysaccharides that can be converted into platform biochemicals. Their ability to produce large amount of hydrocarbons makes them an important and potential source for biofuel applications. The liquid hydrocarbons synthesized by *B.braunii* have more advantage over oils as they do not need any chemical conversion to provide biofuel (Molnár et al., 2012). The hydrocarbons such as tri-methyl botryococcene can be converted into gasoline, kerosene and diesel by existing petroleum refinery and thus do not need any further chemical conversion (Kitazato et al., 1989; Niehaus et al., 2011). Apart from these reasons, hydrocarbons of *B.braunii* have been recovered from various petroleum reserves indicating the potential role of *B.braunii* to produce oils (Cane, 1977; Moldwan and Seifert, 1980; Glikson et al., 1989). However, without the understanding of the evolutionary biology of this microalga along with its biological and biochemical properties, it is still too early to refer it as a viable source for biofuel production.

Furthermore, *B. braunii* biomass is also a valuable source for fatty acids, carotenoids and sterols (Kalacheva et al., 2002; Matsuura et al., 2011; Tanabe et al., 2013). The fatty acids from *B. braunii* have been shown to enhance the drug (flurbiprofen) absorption into and across the skin (Fang et al., 2004). The carotenoid such as echinenone was shown to be accumulated in extracellular matrix (Matsuura et al., 2011) which can be easily accessible via 'milking' process. Besides, high antioxidant activity of phenolic extract from *B. braunii* which accounted for 12.8% of dry biomass (Rao et al., 2006) is another valuable chemical from this alga. Recently, it was reported that the aqueous extracts of *B. braunii* enhance the antioxidant and anti-inflammatory activities and was shown to stimulate adipocytes differentiation, reduce skin dehydration and to instigate the synthesis of new collagen (Buono et al., 2012). Thus, there is a great potential to explore the chemical and metabolites produced by *B. braunii* and thus it is even more necessary to study the biology particularly in terms of its metabolome of this interesting microalga.

In addition, from the environmental aspects as well, it is important to understand the metabolome of *B. braunii* as it was earlier reported that blooms of *B. braunii* was associated with the massive fish mortality near Alexandria in Egypt (Labib et al., 2011). The exact mechanism for fish mortality was not determined in this study, however it was shown that the mortality was associated with the large density of *B. braunii*

colonies (55.3×10^3 colonies L^{-1}) (Labib et al., 2011) and could be because of the excessive hydrocarbon or polysaccharides formation which was responsible for lower dissolved oxygen content. Thus, it is necessary to access the understanding of the different types of the metabolites produced by this alga.

The slow growth rate is one of the main challenges associated with this alga. This slow growth is mainly because of the production of metabolically expensive compounds such as hydrocarbons and polysaccharides and partly because of the colony formation causing hindrance to the gas exchange (Belcher, 1968; Wolf, 1983). Earlier studies have shown that the bacterial consortium associated with *B. braunii* plays an important role in the growth and hydrocarbon production (Chirac et al., 1985) although, a definite answer for the relation between bacteria and *B. braunii* has not been well established. This will require more understanding about the biology especially from the metabolome point of view. Besides, it was also evident that *B. braunii* produces different compounds depending on the respective environmental condition to which they belong. Thus, there are variations not only across different races but also between different strains within a same race. These differences across the races and strains within the same race has contributed to vast and diverse data that was largely focused on hydrocarbons and the production capabilities of *B. braunii*. However very few studies were carried out in order to understand the relation between the physiology, growth behavior and product formation in this alga. Moreover, the variations in the different strains further fueled the research to find an ideal strain of *B. braunii* that can grow faster and also produce large amount of hydrocarbons or polysaccharides. This prompted us to study *B. braunii* and to establish the foundation for physiochemical and biological (metabolome) aspects of this microalga.

2 Objectives

The overall objective of the present work was to understand the relation between the physiological state of cells and its impact on the metabolome profile of *B. braunii* with a special focus on its products such as hydrocarbons and polysaccharides.

In addition, the study aimed at systematically characterizing the metabolome profiles to identify the differences between the race A and race B strains of *B. braunii* and within the race A strains as well. This systematic metabolic characterization includes intracellular primary metabolome profile, lipids and FAME analysis, intracellular photosynthetic pigment profile, and determination of carbohydrates and hydrocarbons. Therefore, this study focused on three different strains of *B. braunii* CCALA 778, CCAP 807/2 and AC 761 in order to understand the differential product formation of this microalga and its response to the different physiological conditions.

The specific objectives and goals of this study were

- What is the influence of different physiological states of the *B. braunii* cell on overall metabolome profile and especially on hydrocarbons and polysaccharides formation?
- When does the synthesis of hydrocarbons and polysaccharides occur in *B. braunii*?
 - Are there any differences between the race A and race B strains with respect to product formation phase?
 - Characterization of lipids, hydrocarbons and polysaccharides produced in race A and B strains.
- Does race A strains differ from each other with regard to growth, metabolome profile and product formation?

3 Materials and methods

3.1 Strains and culture conditions

Botryococcus braunii race A strains CCALA 778, CCAP 807/2 were originally obtained from Serra da Estrela (Baragem da Erva da Fome) Portugal and Grasmere, Cumbria, England respectively. Race B strain AC 761 was isolated from Paquemar, Martinique, France. These strains were grown in the modified Chu-13 medium with pH of 7.2 with following chemical composition:

Table 1 List of chemicals with the quantity used in preparation of modified Chu-13 media. The manufacturer company from which these chemicals are obtained shown in the last column.

No.	Chemical	Quantity	Manufacturer
1	CaCl ₂ .2H ₂ O	734.0 μM	Roth GmbH
2	MgSO ₄ .7H ₂ O	811 μM	Roth GmbH
3	K ₂ HPO ₄	602 μM	Roth GmbH
4	KNO ₃	3.95 mM	Roth GmbH
5	Na ₂ O ₄ Se	50 nM	Alfa Aesar (Thermo Fisher (Kandel) GmbH)
6	FeNaEDTA	50 μM	Alfa Aesar (Thermo Fisher (Kandel) GmbH)
7	CuSO ₄ .5H ₂ O	0.32 μM	AppliChem GmbH
8	ZnSO ₄ .7H ₂ O	0.76 μM	Roth GmbH
9	CoSO ₄ .7H ₂ O	0.32 μM	Roth GmbH
10	MnSO ₄ .4H ₂ O	7.94 μM	Roth GmbH
11	Na ₂ MoO ₄ .2H ₂ O	0.25 μM	AppliChem GmbH
12	H ₃ BO ₃	43.26 μM	Acros Organics
13	H ₂ SO ₄	1 mL	VWR Chemicals

All these chemicals were added in chronological order as shown in the table 1.

Cultures were aerated with 6% CO₂ (Linde AG, München, Germany) under 16:8 light-dark illuminations of 350- 400 μmol photons m⁻² s⁻¹ white light and stirred with a magnetic bar at room temperature 25-28 °C.

Growth of the cells was monitored by measuring optical density at 750 nm and 680 nm. Additionally, measurement of dry biomass weight was carried out for whole culture (cells + supernatant media).

3.2 Measurement of organic dry weight

The determination of organic dry weight (oDW) of whole culture containing cells and medium was performed using 10 mL of culture. This culture was added into the pre-weighed glass vials followed by overnight incubation of the samples at 105°C. The total biomass was gravimetrically measured. To determine the weight of organic matter the following muffle oven (SNOL 3/1100 LHM01, SNOL laboratories) temperature program was used: room temperature ramp to 250°C at 15°C min⁻¹, hold 60 min; ramp to 400°C at 15°C min⁻¹ hold 30 min; ramp to 550°C at 15°C min⁻¹, hold 90 min. After cooling down the samples, weight of the vials was again measured and was subtracted from the initial weight measured after 105°C.

3.3 Chlorophyll measurement and pigment analysis via UV-HPLC

The cell pellet for chlorophyll determination was obtained from 1 mL of culture by centrifugation at 14000 rpm (Eppendorf AG, Hamburg) for 5 mins. The cells were incubated in methanol (VWR International GmbH, Darmstadt, Germany) for 30 mins at 60°C in Thermomixer (Eppendorf AG, Hamburg, Germany) after centrifugation the mixture was vortexed to make sure the cell lysis. Cell debris were removed by centrifugation and optical density was measured at 665 nm, 653 nm and 470 nm using UV spectrophotometer (GENESYS™ 10S UV-Vis-Spectrophotometer, Thermo Fischer Scientific, Germany) for the determination of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids according to Wellburn et al (Wellburn, 1994).

For intracellular pigment analysis, 3-5 mg of lyophilized dry biomass was used. In detail, lyophilized biomass with the mixture of 0.1 mm silica beads, 1 mL of 90 % acetone (v/v, analytical grade saturated with CaCO₃) was homogenized using homogenizer (Precellys 24 homogenizer, Peqlab) for 3 x 45 s at 6500 rpm with 15 s breaks. The particle free supernatant was obtained after centrifugation at room temperature (5 min, 14,000 rpm). Pigments were separated via Thermo Finnigan UV-HPLC with a TSP degasser, TSP AS 3000 auto sampler, TSP P4000 pump, TSP column oven and TSP UV6000 detector. 20 µL of pigment sample was separated on a Accucore™ Polar Premium RP-C18 column (150mmx4,6mm, 2.6 µm particle size, Thermo Scientific) with a pre-column Accucore™ C8 filter (10x4.6 mm, 2.6 µm). For separation, eluent A with 0.1 M ammonium acetate/methanol (15:85, v/v), and eluent B with methanol/acetonitrile/acetone (44:43:13, v/v) were used. The column was equilibrated for 5 min with 100 % A and a flow rate of 0.5 mL min⁻¹. The solvent composition changed at 31 min to 75 % A and 25 % B, and then to 100 % B at 47 min till the end of run at 70 min. The UV-vis-detector scanned the wavelength-

range 190-800 nm with a bandwidth of 1 nm and a scan rate of 1 Hz. Additionally, a discrete channel was recorded at 440 nm. The resulting chromatograms were evaluated with Xcalibur software (Version 2.0.7, Thermo Scientific). Pigments were identified based on commercially available pigment standards (DHI group), and normalized to the dry biomass used. The relative abundance of the individual carotenoid was calculated based on the total identified carotenoids during linear phase (phase II).

3.4 Metabolite extraction and derivatization

Extraction of metabolites from *Botryococcus braunii* strains was performed according to Doebbe et al (Doebbe et al., 2010). For cell harvesting, 50 mL of algal culture was spun down at 13500 rpm for 1 min (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). After centrifugation the biomass was immediately frozen in liquid N₂ and lyophilized. For extraction and derivatization of metabolites 10 mg of freeze-dried cell biomass was used. The mixture of 1 mL of 80% methanol (v/v) containing 10 µM ribitol (internal standard) and 300 mg of silica beads of 0.1 mm diameter (Roth GmbH, Germany) was used for cell disruption. Cell disruption was carried out using Precellys homogenizer (three times at 6,500 rpm for 45 s, Peqlab) and the mixture was centrifuged at 14,500 rpm for 20 min. The supernatant was then extracted leaving glass beads and cell debris behind. This supernatant was dried under the stream of N₂. The samples were then treated with 100 µl of methoxyl-amine hydrochloride (Sigma-Aldrich, Steinheim, Germany) in pyridine (20 mg/ml) (VWR International GmbH, Darmstadt, Germany) for 90 min at 37 °C while stirring. After the incubation, 30 µL of N-Methyl-N-(trimethylsilyl- lyl) trifluoroacetamide (Macherey & Nagel GmbH & Co. KG, Germany) was then added, and the mixture was incubated for 30 min at 37 °C while stirring (Barsch et al., 2004).

3.4.1 GC-MS instrument setting

The extracted metabolites were analyzed on GC/MS. For GC-MS analysis following protocol-settings were used. The analysis was performed with a TraceGC gas chromatograph and a ITQ ion trap mass spectrometer equipped with an AS2000 auto sampler (ThermoFinnigan, Dreieich, Germany). Sample volume of 1 µl was injected at 300 °C injector temperature without split. The gas chromatograph was equipped with a 30-m × 0.25-mm VF-5 ms column having a 0.25 µm 5% diphenyl and 95% dimethyl-siloxane coating (Varian Deutschland GmbH, Darmstadt, Germany). The interface temperature was adjusted to 250 °C and the ion source was set to 200°C. Helium carrier gas was set to a constant flow of 1 mL min⁻¹. Following 1 min of constant heating at 80°C, the oven temperature was raised gradually by 6 °C/min to 300 °C where it was held for 5 min. Mass spectra were recorded at 20 scans s⁻¹ with a scanning range of 50–750 m/z.

The evaluation of chromatogram was performed using *Xcalibur* software from ThermoFinnigan. Respective metabolites were identified by comparison with measured standards and by comparing them

with NIST 05 (National Institute of Standards and Technology) library and Golm metabolome data base (MPI Golm, Germany). All spectra were manually reviewed. The relative abundance was expressed as the percentage of total identified metabolites after normalization to the internal standard ribitol.

3.5 Total lipids and FAME determination

The biomass was harvested at different time points during the cultivation on day 6, day 15 and day 30, the algal cells were lyophilized and then stored at -80°C . The extraction of total lipids was carried out according to modified Folch method (J. Folch, 1957) with 2:1 ratio of chloroform (VWR International GmbH, Darmstadt, Germany) and methanol followed by washing with 3 mL of water. In detail, total lipids were extracted from 50 mg of homogenized dry cell biomass (30s at 6,500 rpm, Precellys 24, Peqlab, Erlangen, Germany). The homogenized sample was then transferred to Pyrex glass tubes (25 mL) and total lipids were extracted using 2:1 ratio of chloroform and methanol. (12 mL in total). The organic phase of chloroform and methanol containing total lipids was washed with 3 mL of water and centrifuged in order to separate the organic phase from the aqueous phase. Excessive washing with water was avoided in order to save the loss of total lipids. The organic phase was collected in a glass vial (7 mL) and organic solvents were evaporated using nitrogen gas. The remaining total lipids were then again dissolved into chloroform and transferred to pre-weighed small glass vials (2.5 mL) for the accurate measurement of total lipids. The extracted total lipids were measured gravimetrically and separated into two fractions as non-polar and polar lipids according to Li et al (Li et al., 2010b). In brief, the non-polar lipids were extracted using silica gel column chromatography using silica gel mesh (Merck Corp., Darmstadt, Germany). Total lipids were loaded onto silica column followed by chloroform eluting on-polar lipids and methanol eluting polar lipids. These separated fraction were collected into pre-weighed glass vials. These separated fractions were then trans-esterified in the presence of methanol and hydrochloric acid (10:1 v/v) for 2 hours at 80°C . The FAMES were separated using hexane and chloroform (4:1 v/v) and subjected to TraceGC gas chromatography coupled with ITQ ion trap mass spectrometer equipped with an AS2000 auto sampler (ThermoFinnigan, Dreieich, Germany). 50 μg C17- triacylglycerol (glyceryl triheptadecanoate, Sigma-Aldrich, Steinheim, Germany) was added to each sample as internal standard. The relative abundance shows the percentage of total identified fatty acids and hydrocarbons after normalization to the internal standard.

3.6 Total sugar/carbohydrates

Amount of total sugars was determined using whole culture broth and only in the supernatant according to Dubois et al (Dubois et al., 1956) using conc. H_2SO_4 and phenol (Roth GmbH, Germany). In brief, the whole culture containing cells and the supernatant and only the supernatant (without cells) was used for the determination of total carbohydrates. 100 μL of conc. H_2SO_4 was added into the acid resistant micro well

plate followed by the addition of 30 µL of ice-cooled samples. The cooling prevents violent boiling when aqueous sample is added to the sulfuric acid, it also increases the sensitivity and reproducibility of the assay. This step was followed by addition of 20 µL of aqueous 5% phenol sample well. The plate was incubated at >90 °C for 5 minutes into the oven (Heraeus instruments, Hanau, Germany). The micro well plate was then cooled on to ice for 5 min with additional incubation at room temperature in order to reach the temperature of reaction mixture at room temperature. The absorbance was measured at 490 nm using Infinite® 200 PRO series reader (Tecan Group Ltd, Switzerland).

3.6.1 Methanolysis and peracetylation of samples

Culture supernatant was frozen in liquid nitrogen and freeze dried. Then 100 µg were used for methanolysis in 0.5 M HCl / MeOH for 45 min at 85°C. After washing with methanol and drying under nitrogen flow, samples were peracetylated using pyridine:acetic anhydride 2:1 v/v for 30 min at 85°C. Samples were washed with chloroform and then taken up in 100 µL of chloroform for GC measurements.

3.7 Hydrocarbon extraction and analysis

Hydrocarbon extraction was performed according to Khatri et al (Khatri et al., 2013) with additional step of heating at 85°C in water bath since thermal pre-treatment increases the recovery of hydrocarbons (Eroglu and Melis, 2010). In brief, 1.5 to 3 mL of culture was mixed with equal volume of acetone and vortexed for 2-3 mins to lyse the algal cells. *n*-Hexane was added into the mixture and vortexed again for 2-3 mins followed by heating the mixture at 85°C in water bath for 5 mins. Thus the extract was centrifuged at 3020 rpm for 5 mins and upper organic phase was collected. This was repeated 2-3 times to ensure the maximum recovery of hydrocarbons. The extracted organic phase containing hydrocarbons was dried under N₂ and resuspend in 500 µL of *n*-hexane with internal standard. *n*-hexatriacontane (C₃₆H₇₂). GC-MS analysis was performed with the Thermo Scientific ITQ ion trap mass spectrometer equipped with an AS2000 auto sampler (ThermoFinnigan, Dreieich, Germany) followed by GC-FID for the quantification of hydrocarbons.

3.7.1 GC-FID analysis for quantification of extracellular hydrocarbons.

Samples were analyzed in a Shimadzu GC-2010 Plus unit with a FID detector operated with hydrogen gas and synthetic air (Linde Gas, quality 5.0) using a Macherey-Nagel OPTIMA FFAPplus column (30 m×0.25 mm ID, 0.40 mm OD and 0.50 µm film) with a 10 m pre-column (carwax-deactivated, 0.25 mm ID, 0.40 mm OD, Macherey-Nagel). The analysis was performed with a constant pressure of 160 kPa with helium as carrier gas and nitrogen as makeup gas (both Linde Gas, quality 5.0). The sample injection volume was 1 µL working in split mode (10). The method consisted of a temperature ramp from 55 to 232

°C at 6 °C min⁻¹ and to 250 °C at 3 °C min⁻¹. The final temperature of 250 °C was kept constant for 29,5 min. For identification of FAME, a Supelco® 37 Component FAME Mix (Sigma-Aldrich), containing FAME from C4:0 to C24:1n9 was used. For identification and quantification of all hydrocarbons external standards (alkane standard solution C21-C40, squalane C₃₀H₆₂ standard and squalene C₃₀H₅₀ standard, all from Sigma-Aldrich/Fluka) were used. All samples were injected twice to average the results.

3.8 Determination of C/N ratio

Total carbon (C) and nitrogen (N) content of the algal cell and whole culture biomass was determined via an element analyzer (VARIO EL III, Elementar Analysesysteme, Hanau, Germany) as described before (Platner et al., 2012)

3.9 Statistical analysis

A two-tailed Student's t-test was performed for statistical analysis, resulting in p-values indicated by asterisks ($p \leq 0.05 = *$, $p \leq 0.01 = **$). Results were shown as mean value, error bars represent standard deviations (\pm SD).

4 Results

Apart from the slow growth rate, variations across different races and strains belonging to the same race in terms of growth behavior, formation of product and biomass yield are some of the main challenges for commercial application of *B.braunii* (Brown et al., 1969; Talukdar et al., 2013). This led to the identification and characterization of novel strains of *B.braunii* which have the ability to perform despite their shortcomings. Fernandes and co-workers showed that, some of the *B.braunii* strains that can produce exopolysaccharides in large quantity were poor hydrocarbon producer (Fernandes et al., 1989). Thus, it was important to study the *B.braunii* race A and race B strains grown under similar physiological conditions to evaluate the distinct and shared features of these races and their product formation abilities. Therefore, the goal of the present study was to investigate the growth behavior, metabolome profile which included non-targeted primary metabolome analysis, intracellular lipid (FAME) content, photosynthetic pigments profile and to access the product formation abilities (exo-polysaccharides, hydrocarbons) of different strains of this alga to gain insights into their intracellular biochemical pathways. This would help to gain information on the differences between the different strains. Additionally, this study also investigated the changes in the metabolome profile during the different physiological state of the cells and its effect on the hydrocarbon and exo-polysaccharide accumulation.

The answers to the above queries will provide significant amount of knowledge and information that will serve as the basis for OMICS (Genome, transcriptome and proteome) based studies to understand the biology and biochemical pathways for production of hydrocarbons and polysaccharides in *B. braunii*. The results of the present study were divided into different sections based on the experimental parameters and comparison of the race A and race B strains with regards to these parameters.

4.1 Growth analysis of *B.braunii* race A strains CCALA 778, CCAP 807/2 and race B strain AC 761

4.1.1 Different growth stages based on total chlorophyll content and C/N ratio

In order to study the different physiological state of the *B.braunii* cells, growth analysis of all the three strains CCALA 778, CCAP 807/2 and AC 761 was determined using different parameters such as organic dry biomass and total chlorophyll content. The organic dry biomass (oDW) content of whole culture yielded $4.3 \pm 0.3 \text{ g L}^{-1}$, $3.3 \pm 0.04 \text{ g L}^{-1}$ and $3.1 \pm 0.2 \text{ g L}^{-1}$ of biomass for CCALA 778, CCAP 807/2 (both race A strains) and AC 761 (race B strain) respectively at the end of cultivation (day 30) (Fig 8A, 8B and 8C). Although all the strains differed in their organic dry biomass yields, they showed steady increase in their organic dry biomass weight from the beginning of the cultivation till the end.

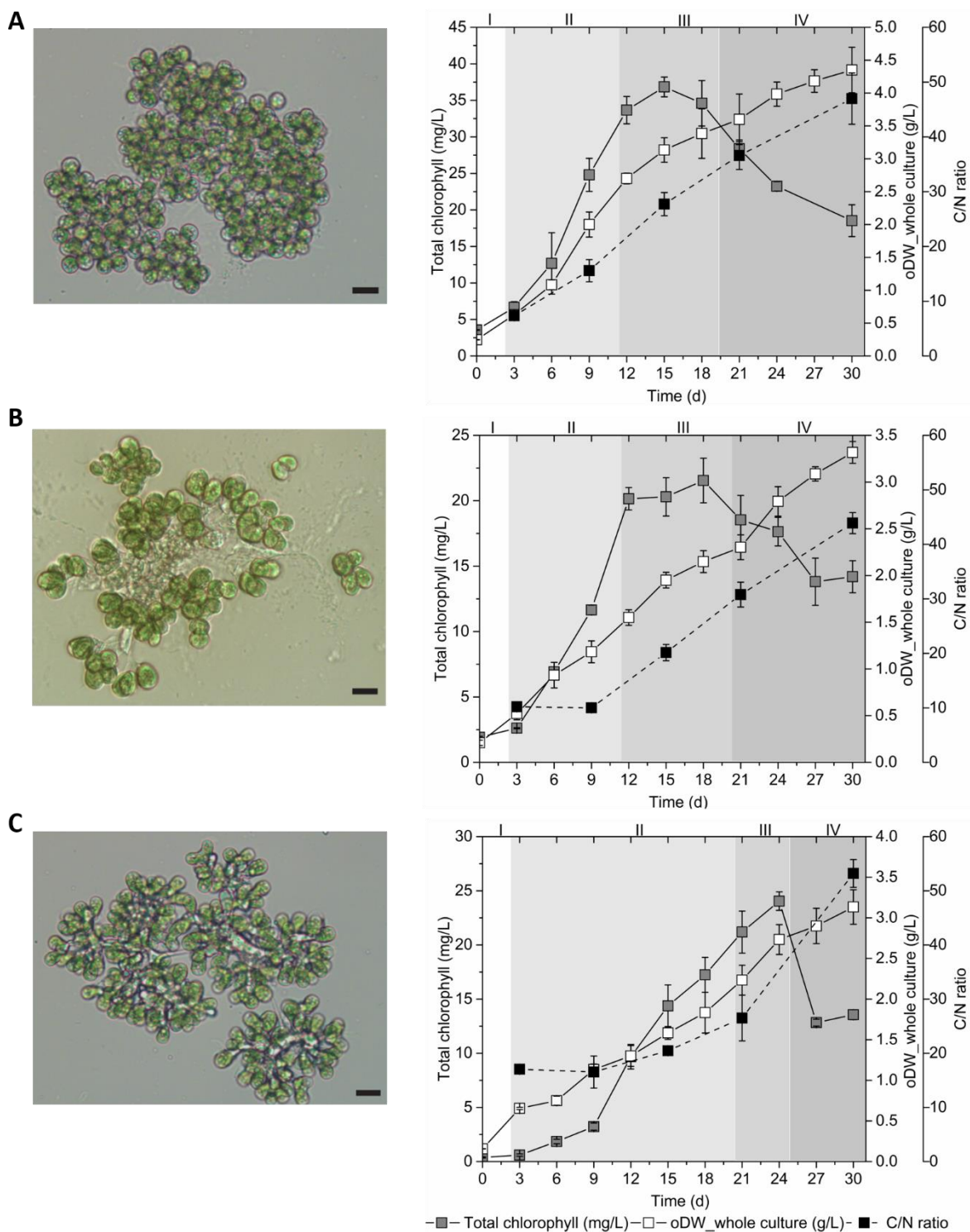


Figure 8 Growth characteristics of *B.braunii* race A and race B strains. Light microscope images and the measurement of total chlorophyll, organic dry biomass and C/N ratio of whole culture of *B.braunii* strains during the entire culture duration of 30 days **A)** CCALA 778 **B)** CCAP 807/2 and **C)** AC 761. Bar size 10 μ m. Numbers at the top of the graph (I to IV) represent different growth stages of *B braunii* cells based on the total chlorophyll content I – lag phase, II – linear phase, III – stationary phase and IV – decline phase. Error bars represent standard error of mean value of three biological and three technical replicates (SE; n=9).

Owing to the continuous increase in organic dry biomass during the cultivation, the measurement of organic dry biomass was insufficient to provide the understanding of the distinct growth stages or physiological states of *B.braunii*. Therefore, to demarcate the growth stages, in the next step, the total chlorophyll content was evaluated which is one of the markers for physiological and biochemical behavior of algal cells in the cultivation. The determination of chlorophyll content revealed that, for race A strains CCALA 778 and CCAP 807/2, except for the first three days which were considered as lag phase (phase I), the total chlorophyll content was significantly increased between day 3 to day 12. (Fig. 8A and 8B). This increase in the chlorophyll content was observed from $3.5 \pm 0.03 \text{ mg L}^{-1}$ to $33.6 \pm 1.8 \text{ mg L}^{-1}$ for CCALA 778 and from $2.6 \pm 0.03 \text{ mg L}^{-1}$ to $20.1 \pm 0.8 \text{ mg L}^{-1}$ for CCAP 807/2 from day 3 to day 12 respectively. This gradual increase in the total chlorophyll content indicates that the cells were in linear growth phase (phase II). After day 12, the total chlorophyll content nearly remained constant for both race A strains till day 18 of the cultivation and this was considered as a stationary phase (phase III). Following the stationary phase, the total chlorophyll content was decreased gradually from $34.5 \pm 3.1 \text{ mg L}^{-1}$ to $18.5 \pm 2.1 \text{ mg L}^{-1}$ for CCALA 778 and from $20.1 \pm 0.8 \text{ mg L}^{-1}$ to $14.1 \pm 1.2 \text{ mg L}^{-1}$ for CCAP 807/2 by the end of the cultivation (Fig. 8A and 8B). Thus, the progressive reduction in the chlorophyll content indicated the decline phase (deceleration phase, phase IV)). However, unlike in race A strains, the total chlorophyll content in race B strain AC 761 was increased from $0.3 \pm 0.05 \text{ mg L}^{-1}$ on day 3 to $24.1 \pm 0.3 \text{ mg L}^{-1}$ on day 24 (Fig. 8C) indicating a more extended linear growth phase compared to race A strains. Thus, the results from this study showed that race A and race B have distinct growth behavior based on their total chlorophyll content.

The detailed analysis of chlorophyll content in race A and B also revealed that, the race A strain CCAP 807/2 had a linear growth phase (phase II) spanning from day 3 till day 12, wherein, continuous increase in its total chlorophyll content with the rate of $1.95 \text{ mg L}^{-1} \text{ day}^{-1}$ was observed (Fig.8B). However, race B strain AC 761 displayed slower rate of accumulation of total chlorophyll with $0.44 \text{ mg L}^{-1} \text{ day}^{-1}$ in its initial period of cultivation from day 3 to day 9 which was elevated by ~ 3-fold between day 9 to day 24 to $1.38 \text{ mg L}^{-1} \text{ day}^{-1}$ (Fig.8C). This implies that race B displayed slow growth at the beginning of the linear phase but its growth was promoted from mid of linear phase (day 9) onwards.

Even though the growth behavior of the race A strains, CCALA 778 and CCAP 807/2 was similar in terms of different growth stages (lag phase, linear growth phase, stationary phase and decline phase), the total chlorophyll content and organic biomass yields were different for these strains. The strain CCALA 778 produced 24% of more organic dry biomass than CCAP 807/2 at the end of culture duration of 30 days. Besides, the biomass productivity during the linear phase was $231 \text{ mg L}^{-1} \text{ day}^{-1}$ for CCALA 778, whereas, it was $114 \text{ mg L}^{-1} \text{ day}^{-1}$ for CCAP 807/2. This difference was also reflected from the maximum total chlorophyll content of both the strains, as it was 41% more in CCALA 778 compared to CCAP 807/2

(Fig.8A and 8B). Taken together, the growth analysis of race A strains showed similar growth behavior in terms of different growth stages, however, they differed significantly in their organic biomass yield with CCALA 778 showing the highest biomass yield.

4.1.1.1 Cellular elemental composition (C/N ratio) of race A and race B strains

The cellular elemental composition of algal culture changes as the cultivation progresses and it has been shown that elemental composition tends to vary with the specific growth rate of algae (Yoshimura et al., 2013). Carbon and nitrogen ratio (C/N ratio) is an indicator of physiochemical state of algal cells in the cultivation since carbon is an energy compound and nitrogen is essential nutrient for the growth. It is well known that under nutrient replete conditions, microalgal cells showed C/N ratio between the range of 5 to 9 (Oh-Hama, T., Miyachi, S., 1988) (Yen and Brune, 2007). At the beginning of the cultivation, the measured C/N ratio for *B.braunii* race A strains CCALA 778 and CCAP 807/2 did not show significant differences. While the C/N ratio in CCALA 778 was measured to be 7.3 ± 0.2 , it was 10.1 ± 0.3 for CCAP 807/2 (Fig.8A and 8B). Contrasting to the race A strains, the race B strain AC 761 revealed a higher C/N ratio of 17 ± 0.03 at the start of the cultivation. A difference in the onset of the linear phase was observed in race A strains. While CCALA 778 displayed a continuous increase in their C/N ratio throughout the linear phase, the C/N ratio was nearly constant for CCAP 807/2 till the late linear and early stationary phase. Therefore, the gradual increase in the C/N ratio for CCALA 778, from initial days of cultivation indicates that CCALA 778 cells were producing carbon compounds in the form of polysaccharides and/or hydrocarbons as well as lipids (Fernandes et al., 1989; Metzger et al., 1985). However, for CCAP 807/2, the C/N ratio increased only from 10.0 ± 0.18 in late linear (day 9) phase to a value of 43 at the end of cultivation (day 30) indicating the nitrogen limitation from day 9 onwards with a simultaneous increase in the carbon storage compounds. The measured C/N ratio for race B strain AC 761 was constant from the beginning of linear phase on day 3 to day 9; however, as the cells approach the mid-linear phase from day 9 onwards, the C/N ratio increased gradually to reach a value of 26.5 ± 4.2 by the end of linear phase on day 21. At the end of the cultivation, CCALA 778 and CCAP 807/2 showed high values for C/N ratio with 46.9 ± 4.6 and 43.9 ± 1.9 respectively. Furthermore, in race B strain, a proximate 2-fold increase in the C/N ratio from day 21 to day 30 (from early stationary phase to the end of the cultivation) indicates the higher accumulation of carbon at the later stages of the cultivation on day 30. The simultaneous rapid decrease in the overall nitrogen content is indicative of its consumption for promoting growth. Altogether, the measurement of C/N ratio supports the different growth stages based on the chlorophyll content. Additionally, the exponential increase in the C/N ratio during stationary and decline phase also indicates that nutrient limitation may have triggered the accumulation of carbon compounds in *B.braunii* in the form

of polysaccharides, lipids and hydrocarbons, as described earlier for other microalgae (Hu et al., 2008; Procházková et al., 2014).

4.2 Non-targeted metabolome profile of *B.braunii* race A and B strains

The non-targeted metabolome profile provides an overall response of the *B. braunii* cells to the changing nutrient availability in the medium during different growth stages. The physiological state of the cells plays an important role in the formation of metabolites as they represent an integrated state of genetic and environmental factors and therefore it is important to study the effect of different growth stages on primary as well as secondary metabolism. The non-targeted primary metabolome analysis was performed using GC-MS and methanol as an extraction solvent resulting in the number of peaks during the different growth stages. The comparison of race A strains CCALA 778 and CCAP 807/2 and the race B strain AC 761 for the detected peaks and identified peaks is shown in the table 1.

Table 2 Summary of the peak detection obtained from non-targeted metabolome analysis via GC-MS during the different growth stages in *B.braunii* race A and B strains. Peaks were identified by comparison with the NIST 05 library (Lib) (ThermoFinnigan), the Golm Metabolome Database (GMD, MPI, Golm, Germany) and verified with purified standards.

	CCALA 778		CCAP 807/2		AC 761	
Growth stages	Total detected peaks	Identified peaks	Total detected peaks	Identified peaks	Total detected peaks	Identified peaks
Phase II	68	33	108	39	66	32
Phase III	106	37	87	26	--	--
Phase IV	86	32	83	15	73	40

4.2.1 Sucrose as the most abundant metabolite in race A strains but not in race B strain

The non-targeted metabolome profile of race A strains CCALA 778 and CCAP 807/2 in this present study showed that sucrose was the most abundant metabolite compared to all the other metabolites. The relative abundance of sucrose in CCALA 778 was $32.7 \pm 1.1\%$ of all the identified metabolites in the linear phase (phase II), which was elevated at the later stages of cultivation to $46.3 \pm 5.5\%$ in the stationary phase (phase III) and finally reached to $47.3 \pm 3.9\%$ by the time the cells entered the decline phase (phase IV; the end of cultivation) (Fig. 9A). Likewise, CCAP 807/2 showed higher sucrose abundance with $31.0 \pm 0.6\%$ in the linear phase (phase II) which was further increased with the progressing stages of cultivation (Fig.9B). Therefore, this results showed sucrose as the most abundant metabolite in race A strains at any growth stage during the entire culture duration. Besides sucrose, raffinose was the other common sugar compound

detected in both race A strains. The relative abundance of raffinose in CCALA 778 increased from $3.0 \pm 0.7\%$ at the linear phase (phase II) to $12.7 \pm 3.2\%$ in the stationary phase (phase III) and finally reached to $16.3 \pm 3.0\%$ in the decline phase (phase IV). At the same time, CCAP 807/2 showed a marginal increase in the relative abundance of raffinose from $7.0 \pm 0.8\%$ to $10.9 \pm 0.1\%$. In addition, the metabolites related to the carbohydrate metabolism revealed differences between race A strains with regard to the relative abundance of α,α -trehalose. This sugar compound was present in lower abundance ($0.1 \pm 0.05\%$) in CCALA 778 than CCAP 807/2 ($7.7 \pm 0.3\%$). Some of the metabolites related to the carbohydrate metabolism were decreased during the cultivation in both race A strains. These included glycerol-3-phosphate which was reduced by 10-fold from linear phase (phase II) to decline phase (phase IV) in CCALA 778 and approximately 2-fold in CCAP 807/2. (Fig.9A and 9B). Moreover, other sugar compounds such as glucose and maltose present in less abundance were also further reduced as the cultivation progressed in CCALA 778 and CCAP 807/2. Apart from these sugar compounds, glycolysis intermediates such as fructose-6-phosphate, glucose-6-phosphate, galactose and glycerate 2-phosphate were detected at very low levels in CCAP 807/2 during the linear growth stage (phase II).

Interestingly, the metabolome analysis of race B strain AC 761, unlike race A strains, revealed very low abundance of metabolites related to carbohydrate metabolism. In addition, these metabolites did not show any significant changes in their abundance from linear phase (phase II) to decline phase (phase IV). The presence of sucrose in higher abundance in race A strains against very low abundance in race B strain highlights the distinct primary metabolome profile of different races in *B.braunii*. This difference was further evident from the result that show sucrose as the most abundant metabolite consisting of $47.3 \pm 3.9\%$ and $34.7 \pm 1.0\%$ of all the metabolites in race A strains CCALA 778 and CCAP 807/2 respectively during the linear growth stages, however, it was measured to be only $0.3 \pm 0.1\%$ in race B strain AC 761. In addition to sucrose, maltotritol, raffinose and α,α -trehalose were the other sugar compounds which were less abundant but present only in race A strain CCAP 807/2 and not in race B strain AC 761. However, similar to race A strains, the relative abundance of glycerol-3-phosphate was decreased from 5.7% in the linear phase to 4.2% at the decline phase (Fig.9C). Therefore, together these results primarily indicate that metabolites related to carbohydrate metabolism were less abundant in race B strain compared to race A strains.

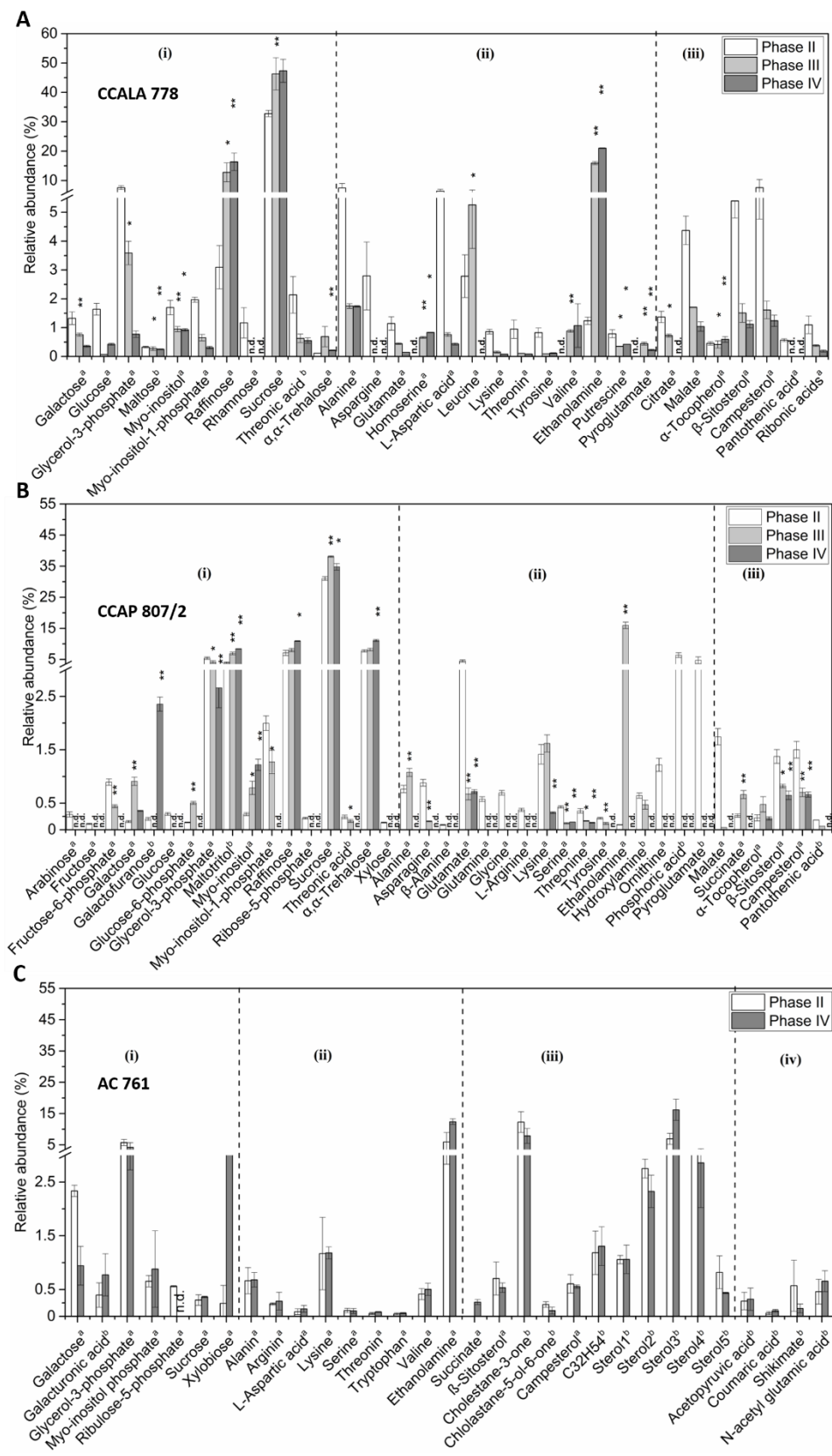


Figure 9 Non-targeted primary metabolome profile of *B.braunii* race A and race B strains. The relative abundance of all the identified metabolites during the different growth stages for **A) CCALA 778 B) CCAP 807/2 and C) race B AC 761**. The identified metabolites were divided into different categories based on their related pathways. These categories are represented in the graph as i) the relative abundance of metabolites related to glycolysis intermediates, sugars and sugar alcohol ii) represents amino acids and related metabolite such as ethanolamine iii) represents the relative abundance of terpenoids, sterols and hydrocarbons and the metabolites related to citric acid cycle intermediates, and iv) represents compounds which are only detected in AC 761 such as coumaric acid, shikimate, N-acetyl glutamic acid. The relative abundance shows the percentage value of total identified metabolites after normalization to the internal standard. Phase II represents linear growth stage, Phase III represents stationary phase and Phase IV represents decline phase. Sterol1- ergosta-5,7,22-trienol, Sterol2- cycloart-24(25)-enol, Sterol3- 4,4-dimethyl-5 α -cholesta-8en- β ol, Sterol4- ergosterol and sterol5- Unknown-C28-sterol Metabolites, marked with (superscript) “a” were identified by comparison with purified standards and the NIST 05 library (Lib) (ThermoFinnigan), the Golm Metabolome Database (GMD, MPI, Golm, Germany) and, the metabolites which are identified only via above mentioned databases with RSI value above 750 and mass spectral analysis are shown with (superscript) “b”. The error bars indicate standard deviation (\pm SD, n= 6). Asterisk represents p-values as determined using Student’s t-test (* = < 0.05, ** = < 0.01). n.d.=not detected.

4.2.2 Decrease in the amino acids levels from linear to decline phase in race A strains

In addition to the metabolites involved in the carbohydrate metabolism, the non-targeted metabolome analysis in this study also detected amino acids. In both race A strains, most of the detected amino acids showed reduction in their relative abundance from linear phase (phase II) to decline phase (phase IV). The N-rich amino acids such as glutamate and asparagine showed significant decrease in their respective abundances (Fig. 9A and 9B). The relative abundance of glutamate was decreased from $1.14 \pm 0.2\%$ in linear phase (phase II) to $0.14 \pm 0.005\%$ in decline phase for CCALA 778 and was from $4.48 \pm 0.4\%$ to $0.71 \pm 0.003\%$ for CCAP 807/2 during the same duration. Therefore, this reduction in their levels may suggest the decrease in amino acid synthesis due to the resulting nutrient depletion especially nitrogen in the growth medium. Thus, this supports the above observations for the distinct growth phases of *B.braunii* strains based on the total chlorophyll content. Furthermore, the reduced level of amino acids could mean the re-partitioning of the nitrogen (via amino acid catabolism) to the cellular functions which are most essential to maintain the cell viability. This study also showed ethanolamine as one of the most abundant compound identified among the amino acids and related compounds for both race A strains CCALA 778 and CCAP 807/2 and race B strain AC 761. It showed a significant increase in its abundance from $1.2 \pm 0.1\%$ during the linear growth phase (phase II) to $21.0 \pm 0.12\%$ at decline phase (phase IV) for CCALA 778 (Fig. 9A, ii). In CCAP 807/2, ethanolamine was increased drastically from $0.09 \pm 0.004\%$ to $15.9 \pm 1.0\%$ from linear (Phase II) to stationary phase (Phase IV) (Fig. 9B, ii). Similarly, AC 761 also displayed increased abundance of ethanolamine from $5.9 \pm 3.0\%$ in linear phase to $12.0 \pm 0.9\%$ at the decline phase.

4.2.3 Decrease in TCA cycle intermediates and sterols

Furthermore, this study also evaluated the response of citric acid cycle intermediates such as citrate, malate and succinate using primary metabolome analysis. In comparison to the other previously mentioned

metabolites, these intermediates were detected in very low abundances. Apart from succinate, these intermediate metabolites showed a decrease in their relative abundances with progressing cultivation. For instance, in CCALA 778, the relative abundance of citrate was $1.3 \pm 0.2\%$ during the linear phase (phase II) which was later reduced by almost half to $0.7 \pm 0.05\%$ in the stationary phase (Fig 9A, iii), however, it was not detected in the decline phase. Unlike CCALA 778, citrate could not be detected in CCAP 807/2 (Fig 9B, iii). In addition to citrate, the relative abundance of malate also decreased from $4.3 \pm 0.5\%$ in linear phase to $1.7 \pm 0.07\%$ at the stationary phase with a further marginal decrease to $1.03 \pm 0.16\%$ by the time the cells were in the decline phase in CCALA 778. Similarly, CCAP 807/2 also showed a reduction in the abundance of malate from $1.7 \pm 0.15\%$ in linear phase (phase II) to 0.04% in the stationary phase (phase III). Succinate was not detected in CCALA 778, however, it was increased by 1.3-fold in its stationary phase (phase III) compared to linear phase (phase II) (Fig. 9B, iii) in CCAP 807/2. Overall, these results showed a decline in the citric acid cycle intermediates at the stationary phase and they remained suppressed in the decline phase. However, the primary metabolome analysis of race B strain AC 761 did not detect citrate and malate although, succinate was present in low abundance in the decline phase.

Additionally, the metabolome analysis in this study also revealed the presence of two sterols, namely β -sitosterol and campesterol in CCALA 778 and CCAP 807/2. Both sterols showed a decrease in abundance from linear phase (phase II) to decline phase (phase IV). β -sitosterol was decreased from $5.3 \pm 0.6\%$ in linear phase (phase II) to $1.1 \pm 0.1\%$ during decline phase (phase IV) in CCALA 778 (Fig.9A, iii) whereas it reduced from $1.4 \pm 0.1\%$ to $0.6 \pm 0.08\%$ in the same culture duration for CCAP 807/2 (Fig. 9B, iii). Similarly, campesterol was lowered from $7.5 \pm 2.7\%$ to $1.2 \pm 0.2\%$ and $1.5 \pm 0.1\%$ to $0.6 \pm 0.04\%$ for CCALA 778 and CCAP 807/2 respectively. Both these sterols were marginally reduced in race B strain AC 761. Of note, the relative abundance and the number of different sterols present in the race B strain differed from race A strains. For example, the relative abundance of sterols such as cholestane-3-one and 4,4-dimethyl cholesta-8-en-3 β -ol (sterol3) was higher in race B strain AC 761 and both these sterols were only detected in race B strain. However, the influence of different growth stages on these sterols was not evident. Additionally, the presence of a hydrocarbon such as $C_{32}H_{54}$ was detected in very small abundance (Fig.9C).

The presence of vitamin E and B family members such as α -tocopherol and pantothenic acid was also observed in the metabolome analysis in this study. α -tocopherol contributes to the integrity of photosynthetic membrane and also influences the response to photo-oxidative stress in *C.reinhardtii* (Trebst et al., 2002). A marginal increase in α -tocopherol from linear phase to stationary phase was observed for both race A strains. In contrast, pantothenic acid showed decreased abundance and was not detected during the decline phase of CCALA 778 and CCAP 807/2 (Fig.9A iii, and 9B iii). However, α -tocopherol and pantothenic acid were not detected at all in race B strain AC 761 in its metabolome profile.

Collectively, the non-targeted metabolome analysis of race A strains in the present study revealed the prevalence of higher abundance of metabolites related to carbohydrate metabolism where sucrose was prominent. The high abundance of sucrose in both the strains indicate its important role in the carbohydrate metabolism of race A strains. In addition, the observed decrease in nitrogen rich amino acids such as asparagine and glutamine suggests that the cells were under nitrogen depletion. The elevation in the abundance of ethanolamine at the later stages of cultivation hints toward its indirect involvement in the fatty acid biosynthesis. Moreover, both race A strains displayed lower abundances of sterols. Interestingly, in contrast to race A strains, the race B strain AC 761 however showed higher abundance of sterols including the presence of different sterols which were not detected in race A strains, but with lower amounts of metabolites related to carbohydrate metabolism.

4.3 Photosynthetic pigment profile of *B.braunii* race A and race B strains

Studies in the past have shown that the increase in the amount of carotenoids was relative to the chlorophyll content (Grung et al., 1994). Therefore, the next aim of the study was to determine the amount of carotenoids in order to assess the relation between carotenoids content and the different physiological state of the cells. The analysis showed that the total amount of carotenoids in CCALA 778 increased continuously from 0.58 ± 0.04 mg L⁻¹ in lag phase (phase I; day 1) to 7.50 ± 0.69 mg L⁻¹ by the end of stationary phase (phase III; day 18). Though the total carotenoid content measured was highest at the end of the stationary phase, it was reduced thereafter gradually to 3.4 ± 0.55 mg L⁻¹ as on day 27 (Fig.10A). Similarly, the total carotenoid in CCAP 807/2 showed a gradual increase from lag phase (phase I; day1) reaching to a maximum of 4.1 ± 0.19 mg L⁻¹ on day 18, the end of stationary phase (Fig.10B). As the cells entered the decline phase, there was a progressive decrease in the amount of total carotenoids which finally reached to 2.2 ± 0.15 mg L⁻¹ as on day 30. Comparably, the total carotenoid content of race B strain AC 761 was lower than race A strains. The maximum total carotenoid content of 3.8 ± 0.4 mg L⁻¹ was observed at the start of stationary phase (phase III) on day 21 which was followed by a gradual decrease to 2.3 ± 0.1 mg L⁻¹ by the end of the cultivation (Fig.10C).

Taken together, the analysis for the presence of total carotenoids in this study showed that the race A and race B strains significantly differ from each other. While a sustained increase in the total carotenoid content until the end of stationary phase was observed for both race A strains, however, for race B, the total carotenoid content was increased only until the end of linear phase.

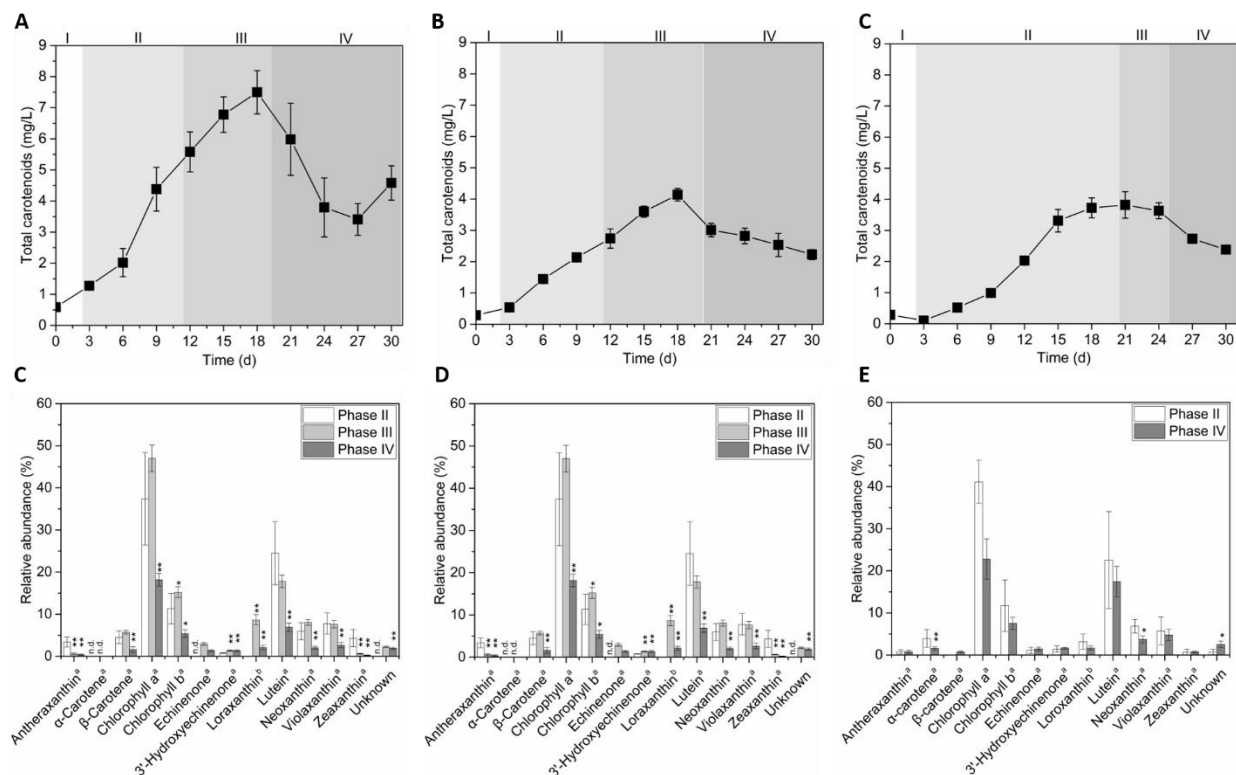


Figure 10 The analysis for total carotenoids and the intracellular pigment profile of *B. braunii* race A and B strains. The measurement of the total carotenoid content using Wellburn's coefficient constant for **A**) CCALA 778, **B**) CCAP 807/2 and **C**) AC 761. The pigment profile of the intracellular carotenoids determined using UV-HPLC for **D**) CCALA 778 **E**) CCAP 807/2 and **F**) AC 761. The relative abundance of intracellular pigments normalized to the total carotenoids at phase II at different growth stages. Phase II represents linear growth stage, Phase III represents stationary growth stage and Phase IV represents decline phase. Numbers at the top of the graph (I to IV) represent different growth stages of *B. braunii* cells, I – lag phase, II – linear phase, III – stationary phase and IV – late stationary (decline phase) during the cultivation **B**) Superscript “a” and “b” shows the identified metabolites using standards. The error bar represents standard deviation (+SD, n=9). Asterisk represents p-values as determined using Student's t-test (* = < 0.05, ** = < 0.01). n.d.=not detected.

The pigment profiling in this study showed the intracellular pigment profile of race A and race B strains to be similar (Fig.10D,10E and 10F). This pigment profile was composed of antheraxanthin, β -carotene, chlorophyll a, chlorophyll b, echinenone, 3'-hydroxyechinenone, loroxanthin, lutein, neoxanthin, violaxanthin and zeaxanthin. From all the identified pigments, chlorophyll a was the most abundant at every growth stage, followed by chlorophyll b and lutein in all the three strains (Fig.10D,10E and 10F). Furthermore, a significant reduction was observed for all the primary pigments such as loroxanthin, neoxanthin and violaxanthin during the decline growth stage (day 30) when compared to the linear phase (phase II). Notably, echinenone, an interesting carotenoid reported to have a light protective function in *B. braunii* (Grung et al., 1994) showed an increase in its abundance from linear to decline phase in all three strains of *B. braunii* used in this study. Lutein, being the next most abundant pigment after chlorophyll a and b in all the strains was further quantified. The quantification of lutein revealed that the race B strain

AC 761 produced the highest amount of lutein $2.8 \pm 0.3 \text{ mg g}^{-1}$ of dry biomass (Fig.11). This was followed by race A strain CCAP 807/2 which had $2.1 \pm 0.3 \text{ mg g}^{-1}$ of biomass of lutein whereas CCALA778 showed 1.1 mg g^{-1} of biomass of lutein content during the linear phase (phase II). However, this lutein content was decreased at the later stages of cultivation i.e. the stationary and decline phases (Fig.11).

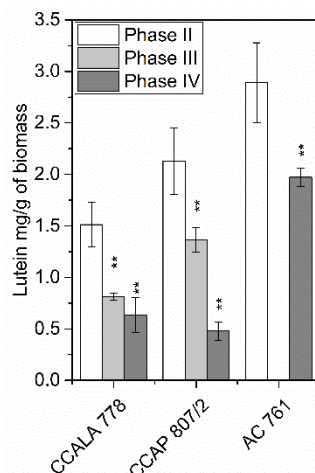


Figure 11 Comparison of lutein content in *B.braunii* race A and race B strains. Phase II represents linear growth stage, Phase III represents stationary growth stage and Phase IV represents decline phase. The error bar shows standard deviation (+ SD, n=9). Asterisk represents p-values as determined using Student's t-test (* = < 0.05, ** = < 0.01).

Together, the results from this study for the intracellular pigment profile of *B.braunii* race A and race B strains showed that the pigment profile in the strains CCALA 778, CCAP 807/2 (both race A) and AC 761 (race B) did not differ significantly. Changes in the abundance of pigments have shown to be dependent on the physiological state of the alga. In addition, most of the pigments except echinenone were decreased in this study during the later stages of cultivation for the three strains. Thus, it seems plausible that the changes in the relative level of carotenoids may be reflective of the different physiological state of the cells.

4.4 Intracellular lipids and fatty acid methyl ester profile of *B.braunii* race A and B strains

4.4.1 Higher intracellular lipid content in race B strain than race A strains

In order to study the influence of different growth stages on the total lipid content in both race A and race B strains, three time-points were selected during different growth stages. The total lipid content was measured gravimetrically and expressed as percentage of dry biomass weight.

The analysis of total lipid content did not show significant increase in the amount of total lipids in race A strains. The total lipid content for CCALA 778 in the linear phase (phase II; day 6) was $10.7\% \pm 0.8\%$, which remained constant at stationary (phase III; day 15), and decline phase (phase IV; day 30) with $10.4\% \pm 1.6\%$ and $10.7\% \pm 1.7\%$ respectively (Fig.12A). Similar to CCALA 778, the evaluation for the total lipid

amounts in the other race A strain CCAP 807/2 revealed only a marginal increase from $21.2\% \pm 1.9\%$ at linear phase to $23.3\% \pm 3.0\%$ in the decline phase (Fig.12B). Thus, the obtained results showed that the total lipid content did not change significantly at different growth stages for race A strains. However, it was evident from this analysis that CCAP 807/2 had nearly 10-fold higher lipid content than CCALA 778. Noteworthy is the higher total lipid content observed in the race B strain AC 761 in comparison to race A strains. During the linear phase (phase II), the total lipid content for AC 761 was $36.8\% \pm 3.5\%$, which was later increased at the decline phase (phase IV) to $42.0\% \pm 3.9\%$ (Fig.12C).

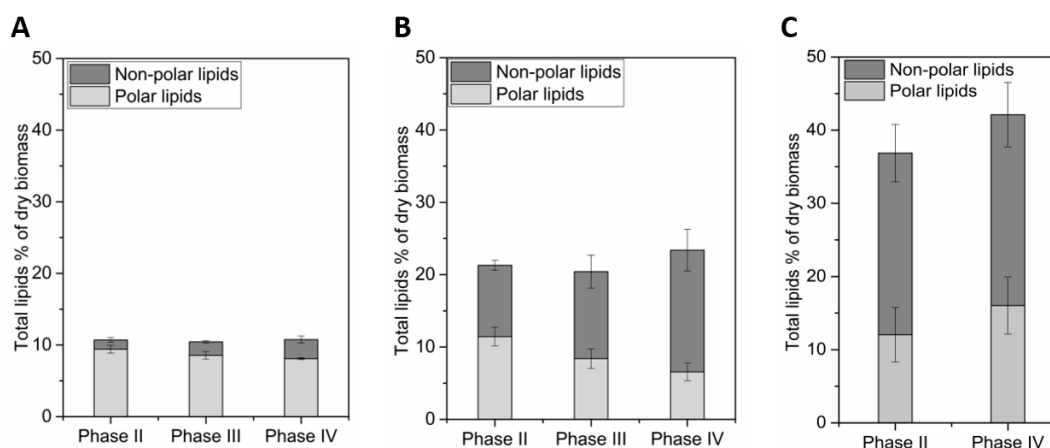


Figure 12 The total lipid content of *B. braunii* race A and race B strains. The total lipid content comprised of polar and non-polar lipid fractions for race A strains **A)** CCALA 778 **B)** CCAP 807/2 and **C)** race B strain AC 761. The total lipid content was measured gravimetrically and expressed as the percentage of dry biomass. Phase II represents linear growth stage, Phase III represents stationary growth stage and Phase IV represents decline phase. The error bar represents standard deviation (\pm SD, n=9).

The total lipids were further divided into polar and non-polar fractions. While the polar lipids were more prominent in the total lipid content of CCALA 778, the non-polar lipids formed only a small part of the total lipid content. In fact, the non-polar lipids were measured to be less than 2% of the total dry biomass at each growth stage of CCALA 778 (Fig.12A). Contrary to CCALA 778, the non-polar lipids of CCAP 807/2 were significantly increased from $9.8\% \pm 0.6\%$ of total biomass in the linear phase (phase II) to $11.4\% \pm 2.2\%$ at the stationary phase (phase III) with a further elevation to $16.8\% \pm 2.8\%$ by the end of cultivation (decline phase) (Fig.12B). In whole, there was an increase of 1.7-fold from linear phase (phase II) to decline phase (phase IV). The overall non-polar lipid productivity for CCAP 807/2 during the stationary phase (phase III) was $3.5 \text{ mg g}^{-1} \text{ day}^{-1}$ which increased to $4.9 \text{ mg g}^{-1} \text{ day}^{-1}$ throughout the decline phase (phase IV). Unlike, the non-polar lipids, the polar lipid content for CCAP 807/2, however was decreased from $11.4\% \pm 1.2\%$ in the linear phase (phase II) to $6.5\% \pm 1.2\%$ at the decline phase (phase IV). Taken together, this study has revealed that race A strains differed significantly in their total lipid contents with CCAP 807/2 producing a 2-fold total lipid content than CCALA 778. Additionally, CCALA 778

showed less than 2% of non-polar lipids which was nearly 10-fold less than non-polar lipid content of CCAP 807/2.

Unlike the two race A strains, the race B strain AC 761 showed a higher percentage of non-polar lipids at every growth stage. In comparison to the polar lipid content which was $12.03\% \pm 3.7\%$ during the linear phase (phase II), the non-polar lipid content was $24.8\% \pm 3.2\%$, nearly 2-fold more than polar lipids. Conversely, AC 761 did not show a drastic increase in its non-polar lipid content which was increased marginally to $26.0\% \pm 4.4\%$ in the decline phase (phase IV). Besides, the polar lipid content also showed only a slight increase with an amount of $16.0\% \pm 3.9\%$ in its decline phase (phase IV) (Fig.12C).

Altogether, the intracellular lipid contents as measured in this study revealed the race B strain AC 761 to produce and accumulate more lipids than race A strains. Additionally, the race A strain CCAP 807/2 produced higher amounts of total lipids than CCALA 778.

4.4.2 Variations in fatty acid methyl ester (FAME) profile of *B.braunii* race A and race B strains

The next aim was to evaluate the composition of the polar and non-polar lipids and therefore these lipids were esterified to fatty acid methyl esters via transesterification process. The FAME profile derived from polar lipids of race A strains CCALA 778 and CCAP 807/2 revealed palmitic acid (C16:0) and oleic acid (C18:1n9c) to be the most abundant fatty acids (Fig.13A and Fig.13C). Interestingly, the FAME profile derived from polar lipids exhibited the presence of very long chain fatty acids (VLCFAs). These VLCFAs included octacosenoate (C_{28:1}), octacosadienoate (C_{28:2}) and tricosenoate (C_{30:1}). Although, when compared to the levels of the overall polar lipids, these VLCFAs were low in abundance. However, they showed an increase in their abundances over the period of cultivation in both strains of race A. The FAME profile derived from polar lipids of race B strain AC 761 also displayed palmitic acid (C16:0), Oleic acid (C18:1n9c) and very long chain fatty acid octacosenoate (C28:1) as their most abundant fatty acids (Fig.13E). The race B strain AC 761 did not show any significant changes in the FAME profile of polar lipids from linear to decline phase. Intriguingly, all the strains in this study showed an increased abundance for fatty acid octacosenoate (C28:1) at the later stages of cultivation than the linear phase.

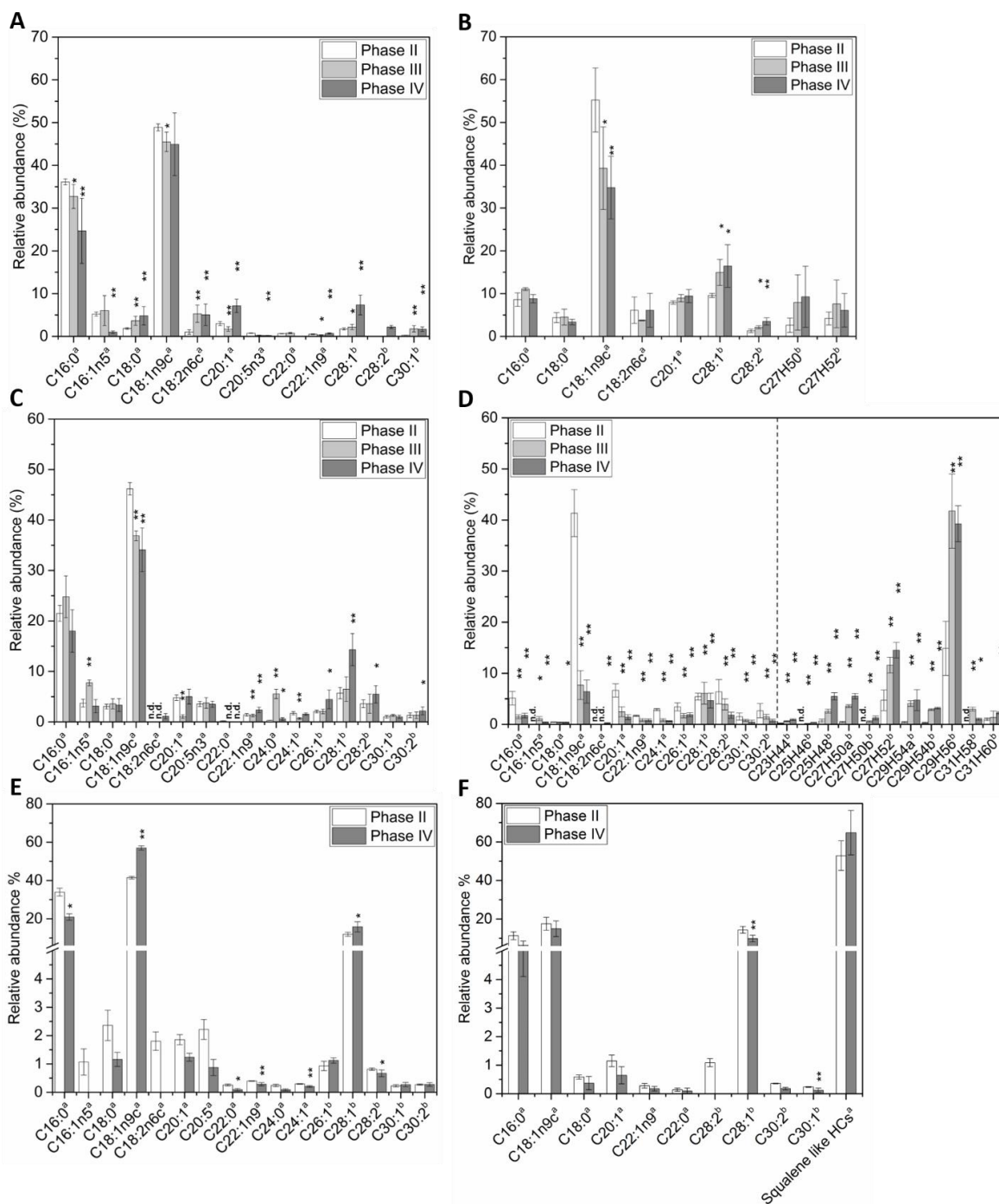


Figure 13 FAME profile of *B. braunii* race A and race B strains. FAME profile derived from polar lipids of **A)** CCALA 778 **C)** CCAP 807/2 and **E)** AC 761. FAME profile derived from non-polar lipids of **B)** CCALA 778 **D)** CCAP 807/2 and **F)** AC 761. Phase II represents linear growth stage, Phase III represents stationary growth stage and Phase IV represents decline phase. Superscript “a” and “b” shows the identified fatty acids using purified standards. The error bar represents standard deviation (\pm SD n=9). Asterisk represents p-values as determined using Student’s t-test (* = < 0.05, ** = < 0.01) n.d.=not detected.

Strikingly, the FAME profile derived from the non-polar lipid fraction composed of both fatty acids as well as hydrocarbons. One of the reason for the presence of the hydrocarbons was that they cannot be derivatized.

The detailed evaluation of the FAME profile derived from non-polar lipids of CCALA 778 comprised of palmitic acid (16:0) and oleic acid (18:0) as major fatty acids. Moreover, the presence of hydrocarbons such as $C_{27}H_{52}$ (heptacosadiene) and $C_{27}H_{50}$ (heptacosatriene) was also detected in very low abundance (Fig. 13B) in CCALA 778. These hydrocarbons were only a very small part of total non-polar lipid fraction, however, it is worth to note that the relative abundance of these hydrocarbons increased proximately by 2-fold from linear phase (phase II) to decline phase (phase IV) (Fig. 14A). However, for CCAP 807/2, its FAME profile of non-polar lipids was changed drastically. During the linear phase (phase II), fatty acids were the most abundant accounting to 72% of its total derivatized non-polar lipids. However, the relative abundance of fatty acids was decreased significantly as the cells entered into the stationary phase (phase III) amounting to 27% and 21% at the end of cultivation (phase IV) (Fig. 14B). Conversely, the relative abundance of hydrocarbons in the derivatized non-polar lipid fraction was increased from 28% in the linear phase (phase II) to 73% in the stationary phase (phase III) and even increased further to reach at 78% at the end of the cultivation (decline phase, Phase IV) (Fig. 14B). This was nearly 2.6-fold increase in the relative abundance of hydrocarbons in the non-polar lipid fraction. The thorough analysis of this derivatized non-polar lipid fraction revealed that the abundance of oleic acid was decreased significantly from 41.3% at the linear phase (phase II) to 7.6% in the stationary phase (phase III) with further reduction in its abundance to reach at 6.4% by the end of the cultivation (decline phase; phase IV) (Fig. 14B). Concurrent to this drastic decrease, the abundance of all the hydrocarbons was elevated from linear to decline phase. The most prominent increase was observed for $C_{29}H_{56}$ (nonacosadiene) followed by $C_{27}H_{52}$ (heptacosadiene) alkadienes. Apart from these hydrocarbons, the derivatized non-polar lipid profile also composed of alkatrienes such as $C_{27}H_{50}$, $C_{25}H_{46}$ and few smaller chain alkadienes like $C_{25}H_{48}$ and $C_{23}H_{44}$. An elevated level was observed for all of these hydrocarbons at the later stages of growth. Unlike CCAP 807/2, the derivatized non-polar lipids from race B AC 761 did not change significantly although the increased relative level of hydrocarbons was obvious. Although these hydrocarbons were triterpenes especially squalene and its derivatives consisting of nearly 53% and 67% of the total derivatized non-polar fraction. Besides these squalene derivatives, palmitic acid (C16:0), oleic acid (C18:1n9c), and octacosenoic acid (C28:1) were also abundant in AC 761, however, they did not show prominent changes.

Taken together, these results imply that the major parts of non-polar lipids in race A strain CCAP 807/2 and race B strain AC 761 were hydrocarbons. The other race A strain CCALA 778 showed very less abundance of hydrocarbons in their non-polar lipid fraction indicating it to be a poor hydrocarbon producer. In addition, a significant reduction in oleic acid (C18:1n9c) in the derivatized non-polar lipid fraction of CCAP 807/2 from linear to decline phase was observed with a simultaneous increase in hydrocarbons. This may suggest a potential link and/or interdependent relation between fatty acid and hydrocarbon biosynthesis

in *B. braunii*. Thus, all these results indicate that both strains from race A produced and accumulated lipids differently as reflected in their lipid content and the FAME profile derived from non-polar lipids.

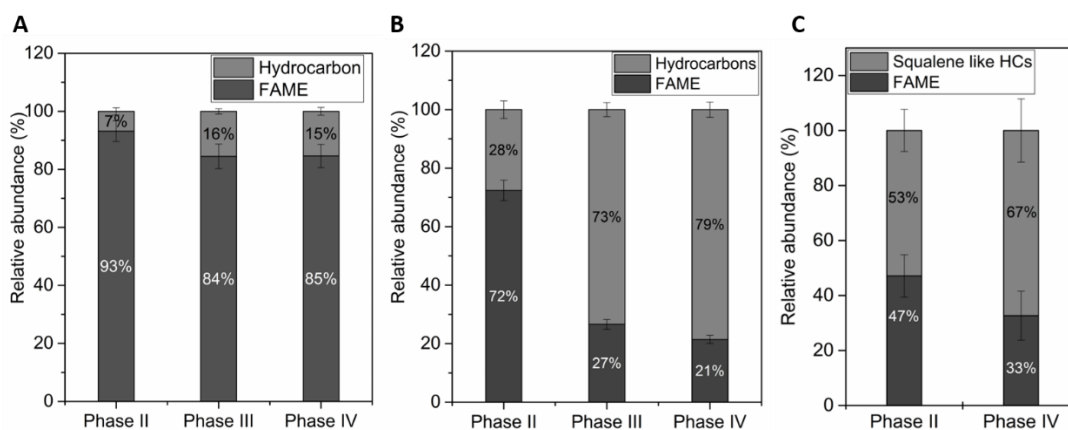


Figure 14 Derivatized non-polar lipid fraction of *B. braunii* race A and race B strains. The relative abundance of fatty acid methyl ester and hydrocarbons as a part of esterified non-polar lipid fraction, considering the total esterified non-polar lipid fraction as 100% during each growth stage for **A)** CCALA 778, **B)** CCAP 807/2 and **C)** AC 761. Phase II represents linear growth stage, Phase III represents stationary growth stage and Phase IV represents decline phase. The error bar represents standard deviation (\pm SD n=9).

4.5 Extracellular product formation in *B. braunii* race A and race B.

B. braunii, especially race A strains are able to produce large amounts of hydrocarbons and polysaccharides in the extracellular matrix (ECM) (Largeau et al., 1980a; Casadevall et al., 1985; Díaz Bayona and Garcés, 2014; Ranga Rao et al., 2012) which can be potentially used for industrial applications. Therefore, an important goal of this study was to determine and to quantify the extracellular product formation in the strains used in the present work. Besides from the biotechnological aspects, it is also important to understand the exact culture duration during which these strains synthesize hydrocarbons and exopolysaccharides. Thus, the following section presents the results wherein the easily accessible products such as carbohydrates (exo-polysaccharides) and hydrocarbons from different *B. braunii* strains CCALA 778, CCAP 807/2 (both race A) and AC 761 (race B) were studied.

4.5.1 Higher carbohydrate production in *B. braunii* race A strains compared to race B

B. braunii produces large amounts of exopolysaccharides (Fernandes et al., 1989). Therefore, to study the effect of different physiological state of the cells on carbohydrate production in *B. braunii* race A and B strains, the total carbohydrates content was determined at each time-point during the cultivation. The analysis of total carbohydrates was determined using whole culture (cells + supernatant) as well as in the supernatant fraction (without cells) as well as.

Indeed, the results for the total carbohydrates content in the whole culture fraction showed CCALA 778 producing as much as $3.2 \pm 0.22 \text{ g L}^{-1}$ of total carbohydrates on day 27 of cultivation which was 78% of the total biomass (Fig. 17A). While the other race A strain CCAP 807/2 produced a maximum of $1.7 \pm 0.02 \text{ g L}^{-1}$ at the end of the cultivation accounting to 51% of the total biomass. However, for race B strain AC 761, the carbohydrate content in the whole culture was $1.1 \pm 0.1 \text{ g L}^{-1}$, comparatively less than race A strains. This measured carbohydrate content was 36.6% of the total biomass produced by AC 761. Therefore, it was evident from the results that race B is a poor carbohydrate producer compared to race A strains. Interestingly, the carbohydrates content in the race A strains differed significantly. Upon detailed evaluation for the accumulation of these carbohydrates during different growth stages revealed that the production of carbohydrate was elevated when the cells entered into stationary phase (phase III) from $1.4 \pm 0.20 \text{ g L}^{-1}$ on day 15 to $3.0 \pm 0.01 \text{ g L}^{-1}$ on day 21 for CCALA 778. This observed increase was in accordance with the previously mentioned C/N ratio (Fig.8B). However, from the late stationary phase (day 21) onwards, the production of total carbohydrates slowed down remaining almost constant and finally reducing at the end of cultivation. Likewise, in the other race A strain CCAP 807/2, the amount of total carbohydrates in the whole culture also increased progressively from the beginning of the cultivation till the end of the cultivation. In comparison to race A strains, the carbohydrate accumulation in race B strain was moderate, although, it showed progressive increase in the linear phase from $0.14 \pm 0.05 \text{ g L}^{-1}$ to $0.9 \pm 0.05 \text{ g L}^{-1}$. Unlike race A, the amount of carbohydrate remained constant in stationary and decline phase for race B strain AC 761.

Furthermore, a detailed analysis for the assessment of the rate of carbohydrate accumulation in the whole culture showed that both race A strains accumulated carbohydrates differently during the linear and stationary phase. While CCAP 807/2 achieved a maximum rate of $82 \text{ mg L}^{-1} \text{ day}^{-1}$, CCALA 778 accumulated $70 \text{ mg L}^{-1} \text{ day}^{-1}$ of carbohydrates in the linear phase. However, the rate of carbohydrate accumulation changed drastically with the entry into their respective stationary phases. Surprisingly, a higher accumulation was observed for CCALA 778 with $193 \text{ mg L}^{-1} \text{ day}^{-1}$ whereas a slight decrease in carbohydrate accumulation with $63 \text{ mg L}^{-1} \text{ day}^{-1}$ was observed for CCAP 807/2. Together, the analysis for assessing the dynamics of carbohydrates in this study revealed that the two strains CCALA 778 and CCAP 807/2 not only did differ in their total carbohydrates content but also in their rate of accumulation of these products in the linear phase.

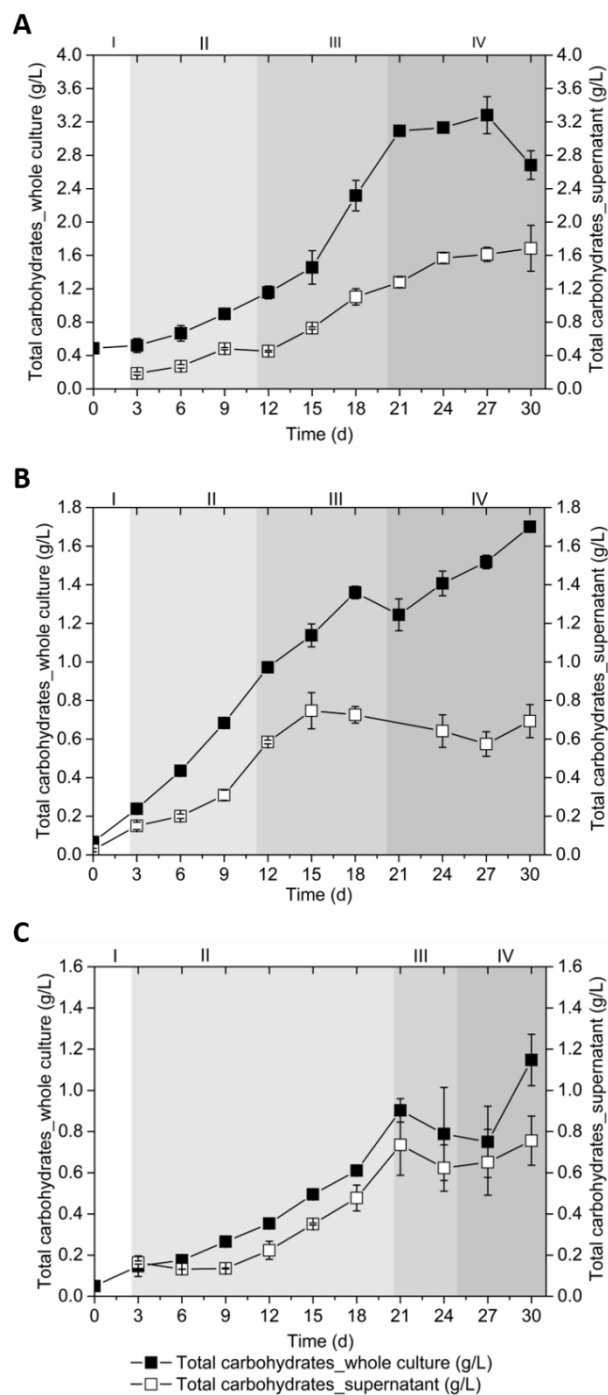


Figure 15 Determination of the total carbohydrate content in *B. braunii* race A and race B strains. The amount of total carbohydrates determined in whole culture and only the supernatant at each time-point during the entire duration of cultivation of 30 days for **A)** CCALE 778 **B)** CCAP 807/2 and **C)** AC 761. Numbers at the top of the graph (I to IV) represent different growth stages of *B. braunii* cells, I – lag phase, II – linear phase, III – stationary phase and IV – decline phase. Error bar represents the standard error of mean (\pm SE, n=18).

4.5.1.1 The extracellular carbohydrates and their monomers in *B.braunii* race A and race B strains

In addition to the analysis of the total carbohydrate content in the whole culture, the total carbohydrates were also measured in only the supernatant fraction in all the three strains in this study. These analyses displayed significant differences between these strains. The total carbohydrates content of only the supernatant fraction for CCALA 778 increased continuously from $0.1 \pm 0.01 \text{ g L}^{-1}$ on day 1 to $1.6 \pm 0.27 \text{ g L}^{-1}$ on day 30 (Fig.15A). However, for CCAP 807/2, the measurement of total carbohydrates in only the supernatant showed relatively slower increase at the initial stages of cultivation (lag phase and early linear phase), and as the cells approached the late linear and early stationary phase, a linear increase was observed (Fig.15B). In contrast to CCALA 778, where the supernatant (external) carbohydrates elevated even during the late stationary and decline phase, the amount of these carbohydrates remained constant for CCAP 807/2 till the end of the cultivation. Hence, the maximum amount of carbohydrates in the supernatant media for CCAP 807/2 reached to $0.74 \pm 0.09 \text{ g L}^{-1}$ on day 15 (Fig.15B). Therefore, the amount of external carbohydrates produced by CCALA 778 accounted to 39% of its total organic dry biomass while for CCAP 807/2 it was 20% only. Similar to its low carbohydrate amounts in the whole culture, the race B strain AC 761 also showed very low levels of the external carbohydrates (Fig.15C). The slight increase observed in its carbohydrate content occurred in the linear phase (phase II) from $0.06 \pm 0.01 \text{ g L}^{-1}$ on day 3 to $0.73 \pm 0.14 \text{ g L}^{-1}$ on day 21. However, with the entry into its stationary phase, the total carbohydrates content did not change largely and had only a marginal increase at the end of cultivation.

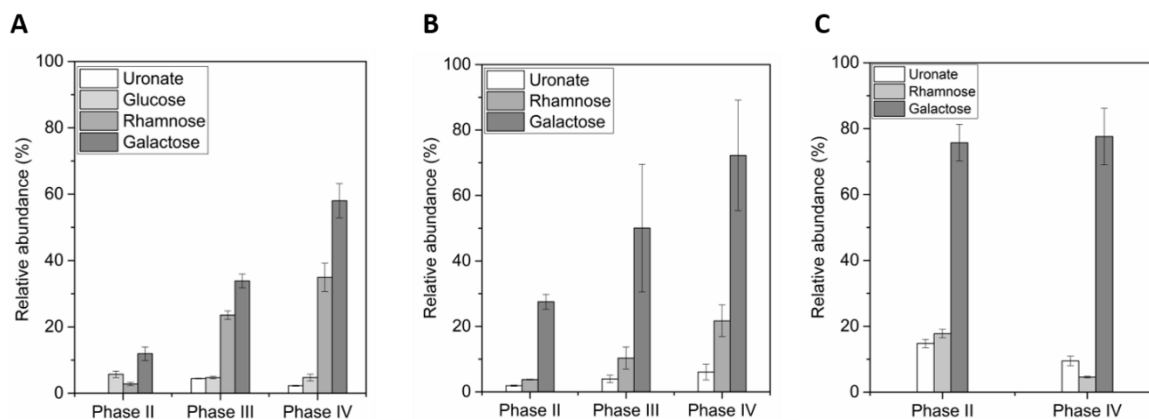


Figure 16 Composition of sugar monomers detected in the supernatant (external) carbohydrates of *B.braunii* race A and race B strains. The relative abundance of sugar monomers detected only in the supernatant of *B.braunii*. A) CCALA 778 B) CCAP 807/2 and C) AC 761. The relative abundance expressed as a percentage value after normalization to the total peak area of identified sugar monomers at phase IV.

For further evaluation, the carbohydrates obtained from the supernatant fraction were later derivatized into monomers and assessed using GC-MS. No major changes were observed in the composition of external

carbohydrates of *B.braunii* race A and race B strains. The analysis showed that the external carbohydrates from both the races of *B.braunii* comprised of monomeric sugars such as uronate, rhamnose and galactose. Galactose was the most abundant form of sugar followed by rhamnose in race A as well as in race B strains (Fig.16). An increased in the relative levels of these monomers was observed as the cultivation progressed in race A strains. However, glucose was only found in race A strain CCALA 778 but its relative abundance did not change significantly over the period of cultivation (Fig.16A).

In conclusion, these results revealed that race A produces more carbohydrates than race B strains. The race A strain CCALA 778 yields carbohydrates as its main product and large amount of these carbohydrates were excreted out of the cells as evident from the measurement of carbohydrates in the supernatant. In addition, high rate of accumulation of carbohydrate was observed during stationary phase for race A strains however, the production rate of carbohydrates was reduced in the stationary phase in race B strain. Furthermore, galactose was the main component of the external carbohydrates in race A and race B strains.

4.5.2 Extracellular hydrocarbon production differs in race A and B strains

The extracellular hydrocarbons produced by *B. braunii* can be easily extracted using short exposure to n-hexane, a non-polar solvent. The analysis of these extracted hydrocarbons from ECM however, showed that CCALA 778 had only detectable amount of hydrocarbons present in its ECM. Moreover, the amount detected was so small that these hydrocarbons could not be quantified using GC-FID. This results were not surprising considering the fact that this strain produced very high amount of carbohydrates, besides, aforementioned FAME profile derived from non-polar lipids displayed only minute abundance of alkadienes. Overall, these results suggest that the race A strain CCALA 778 was able to produce only detectable amount of hydrocarbons ($\sim < 1\%$ of total biomass) (Fig.18) and is mainly a carbohydrate producer. In contrast to CCALA 778, the other race A strain CCAP 807/2 was found to be a moderate hydrocarbon producer (Fig.19). The total amount of extracellular hydrocarbons in CCAP 807/2 increased progressively from $0.08 \pm 0.01 \text{ g L}^{-1}$ on day 3 to $0.66 \pm 0.11 \text{ g L}^{-1}$ on day 24 (Fig.19), where it was the highest during the cultivation. Intriguingly, a 2.3-fold increase was observed in the amount of hydrocarbons from late linear (phase II; day 9) to mid-stationary phase (phase III; day 15) (Fig.19). Correlating this results is the above mentioned increase observed in the C/N ratio in the same culture duration (Fig.8D). The detailed evaluation of hydrocarbons revealed $\text{C}_{29}\text{H}_{56}$ (nonacosadiene) as the most abundant hydrocarbon followed by $\text{C}_{27}\text{H}_{52}$ (Heptacosadiene) (Fig.19). Apart from the above-mentioned alkadienes, few other hydrocarbons such as $\text{C}_{29}\text{H}_{54}$, $\text{C}_{25}\text{H}_{48}$ and $\text{C}_{23}\text{H}_{44}$ were also detected in trace amounts. This is the first study where extracellular matrix hydrocarbons and the composition of CCAP 807/2 is reported for different time-points throughout the cultivation. The hydrocarbon analysis at each time-point in the present study suggests

that the hydrocarbon biosynthesis occurred when the cells were in the linear phase and was further promoted in the late linear and early stationary phases. However, towards the end of the cultivation, the amount of hydrocarbons reduces. The possible reasons for reduced hydrocarbon amount towards the end of cultivation are discussed in details in discussion section.

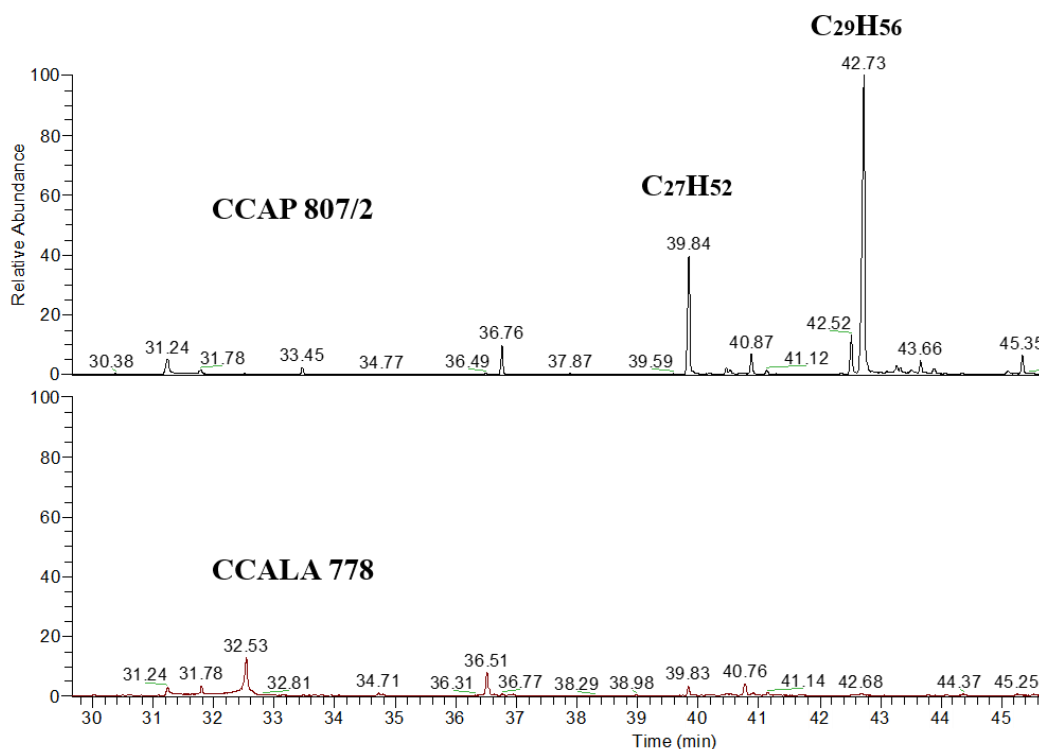


Figure 17 Chromatogram obtained from GC-MS analysis of extracellular hydrocarbons from *B. braunii* race A strains. The differences between chromatogram of extracellular hydrocarbons produced by CCAP 807/2 and CCALA 778. Very small peaks for hydrocarbons were detected in CCALA 778 compared to CCAP 807/2 from retention time of 36 min to 46 min.

In conclusion, this results show that the *B. braunii* CCAP 807/2 was able to produce extracellular hydrocarbons with maximum amount reaching to 23.6 %. In contrary, only detectable amount of extracellular hydrocarbons (< 1%) were present in CCALA 778.

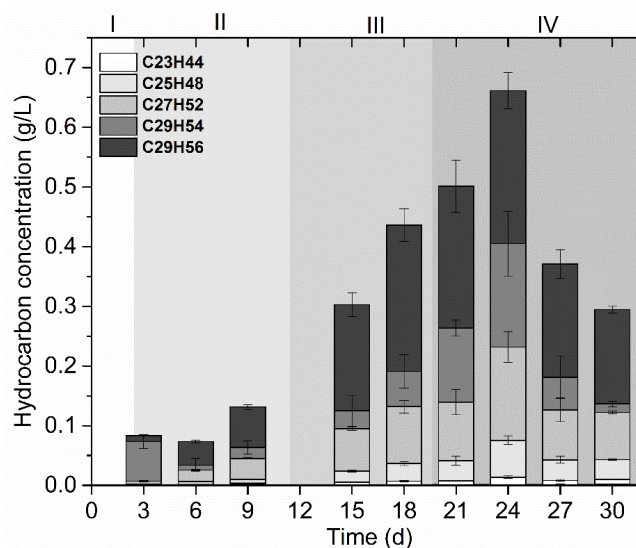


Figure 18 Extracellular hydrocarbons content of *B. braunii* CCAP 807/2 (race A). Measurement of total hydrocarbons was performed using extraction solvents hexane and acetone at each time-point during cultivation via GC-FID. Numbers at the top of the graph (I to IV) represent different growth stages of *B. braunii* cells I – lag phase, II – linear phase, III – stationary phase and IV – decline phase. Error bars represent standard deviation (\pm SD, n=9).

4.5.2.1 Race B produces more hydrocarbons than race A strains

As previously mentioned, race B strains of *B. braunii* are known to produce large amount of triterpenes and methylated squalene from C31-C37 (Metzger et al., 1986a). The further evaluation of these hydrocarbons using GC-FID revealed AC 761 to be producing large amount of hydrocarbons. The total yield of hydrocarbons produced at the end of cultivation was 1.37 ± 0.04 g L⁻¹, although highest amount of hydrocarbon was measured on day 24 which was 1.56 ± 0.22 g L⁻¹ (Fig.20A). Moreover, the hydrocarbon biosynthesis increased when the cells were in the linear phase. In addition, GC-MS analysis was performed to study the type (chain length) of hydrocarbons produced by AC 761. This analysis showed C₃₃H₅₆ as the major hydrocarbon produced by this strain followed by C₃₄H₅₈, (isobotryococcene) and C₃₂H₅₄ (Braunicene) (Fig.20B). The relative abundance of these hydrocarbons increased from phase II to phase IV. This is the first study where hydrocarbon analysis at each time-point throughout the cultivation was performed. Thus, the present study indicates that the race B strain AC761 was producing hydrocarbons from the beginning of the cultivation. Moreover, this results also show the rate of the hydrocarbon biosynthesis was maximum during the linear phase and provides valuable insights into the different chain length of hydrocarbons produced by AC 761.

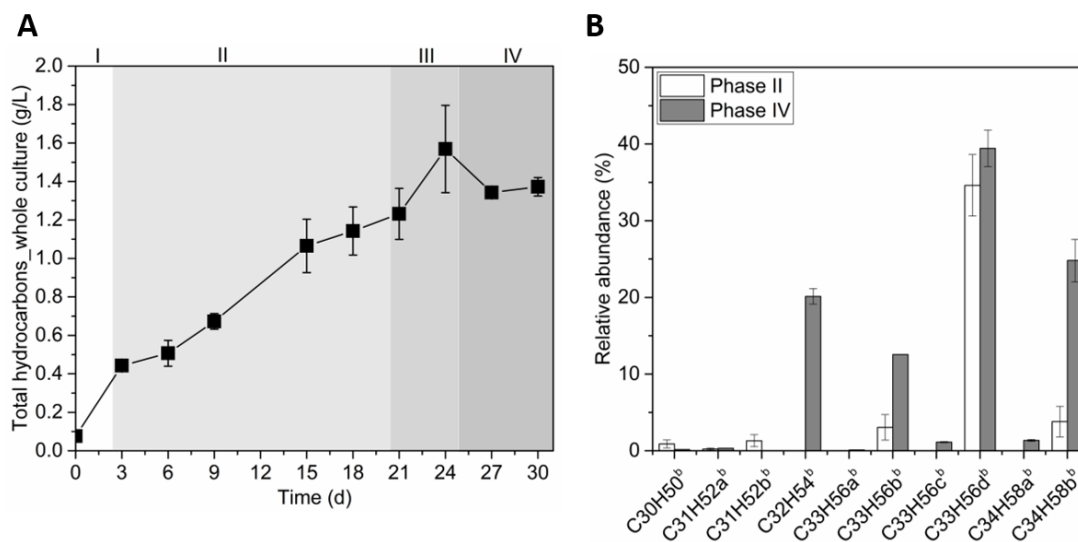


Figure 19 Extracellular hydrocarbons content of *B. braunii* AC 761 (race B). **A)** Measurement of total hydrocarbons was performed using extraction solvents hexane and acetone at each time-point during cultivation via GC-FID. Numbers at the top of the graph (I to IV) represent different growth stages of *B. braunii* cells I – lag phase, II – linear phase, III – stationary phase and IV – decline phase. **B)** Relative abundance of different chain length of hydrocarbons and their isomers normalized to total identified hydrocarbons at phase IV. Error bars represent standard deviation (+SD, n=6).

Table 3 Summary of mean, maximum productivities and yields of organic dry biomass, carbohydrates and hydrocarbons determined for *Botryococcus braunii* race A and race B strains. The maximum productivities for biomass carbohydrates and hydrocarbons were obtained during different time-points. The superscript alphabet represents the duration of cultivation when the maximum productivities are obtained. a days 3-12, b days 3-15, c days 18-24, d days 15-21, e days 3-18, f days 12-18, g days 6-24 and h days 6-15.

		Race A		Race B
		CCALA 778	CCAP 807/2	AC 761
Biomass	Max. Yield mg L ⁻¹	4300	3300	3133
	Max. Productivity mg L ⁻¹ day ⁻¹	231 ^a	118 ^b	150 ^c
	Mean Productivity mg L ⁻¹ day ⁻¹	143	110	104
Carbohydrates	Max. Yield mg L ⁻¹	3200	1700	1140
	Max. Productivity mg L ⁻¹ day ⁻¹	273 ^d	74 ^e	43.3 ^f
	Mean Productivity mg L ⁻¹ day ⁻¹	86	56.6	4.9
Hydrocarbons	Max. Yield mg L ⁻¹	--	661	1568
	Max. Productivity mg L ⁻¹ day ⁻¹	--	32 ^g	62 ^h
	Mean Productivity mg L ⁻¹ day ⁻¹	--	27	45.6

Conjointly, the results from the present study reveal that race A and race B strain of *B. braunii* are different in their growth behavior, intracellular metabolome profile and extracellular product formation. Furthermore, the results from this study also suggest that the hydrocarbon biosynthesis for both the races of *B. braunii* differs with respect to the physiological state of cells (growth stages). In addition, the study further provides the observed precise time duration of the product formation in different races of *B. braunii*. Besides, it gives valuable information regarding the chain length of the hydrocarbons produced by CCAP 807/2 and AC 761.

Collectively, the systematic metabolic characterization of the *B. braunii* strains, CCALA 778, CCAP 807/2 and AC 761 in the current study was essential from the biotechnological point of view wherein the strain-specific product formation of *B. braunii* races could be investigated. The comparison of maximum yield, maximum and mean productivity between the three strains in this study have revealed race A strain CCALA 778 to produce the highest yield of biomass. Additionally, this strain was also producing the highest amount of carbohydrates when compared to the other strains. Furthermore, the hydrocarbon production was the highest in the race B strain AC 761 followed by CCAP 807/2.

5 Discussion

The systematic metabolic characterization of three strains of *B.braunii* was accomplished during this work in order to understand the relationship between the growth/physiological condition and the product formation of this alga. Defining the metabolome profile of *B.braunii* is vital for the understanding and evaluation of the biochemical pathways and cellular mechanisms of hydrocarbon and polysaccharides biosynthesis. Additionally, the effect of the different physiological conditions and nutrient availability plays an important role in microalgal metabolism as reported in earlier studies (Park et al., 2015; Msanne et al., 2012; Lee et al., 2012; Lupi et al., 1994). Because of the range and functions of primary and secondary metabolites in overall metabolism, the metabolome study has a special importance in plants and algae. Thus, the present study focused on the metabolome profiling of *B. braunii*, which has captured the attention of researchers mainly because of its ability to produce large amounts of hydrocarbons and polysaccharides. Besides, the variations across the strains of *B.braunii* with regard to their ability to synthesize different products have generated a lot of interest propelling the biological and biochemical based studies of this alga. In this study, three different strains belonging to races A and B have been characterized and the obtained results are discussed in detail in terms of algal growth behavior, biomass yields, the differences in their metabolome profiles and the differential ability to produce hydrocarbons and polysaccharides.

5.1 The differential growth behavior of *B.braunii* race A and race B

One of the important objectives of the present study was to compare race A and race B strains to evaluate the dynamics of product formation (e.g. hydrocarbons and/or polysaccharides) and to generate their respective metabolome profiles for the different growth stages. Furthermore, the aim was to study the biotechnological relevance of these strains for their future use as hydrocarbon and/or exo-polysaccharide sources. Although, race A and B both produces hydrocarbons, they differ not only in the type of hydrocarbons they produce but also in the hydrocarbon biosynthesis (Templier et al., 1984; Molnár et al., 2012; Baba et al., 2012; Ioki et al., 2012a). Therefore, it was crucial to study the duration when these products are formed and their differing metabolome profiles to gain an insight into the bottlenecks of the product formation with taking into account the existing similarities and differences amongst the different races.

One of the vital and interesting findings of the present study was the identification of different growth stages in CCALA 778, CCAP 807/2 and AC 761 based on their respective total chlorophyll content. Apart from photosynthesis, chlorophyll is important for the nitrogen content as 1 mol of chlorophyll contains 2 mol of elemental nitrogen and thus, changes in chlorophyll amounts reflect the nitrogen availability in the microalgal biomass (Sirisansaneeyakul et al., 2011). It was evident from the total chlorophyll content that

the growth behavior of race A strains CCALA 778 and CCAP 807/2 used in this study was comparable, however the race B strain differed in the duration of its different growth stages (Fig.8). In race B, the total chlorophyll content increased until day 24, however, for race A strains this elevation was observed only upto day 12. This increase in the total chlorophyll content for longer period in race B strain could be due to the increase in the total flux towards the MEP pathway (Molnár et al., 2012). The chlorophyll is made up of phytol, an isoprenoid synthesized via MEP pathway (Lohr et al., 2012). Since the MEP pathway is described to be responsible for the synthesis of hydrocarbons in race B (Baba and Shiraiwa, 2013; Ioki et al., 2012b) and is very active in race B strains than in race A (Ioki et al., 2012b), the possibility of metabolic flux towards MEP pathway resulting in increased chlorophyll content for extended period is plausible. Interestingly, at the initial growth stages of race B (AC 761) strain, the total chlorophyll content was comparatively less than both race A strains. It is conceivable that the moderate increase therefore could be due the slow growth rate of race B strain during the initial stages of growth than the race A strains. This slow growth rate of race B at initial stages of growth may be attributed to the high rate of hydrocarbon biosynthesis which is metabolically expensive.

The growth analysis from the whole culture biomass for all the strains of race A and race B showed a continuous increase in their respective organic biomass yields. The continuous increase in the biomass was earlier observed for different strains of *B.braunii* belonging to different races with Belcher and co-workers reported steady increase in the biomass for 16 weeks (Talukdar et al., 2013; Díaz Bayona and Garcés, 2014; Belcher, 1968). The constant increase in the biomass could be due to the formation of metabolites especially water soluble exo-polysaccharides as well as the insoluble hydrocarbons (Casadevall et al., 1985; Largeau et al., 1980a). The detailed growth analysis of all the three strains in the present study revealed that, although all the strains had a gradual increase in their biomass during the cultivation, the final biomass yield at the end of the cultivation was different for race A strain CCALA 778 and race B AC 761 wherein, the AC 761 was the lowest biomass producer (table 2). However, the difference in the biomass yield was only marginal between another race A strain CCAP 807/2 and AC 761. This difference in the biomass yield between race A and race B strains may exist due to the differential ability of these strains to adapt to the experimental conditions used. In principal, further improvement in the biomass yield and productivity can be possible, since the growth rates and biomass yields in *B. braunii* could significantly be influenced by the light intensity or irradiance (Yoshimura et al., 2013; Li and Qin, 2006), availability of CO₂ (Ge et al., 2011) and salinity (Ruangsomboon, 2012; Ranga Rao et al., 2007). Significant improvement in the growth rate and product formation (especially hydrocarbon productivity) was reported for *B.braunii* race B Showa strain by Yoshimura and co-workers (Yoshimura et al., 2013) by applying optimized growth conditions which includes 0.2-5% CO₂ supply, with temperature at 30°C and light intensity of 850 μmol photons m⁻² s⁻¹. The same study also reported that optimal conditions for improved growth and product formation are

strain specific (Yoshimura et al., 2013). Therefore, it is most likely that these strains may have required alternate optimal conditions and growing these different strains in their optimized conditions may further enhance their growth and product formation.

Strikingly, even though belonging to the same race, the growth analysis of the race A strains CCALA 778 and CCCAP 807/2 in this study resulted in significant differences in their organic biomass yields. The growth analysis of race A strain CCALA 778 revealed that the organic biomass yield obtained from the whole culture at the end of 30 days of cultivation was 4.3 ± 0.34 g/L (Fig.8A). The average biomass productivity of the strain was $143 \text{ mg L}^{-1} \text{ day}^{-1}$, which was reached to a maximum of $231 \text{ mg L}^{-1} \text{ day}^{-1}$ from day 3 to day 12 (linear phase) (Fig.8A). The mean biomass productivity of CCAP 807/2 over the entire duration of cultivation was $110 \text{ mg L}^{-1} \text{ day}^{-1}$ with a marginal increase in its maximum biomass productivity of $118 \text{ mg L}^{-1} \text{ day}^{-1}$ (Fig.8B). Similar differential biomass productivities for race A strains grown under the same conditions was reported earlier for strains LB 572 ($110 \text{ mg dw L}^{-1} \text{ d}^{-1}$), Yamanaka ($135 \text{ mg dw L}^{-1} \text{ d}^{-1}$), UTEX 2441 ($60 \text{ mg dw L}^{-1} \text{ d}^{-1}$) (Eroglu et al., 2011). Hence, the maximum biomass productivity for the characterized strains in this present work was well within the range of $60 \text{ mg L}^{-1} \text{ day}^{-1}$ to $304 \text{ mg L}^{-1} \text{ day}^{-1}$ as shown in the earlier studies (Eroglu and Melis, 2010; Tran et al., 2010). The total organic biomass obtained by CCAP 807/2 at the end of cultivation was 3.3 ± 0.11 g/L, 24% lesser than the other race A strain CCALA 778 (Fig.8A and 8B). Notably, the obtained biomass yield in this study for CCALA 778 was higher than the previous studies for race A strains (Ranga Rao et al., 2007; Vazquez-Duhalt and Arredondo-Vega, 1991). Although, the organic biomass increased continuously throughout the entire duration of cultivation, it is worth to note that the maximum biomass productivity was achieved during the linear phase (phase II) of cultivation for race A strains (Fig.8) as shown by different growth stages based on the total chlorophyll content. *B.braunii* produces large amounts of hydrocarbons and polysaccharides (Banerjee et al., 2002) (Metzger and Largeau, 2005) and hence, a promising alga as a source for renewable energy. Therefore, the study for the productivity and biomass yield are of great importance to maximize the biomass productivity, to enhance the product formation and thereby reduce the overall cost.

Collectively, based on the results for biomass yield and chlorophyll content from the three strains, this present study has shown the race A strain CCALA 778 to be better adapted to the used experimental conditions than the other race A strain CCAP 807/2 and race B strain AC 761. However, it is well known that the product formation also plays an important role in the growth of microalgae as the metabolically expensive compounds such as hydrocarbons, lipids, and polysaccharides can severely impact the growth rate (Belcher, 1968). The potential reasons for the different growth behavior and its relation to the product formation are not so well understood. Therefore, the present study dealt with investigating the causal links

between growth and product formation by using *B.braunii* race A strains CCALA 778, CCAP 807/2 and race B strain AC 761. The following section discusses the overall metabolome profile for the products obtained during the characterization of these strains at different growth stages.

5.2 The intracellular metabolome profile for race A and B strains: differences and similarities

The intracellular metabolome profiling in this study included the non-targeted metabolome analysis, FAME analysis and photosynthetic pigment profiling. The non-targeted metabolome profile using GC-MS detected number of peaks which were further identified and categorized according to the pathway to which these metabolites are closely related.

5.2.1 Influence of the different physiological state of the cells on the metabolites involved in the carbohydrate metabolism

Both race A strains CCALA 778 and CCAP 807/2 showed sugar and sugar alcohol compounds as their most abundant metabolites compared to any other class of metabolites. The relative abundance of sucrose was the highest among all the identified metabolites during different growth stages in both strains. This abundance increased over the time-course of cultivation for both the race A strains. However, the abundance of sucrose in race B was very less compared to race A strain. For example, in the decline phase (phase IV) the relative abundances of sucrose in race A strains were 47.3 ± 3.9 % for CCALA 778 and 34.7 ± 1.0 % for CCAP 807/2 of all the identified metabolites whereas for race B strain AC 761 it was only 0.36 ± 0.01 % during the decline phase (Fig.9). Additionally, the abundance of glycolysis intermediates, sugars and sugar alcohol was also found to be less in race B strain AC 761 as compared to race A strains. These significant differences in the levels of metabolites related to carbohydrate metabolism in both the races (A and B) of *B.braunii* may be attributed to their preferences in differential regulation of their respective intracellular metabolic pathways. A plausible explanation for this theory emerges from previous studies on the transcriptome and enzymes involved in the hydrocarbon biosynthesis in race B strains that have shown the flux of carbon compounds that are synthesized from photosynthesis are more towards the MEP pathway in race B (Ioki et al., 2012b, 2012a; Molnár et al., 2012; Okada et al., 2004). Furthermore, the presence of three isoforms of 1-deoxy-D-xylulose 5- phosphate synthase (DXS) enzyme, which catalyzes the first reaction of MEP pathway in *B.braunii* race B strain (Molnár et al., 2012) could be the reason for more carbon flux towards MEP pathway. Microalgae such as *C. reinhardtii* and others have only one copy of DXS (Lohr et al., 2012) thus limiting the carbon flux towards MEP pathway. Therefore, it seems plausible that the more carbon flux towards MEP pathway may be a potential cause for the less abundance of sugar compounds in race B strain.

On the other hand, both the race A strains (CCALA 778 and CCAP 807/2) showed higher abundance of sucrose during the entire duration of cultivation. Sucrose is a non-reducing disaccharide which is usually produced as a compatible solute under stress conditions such as osmotic pressure, high temperature in microalgae (Hagemann, 2005). Therefore, the presence of sucrose under normal growth conditions (linear phase) in race A strains in this study was interesting since the sucrose metabolism in microalga is not well understood (Valledor et al., 2014). Sucrose phosphate phosphatase, a gene responsible for sucrose biosynthesis has been shown to be upregulated following nitrogen starvation in *C.reinhardtii*, however it was quickly downregulated during N-replete conditions (Valledor et al., 2014). Besides, *Chlorella vulgaris*, a member of Trebouxiophyceae, is reported to have enzymes related to sucrose metabolism (Duran and Pontis, 1977; Salerno, 1985) though, under normal growth conditions, both these microalgae did not show high abundance of sucrose. However, *Euglena gracilis*, a photosynthetic flagellated eukaryote can produce sucrose as a photosynthetic product (Codd and Merrett, 1971). Additionally, microalga such as *Scenedesmus sp.* have shown sucrose as the principle osmoregulator during salt stress (Bremauntz et al., 2014). Sucrose biosynthesis and its role as a stress tolerant is very well studied in cyanobacteria and it has been reported that the enzymes for sucrose biosynthesis in green algae and land plants were acquired from cyanobacteria (Lunn and MacRae, 2003; Kolman et al., 2012). Several studies have also shown that apart from being a compatible solute, sucrose plays an important role in nitrogen fixation, glycogen synthesis and in the biosynthesis of insoluble polysaccharides in cyanobacteria (Kolman et al., 2015). Considering the fact that, sucrose is the major photosynthetic product in plants and has an important role in translocation and storage (Porchia et al., 1999), it is plausible that sucrose may also have a similar role in *B. braunii*. Furthermore, similarly to plants, *B. braunii* has been shown to possess three distinct 1-deoxy-D-xylulose 5-phosphate synthases (DXS),(the first enzyme in MEP pathway) (Matsushima et al., 2012) indicating some common features between the two. Therefore, taking together, the presence of sucrose in large abundance in *B.braunii* race A strains *perhaps related to its* normal cellular function since it was most abundantly present even during the linear phase (phase II) although, the possibility of its functional role in stress conditions cannot be ignored. In addition, it is worth to note that, *B.braunii* are known to produce exopolysaccharide (Fernandes et al., 1989; Banerjee et al., 2002) and thus, the potential role of sucrose as a precursor for polysaccharides cannot be ruled out.

Besides sucrose, raffinose was detected as another sugar compound in both race A strains, however it was not detected in race B strain. Raffinose, a trisaccharide composed of galactose, glucose and fructose is well known for inducing stress tolerance in plants. Additionally, raffinose and the raffinose family oligosaccharides have many been implicated in cellular functions of transport and storage of carbon, signal transduction, and membrane trafficking (Sengupta et al., 2015). Recently, raffinose has been reported to have an important role in stabilizing photosystem II during the cold stress. (Knaupp et al., 2011). With very

few studies existing which have demonstrated the presence of raffinose in microalgae such as *Chlorella vulgaris* (Salerno and Pontis, 1989), the exact role of raffinose and the presence of enzymes involved in raffinose biosynthesis remains largely unclear.

Interestingly, trehalose, a disaccharide, usually produced as an osmotic and desiccation tolerant compatible solutes in cyanobacteria and plants (Hershkovitz et al., 1991; Müller et al., 1995) was found relatively in higher abundance in CCAP 807/2 than in CCALA 778. Trehalose is a non-reducing N-compatible solute (Galinski and Herzog, 1990). Recently, it was reported that trehalose accumulates during nitrogen starvation in *C.reinhardtii* (Valledor et al., 2014). Trehalose is produced not only as a salt osmolyte but it can also act as a high protective potential for enzymes under desiccation and high temperature (Hagemann, 2005). Although, the physiological conditions pertaining to desiccation and use of high temperature were absent during the present experimental set up, the synthesis of trehalose by *B. braunii* race A strains for protective mechanism to overcome stress conditions for example, nutrient depletion could be possible. Furthermore, the varying trehalose levels observed in both these strains may also be due to the different intracellular enzyme activities as earlier reported in plants and yeast (Lunn, 2002). This selective accumulation between trehalose and sucrose was also observed in cyanobacteria *Anabaena sp.* PCC 7119 (Porchia and Salerno, 1996). It was noted that despite having genes for biosynthesis of trehalose and sucrose, the salt stressed cells of *Anabaena sp.* PCC 7119 accumulated sucrose only, though the genes for trehalose were also activated under desiccation stress (Porchia and Salerno, 1996). Another potential reason for the differences in the abundance of trehalose in both *B. braunii* race A strains could be also their origin of habitat. CCALA 778 was isolated in Portugal having warm weather conditions than England, from where CCAP 807/2 was isolated. The cold temperatures during winters in England could have programmed the cellular machinery for high trehalose production in CCAP 807/2. This is in line with a report that shows *Klebsormidium flaccidum*, a green alga to accumulate compatible solutes such as sucrose following cold stress (Nagao et al., 2008). *B.braunii* has been shown to be tolerant to desiccation (Demura et al., 2014) and this could be very well attributed to the fact that this microalga contains high amount of sucrose, trehalose as shown in the present study (Fig.9A and 9B). A further possible explanation for the increased relative level of trehalose in CCAP 807/2 over the period of cultivation can be attributed to the nitrogen depletion. A stressed induced accumulation of trehalose during nitrogen starved conditions in *C.reinhardtii* was reported to stabilize the cellular membranes and proteins for cellular integrity (Wase et al., 2014a). In line with the observed high trehalose levels in *B. braunii* race A strains in the late stationary phase, Porchia and co-workers have also shown in the same for *Euglena gracilis* (Porchia et al., 1999).

Altogether, the non-targeted metabolome analysis for race A strains showed an increase in the abundances of sugar compounds such as sucrose, raffinose and trehalose during the different growth stages. The

accumulation and the subsequent increase may have been primarily due to nutrient depletion especially nitrogen, although the possibility of sucrose as the product of cellular carbohydrate metabolism owing to its presence during the linear phase (phase II) cannot be ruled out. In addition, it is important to mention that the light-dark conditions used in this experiment could have an influence on the overall carbohydrate metabolism in *B. braunii*. Furthermore, race A strains are known to produce large amounts of exopolysaccharides and thus it can be perceived that the sugar compounds detected in this study may have a potential role in the synthesis of polysaccharides. The genes involved in sucrose biosynthesis in green algae and land plants were shown to be acquired from cyanobacteria during endosymbiotic events (Lunn and MacRae, 2003; Hagemann, 2005) and also shares many common cellular mechanisms. Therefore, it is possible that the presence of all these sugar compounds could be attributed to these shared features acquired from plants and cyanobacteria in *B. braunii*. Additionally, the probable cause for race B to have low abundance of metabolites related to the carbohydrate metabolism could be the high carbon flux towards MEP pathway than carbohydrate metabolism as observed in its transcriptome (Matsushima et al., 2012; Ioki et al., 2012a).

5.2.2 Influence of different growth stages on sterols, vitamins in race A and B

Sterols are essential as they play a major role in the regulation of membrane structure and fluidity (Davies et al., 2014). One of the major differences in the non-targeted metabolome profile of race A and race B strains was the existence of sterols. The non-targeted metabolome analysis revealed high abundance of sterols in race B strain AC 761 compared to race A strains (Fig.9A,9B and 9C). In race B strain, cholestane-3-one was the most abundant sterol followed by 4,4- dimethyl cholesta-8en-3 β -ol (sterol3). Interestingly, these sterols were not detected in race A strains. In addition to these sterols, the race B strain AC 761 also possessed campesterol and β -sitosterol which were also detected in race A strains CCALA 778 and CCAP 807/2. Although, 24- methylenecholesterol and iso-fucoesterol are the main sterols reported in *B.braunii* strains (Metzger et al., 1990), the presence of campesterol and β -sitosterol in *B.braunii* was reported earlier using conventional and pyrolysis GC-MS (Metzger et al., 1990; Barupal et al., 2010). Of note is the decrease in the relative abundance of these sterols over the period of cultivation for both race A and race B strains except 4,4- dimethyl cholesta-8en-3 β -ol (sterol3).

This decrease in the relative level of sterols from linear to decline phase could be due nutrient depletion, especially nitrogen as previously reported for the microalga *Nannochloropsis oceanica* (Lu et al., 2014). Although, the mechanism underlying the decrease in the relative level of sterols was not explained in the *Nannochloropsis oceanica*, differential responses of squalene methyl transferases (SMTs) during nitrogen depletion was observed (Lu et al., 2014). Importantly, the catalytic activity of SMTs in *B.braunii* remains unknown, although three triterpene methyl transferases genes exhibiting sequence similarity to sterol

methyltransferases were identified. Two of these genes showed specificity for squalene methylation whereas and the third gene was specific for botryococcene synthesis (Niehaus et al., 2012). The recent study of *B.braunii* squalene epoxidases, enzymes catalyzing the first reaction of oxygenation in sterol biosynthesis, reported two squalene epoxidases (*BbSQE*-I and II) in race B strain, showing different regulation over the period of cultivation (Uchida et al., 2015). Furthermore, these squalene epoxidases were different to each other where *BbSQE*-II displayed amino acid sequence similarity to plant squalene epoxidases than *BbSQE*-I (Uchida et al., 2015). Therefore, given the complex nature of regulation of the sterol biosynthesis and their close relation to the hydrocarbon biosynthesis, the exact mechanism of sterol downregulation during the later stages of cultivation requires further investigations into the sterol biosynthesis in *B.braunii*. Nevertheless, with race B exhibiting more sterols with higher abundance than race A strains in this study indicates a differential regulation of the sterol biosynthesis.

The presence of α -tocopherol, a member of vitamin E family was found to be marginally increased in CCALA 778 during different growth stages, however for CCAP 807/2 a significant increase over the time of cultivation was observed (Fig.9A and 9B). Conversely, α -tocopherol was not detected in race B strain AC 761 (Fig.9C). The metabolite α -tocopherol contributes to the integrity of photosynthetic membrane and also influences the response to photo-oxidative stress in *C. reinhardtii* (Trebst et al., 2002). The presence of antioxidants such as α -tocopherol in the later stages of cultivation in race A strains suggests that the induction of photo-oxidative stress. Recently, the impact of nutrient stress on the production of antioxidant was reported by Goiris and co-workers for three microalgal species *Phaeodactylum tricornutum*, *Tetraselmis suecica* and *Chlorella vulgaris* (Goiris et al., 2015a) whereby higher tocopherol levels were measured during the nutrient depleted conditions (Goiris et al., 2015b). A similar response was earlier reported for *Nannochloropsis oculata* (Durmaz, 2007) and *Dunaliella salina* (El-Baky et al., 2004). During nutrient depletion, reactive oxygen species (ROS) are formed as a result of impaired electron flow from photosystem to electron transport chain (ETC) (Apel and Hirt, 2004). Therefore, microalgae usually produces antioxidants such as carotenoids, α -tocopherol and ascorbic acid to counter the effects of ROS (El-Baky et al., 2004; Pirastru et al., 2012; Durmaz, 2007). Therefore, in accordance with these reported studies, the presence of α -tocopherol at the later stages of cultivation in race A strains may have been to counteract the oxidative stress induced by depletion in nutrients in the culture. **Influence of different growth stages on amino acid metabolism and citric acid cycle intermediates**

Amino acids play an important role in cells in response to the changing environmental and nutrient conditions (Matthew et al., 2009). In this study, all the amino acids in CCALA 778 had reduced levels during the later stages (phase III and phase IV) of cultivation compared to phase II (linear phase). Similar response was observed for CCAP 807/2 strain, where all the amino acids except alanine and lysine (phase

III) showed declined levels compared to phase II, i.e. linear phase (Fig.9A and 9B). The reduction in the levels of glutamate during linear phase compared to phase III (day 15) and phase IV (day 30) of cultivation indicates an increase in the levels of citric acid cycle intermediates (Fig 9A and 9B). This is because citric acid cycle and glutamate synthesis are interconnected via α -ketoglutarate, a citric acid cycle intermediate (Doebbe et al., 2010). Decrease in the amino acid during nitrogen depletion was previously reported in microalgae such as *C. reinhardtii* (Goncalves et al., 2016), *Pseudochoricystis ellipsoidea* (Ito et al., 2013) and for diatom *Phaeodactylum tricornutum* (Yang et al., 2014). The catabolic pathways of amino acid, putrescine would also help for the formation of urea or ammonia which serves as nitrogen sources and in turn adjusts the metabolic homeostasis during the nitrogen depleted condition (Huppe and Turpin, 1994). Therefore, the decline in the amino acids in this study indicates that these amino acids may have probably broken down or incorporated/assimilated into proteins to counter balance the nitrogen depletion during phase III and phase IV as previously observed in other microalgae (Lee et al., 2012; Ikarin et al., 2015).

Ethanolamine, a primary amine compound and an integral part of phosphatidylamine was one of the most abundant metabolites found in both races. The relative abundance of this metabolite increased over the period of cultivation in race A strain CCALA 778 and race B strain AC 761. In race A strain CCAP 807/2, the metabolite showed increased levels during phase III, however the compound could not be detected in phase IV (Fig.9A,9B and 9C). Recently, Cheng and co-workers suggested that ethanolamine is a marker for lipid accumulation in *S. obliquus* (Cheng et al., 2012). Moreover, an increase in triethanolamine levels was also reported in *C.reinhardtii* following nitrogen depletion (Wase et al., 2014b). Thus, the elevated abundance of ethanolamine may be an indicator for the increased total non-polar lipid content. However, CCALA 778 did not show any changes in the total lipid content during the later stages of cultivation. Phosphatidylethanolamine is an important constituent of polar lipids (Thompson, 1996). The higher abundance of ethanolamine in CCALA 778 possibly could be due to the higher amount of polar lipids in the total lipid content.

Taken together, it was evident that the reduction in the amino acids abundance over the period of cultivation was primarily because of the reduced nutrient content such as nitrogen. Besides, the higher abundance of ethanolamine may also indicate the active role of this compound in the carbon (lipid) metabolism especially during the nutrient depletion.

The citric acid cycle intermediates such as malate and citrate reduced with the progressing cultivation in both race A strains, however, these intermediates could not be detected in race B strain AC 761 (Fig.9A,9B and 9C). The decreased levels of metabolites associated with citric acid cycle and glyoxylate cycle (except succinate) were reported earlier in *C. reinhardtii* (Park et al., 2015) and for *P. ellipsoidea* (Ito et al., 2013) under nitrogen starvation. In addition, citrate can be converted to acetyl-Co-A via the ATP citrate lyase

(ACL1) and thus can enter into the fatty acid biosynthesis (Park et al., 2015). Therefore, the possibility of a similar interconnection between citrate and fatty acid biosynthesis during nutrient depletion in *B. braunii* seems plausible. Succinate is one of the intermediate of citric acid cycle and glyoxylate cycle. While the metabolome analysis for CCAP 807/2 showed an increase in the abundance of succinate, in race B strain AC 761, succinate was only detected during decline phase. Wase and co-workers have shown in *C. reinhardtii* that the abundance of succinate was increased following nitrogen depletion (Wase et al., 2014b). Hence, a similar mechanism propelling the production of succinate in *B. braunii* due to nutrient depletion can be perceived in this study.

5.2.3 Similar photosynthetic pigment profile of race A and race B strains

The intracellular photosynthetic pigments are generally divided into two categories, primary carotenoids and secondary carotenoids. Primary carotenoids are defined as those that are structural and functional components of the photosynthetic apparatus of the cell and therefore essential for cellular survival. Secondary carotenoids are produced in large quantities only after having been exposed to specific environmental condition (Jin et al., 2003). The intracellular pigment profile was similar for race A and race B strains. Decrease in the abundance of all the primary carotenoids such as α -carotene, loraxanthin, neoxanthin, lutein, violaxanthin and including chlorophyll a and b was observed for both the races from linear phase (phase II) to decline phase (phase IV). The transcriptome study on Showa, a race B strain reported that the presence of large and complex network of enzymes involved in the synthesis of variety of carotenoids (Molnár et al., 2012). Some of these carotenoids are present in the insoluble extracellular matrix along with ether polymers suggesting their structural role (Banerjee et al., 2002; Metzger and Largeau, 1999; Metzger et al., 2007). Apart from chlorophyll a and b, lutein was detected as the next major pigment in all the strains. A decrease was observed in the amounts of lutein over the period of cultivation as the cells entered into later stages of growth where nutrients were depleted especially nitrogen. Lutein was also detected as the abundant carotenoids in other strains of *B. braunii* as well (Dayananda et al., 2007; Rao et al., 2006; Velichkova et al., 2012). The other intracellular primary carotenoids such as neoxanthin, violaxanthin and loraxanthin were found to be decreased as well from linear to stationary phase. This is in accordance with previous studies where a similar decrease in primary carotenoids was described (Grung et al., 1994). Belcher (Belcher, 1968) reported β -carotene as the major component of pigments, however, in the present study, β -carotene was found to be less abundant than most of the other carotenoids suggesting strain specificity. In addition, nitrogen deficiency usually increases the amount of secondary carotenoids such as astaxanthin in *Chlorella zofingiensis* and *Haematococcus pluvialis* (Del Campo et al., 2004; Boussiba, 2000), however, the primary carotenoid such as lutein was demonstrated to be lower during nitrogen depletion since it is essential for the structure and function of the light-harvesting complexes in

photosynthesis (Cordero et al., 2011). A similar influence of nutrient depletion on the pigments was also observed in the present study whereby the primary carotenoids were significantly reduced at the later stages of cultivation. Interestingly, in contrast to other primary carotenoids, echinenone was elevated during the stationary phase. Echinenone is a secondary ketocarotenoid which imparts red color to *B.braunii* cells (Matsuura et al., 2011). The exact mechanism how echinenone provides protection is not known yet, however, it has been proposed that echinenone translocated to the lipid rich matrix where it exerts its light protective mechanism for the colonies exposed at surface (Grung et al., 1994). Additionally, it is worth to note that echinenone has 23 times higher antioxidant activity than astaxanthin (Miller et al., 1996). Therefore, the increased abundance of echinenone at the later stages of cultivation in this study suggests the installation of protective mechanisms from light and reactive oxygen species upon nutrient starvation.

5.2.4 The intracellular lipid content varies in race A and race B strains

The intracellular lipid profiling for race A and B strains resulted in significant differences in total lipid content, revealing ~ 3.4-fold and ~ 1.7-fold higher lipid content in race B strain AC 761 compared to race A strains CCALA 778 and CCAP 807/2 respectively during the linear phase (phase II) of growth. Recent study on transformation of lipid bodies related to hydrocarbon accumulation showed that race B accumulated more lipids than race A (Suzuki et al., 2013). Furthermore, the accumulation of lipid bodies in race B occurred during maturation of daughter cells and race A did not show formation of lipid bodies at this stage (Suzuki et al., 2013). Additionally, for race A, the extracellular lipid accumulation in basolateral region occurs only after recovery of chloroplast volume, nonetheless however it was described to start much earlier for race B (Suzuki et al., 2013). These discrepancies in the formation of lipid bodies and their relation with cell development has given more evidences for the variation in the lipid content in race A and B. It was earlier reported that race B produces more amount of hydrocarbons, since these represent a part of total lipid content (Yamaguchi et al., 1987; Okada et al., 1995). Although, most of the studies in the past have focused on hydrocarbon productivity, some of them reported total lipid content for the different strains (Talukdar et al., 2013; Li and Qin, 2006; Ge et al., 2011; Ruangsomboon, 2012). The total lipid content of race B strain (36.8 % in phase II and 42.1 % in phase IV) (Fig.12C) was in the same range of higher lipid producing microalgae such as *Scenedesmus sp.*, *Chlorella vulgaris*, *Chlorella protothecoides* and *Nannochloropsis sp.* (Xin et al., 2010) (Illman et al., 2000; Li et al., 2014; Suen et al., 1987). Therefore, the differences in the intracellular lipid content can be attributed to the presence of hydrocarbons in the non-polar fraction of total lipids. For detail evaluation, total lipids were further divided into polar and non-polar fraction and subsequently trans-esterified to FAME.

It is worth to note that the amount of polar lipid fraction for both race A strains and race B strain during the linear phase (phase II) was nearly same, however, the non-polar lipids differed hugely. The race B AC 761

had ~2.5-fold more non-polar lipids than race A strain CCAP 807/2 ((Fig.12B and 12C). Similar observations were made by Yamaguchi and co-worker for Austin (race A) strain and Berkeley (race B) strain (Yamaguchi et al., 1987). The polar lipid fraction consists of main intracellular membrane lipids of algae, which are associated with thylakoids of chloroplast (Harwood, 2004) and phospholipids of the cell wall. Although, the morphological structure of cell wall for both the races was shown to be fundamentally same (Berkaloff et al., 1984), the major phospholipids such as phosphatidylcholine and phosphatidylethanolamine were not detected in polar fraction of race B (Yamaguchi et al., 1987). Therefore, further study into understanding the distribution of cell wall phospholipids in *B. braunii* will be helpful, however it was not the focus of the present study.

The esterification of polar and non-polar lipid fraction provides more details into the fatty acid profile of *B. braunii*. FAME profile derived from polar lipid for both races were almost similar with palmitic acid (C16:0) and oleic acid (C18:1) being the most abundant fatty acids (Fig 13A,13C, and 13E). This fatty acid composition, along with the common polar fatty acids such as palmitic acid C16:0), oleic acid (C18:1), eicosenoic acid (C20:1), eicosapentaenoic acid (C20:5), behenic acid (C22:0) and erucic acid (C22:1n9c) was found to be similar to most of the previous studies. (Kalacheva et al., 2002) (Kalacheva et al., 2001) (Yamaguchi et al., 1987) (Zhila et al., 2005). The FAMES derived from polar lipid fraction of race B strain AC 761 also composed of VLCFAs such as lignoceric acid (C24:0), nervonic acid (C24:1), hexacosenoic acid (C26:1), octacosanoic acid (C28:0), octacosenoic acid (C28:1), tricosenoic acid (C30:1) and tricontadienoic acid (C30:2). These VLCFAs were also present in race A strains CCAP 807/2 and CCALA 778 (not all of them), therefore suggesting that both the races of *B. braunii* may share common pathways for the bio-synthesis of VLCFAs. VLCFAs play an important role in the synthesis of resistant biopolymer PRB-A and PRB-B in race A and B, respectively (Laureillard et al., 1986). The transcriptome study of race B strain Showa also showed the presence of multiple contigs with moderate to high sequence coverage that encodes for very long chain fatty acid elongase, β -ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydratase, and enoyl-CoA reductase enzymes (Molnár et al., 2012). Besides, Laureillard and co-workers also reported oleic acid as a common precursor for both races for the synthesis of resistant polymers (PRB-A and PRB-B) though the larger part of VLCFAs is diverted to the formation of non-isoprenoid hydrocarbon biosynthesis in race A (Laureillard et al., 1988). The presence of VLCFAs in the present study in both races was in conjunction with the transcriptome studies carried out earlier on race A (Baba et al., 2012; Ioki et al., 2012a) and race B strains (Molnár et al., 2012). However, further metabolism of these VLCFAs seems to be regulated differentially in race A and B. The absence of very long chain fatty acyl-CoA hydrolase, which may act as a thioesterase in race B (Molnár et al., 2012) highlights the difference between race A and B. Thus, detailed analyses on the formation of VLCFAs and its diversion to resistant

polymer and hydrocarbon biosynthesis may be helpful to understand the rate limiting steps during hydrocarbon biosynthesis in race A.

As mentioned earlier, the FAME profile of non-polar lipids differed in race A strain CCAP 807/2 and AC 761, since race A strains showed higher abundance of oleic acid (C18:1n9c) compared to hydrocarbons during the linear phase. However, in race B AC 761 triterpene hydrocarbons (squalene and its methylated derivatives) were prominent in the linear phase. Thus, this indicates that the race B **was may have been** probably producing hydrocarbons from the beginning of the cultivation and these hydrocarbons formed the major part of its non-polar lipid fraction. Unlike the race B strain, in race A strains the abundance of hydrocarbons increased during the later stages of cultivation. Conjointly, these results suggest that the active hydrocarbon accumulation is most likely dependent on the different growth stages for hydrocarbon biosynthesis. Moreover, the productivity of hydrocarbons is possibly also different for race A and B strains. Furthermore, the study has shown that race B produced large amounts of hydrocarbons during their active growth phase (phase II). The higher abundance of hydrocarbons in the non-polar lipid fraction also indicates the presence of lower amount of TAGs in race B strain, as TAGs usually represent a dominant the part of non-polar lipid fraction in microalgae (MacDougall et al., 2011). The transcriptome study on race B Showa strain reported the lower coverage of enzymes involved in TAG biosynthesis supporting the lower TAG levels in race B strain (Molnár et al., 2012; Metzger et al., 1990). However, the observation made from the FAME profile of non-polar lipid fraction needs to be confirmed at the level of crude hydrocarbon extract from extracellular matrix (external pool). *B.braunii* stores 95% of their hydrocarbons into extracellular matrix (Largeau et al., 1980a), and thus it is extremely important to study these hydrocarbons in order to understand the duration of cultivation during which the active biosynthesis of hydrocarbons occurred. This is further discussed in more detail in the section of extracellular product formation below. The existence of internal and external pool for hydrocarbons in *B.braunii* and the presence of hydrocarbons in chloroform and methanol extract was earlier studied for race A (Largeau et al., 1980b). However, Metzger and co-workers reported the presence of large abundance of botryococcenes in the internal pool for race B, further the composition of these botryococcenes in the internal and external pool was similar (Metzger et al., 1986a). Together, these above observations suggest that the photosynthetic carbon which was supposed to be used primarily as storage energy in *B. braunii* instead may have metabolized into hydrocarbons, TAGs and carbohydrates. Additionally, a possible competition between hydrocarbon and TAGs biosynthesis cannot be ruled out in this aspect.

5.2.4.1 Differential intracellular lipid accumulation for race A strains

One of the distinct features of both the race A strains, CCALA 778 and CCAP 807/2 was the difference in their total lipid content. The average total lipid content for CCALA 778 when measured during the different

growth stages, showed only marginal changes with $11.8 \% \pm 2.1 \%$ compared to $21.7 \% \pm 1.5 \%$ of CCAP 807/2 (Fig.12). The difference in the total lipid content in *B.braunii* strains was previously shown in several studies (Metzger et al., 1990; Metzger and Largeau, 1999; Kalacheva et al., 2002), however these differences were largely attributed to the different growth conditions used. The strains used in the present study were grown under similar optimal conditions, therefore, the observed differences in their total lipid content rather can be due to genetic factors (differential gene expression), different product formation abilities and strain origin.

The strain producing higher yield of exo-polysaccharides are poor hydrocarbon producer (Fernandes et al., 1989). The race A strain CCALA 778 used in this study was shown to be a high carbohydrate producer (Fig.13). Thus, the possibility of more carbon sink towards carbohydrate metabolism than fatty acid synthesis in CCALA 778 cannot be ignored. Although, the degree of genetic variation across different strains in *B.braunii* from same race is not reported yet. Nevertheless, the diversity between hydrocarbons, their productivities and morphology of strains was studied earlier (Blackburn, 1936). Recent transcriptome study on *B.braunii* CCALA 779 reported the highest level of similarity between transcriptome of same race (CCALA 779 and Bot-88) however, there are still some EST (expressed sequence tags) which are not homologous in those strains (Fang et al., 2015).

Variation among the strains isolated from different places and grown under similar conditions varied in their lipid and carotenoid content (Belcher, 1968). Cell composition, including total lipid content of two race A strains Austin and Göttingen grown under same condition was different (Vazquez-Duhalt and Arredondo-Vega, 1991). All studies thus suggest the diversity in the intracellular metabolite contents among the different strains of *B.braunii* belonging to the same race. Similar variations were also observed between the different strains in the present study. Possible explanations for the existence of these variations could be the existence of differential gene regulation among these strains. In addition, the different origin of habitat may have also contributed to the observed differences between these strains. Supporting such a theory are the studies that underline the importance of strain origin for the variations between race A strains with regard to chemical content such as lipids, hydrocarbons and exo-polysaccharides (Metzger et al., 1989, 1991; Fernandes et al., 1989).

5.2.4.2 Influence of different growth stages on FAME profile of race A strains

Interestingly, the detailed evaluation performed in this study on the total lipid with polar and non-polar fraction and their subsequent conversion to fatty acid methyl ester (FAME) revealed huge differences in the non-polar lipid content (expressed as percentage of dry biomass) between CCALA 778 and CCAP 807/2 strains. CCAP 807/2 produced nearly 5.4, 6.3 and 6.3-fold more non-polar lipids than CCALA 778 during

linear phase (phase II), stationary phase (phase III) and late stationary phase (phase IV) respectively (Fig.12). The polar lipids were more prominent in CCALA 778 at all the growth stages. *B.braunii* Kutz No LB 807/1 Droop 1950 H-252 strain have been reported to produce polar lipids in all the growth stages (Kalacheva et al., 2001). The presence of more polar lipids in conjunction with only marginal amounts of non-polar lipids in the total intracellular lipid content of CCALA 778 suggest this strain to produce only marginal levels of TAGs or hydrocarbons. This may also hint that the photosynthetic carbon flux to be more towards the carbohydrate metabolism.

Although, the FAME profile derived from polar lipids was similar for both race A strains (CCALA 778 and CCAP 807/2), the FAME profile from non-polar lipids was very different. In FAME profile obtained from polar lipids of both strains, palmitic acid (C16:0), oleic acid (C18:1n9c) and octacosenoic acid (C28:1) were the most abundant fatty acids during the different growth stages (phase II, phase III and phase IV) (Fig.13A and 13C). The presence of similar fatty acids in all the races (A,B and L) of *B.braunii* was shown in earlier studies as well (Metzger and largeau, 1999). Several studies on the lipid content of *B.braunii* race A strains showed palmitic acid and oleic acid to be majorly present (Yamaguchi et al., 1987; Kalacheva et al., 2002; Dayananda et al., 2007). This study have also showed that the higher abundance of oleic acid in race A strains may be related to the precursor role of this compound in hydrocarbon biosynthesis and formation of VLCFAs (Templier et al., 1984; Laureillard et al., 1986). Apart from the above mentioned fatty acids, very long chain fatty acids (VLCFA) such as hexacosenoate (C26:1), tricontenoate (C30:1) and C30:2 were absent or could not be detected in the FAME derived from the non-polar lipid fraction of CCALA 778 (Fig. 13B). The absence of these VLCFAs could be perhaps related to the absence of hydrocarbons especially C29 and C25 dienes and trienes in its FAME profile. This is because race A strains produce odd chain hydrocarbons via elongation-decarboxylation pathway (Templier et al., 1984). The last step of decarboxylation in hydrocarbon biosynthesis is the removal of carbon dioxide which requires high activation energy and some activating group. This required activation energy could be provided by a β -OH group present in the long chain fatty acyl derivatives (Yong et al., 1986). Other possibility for the absence of some of these VLCFAs can also be the proposed hypothesis on the formation of resistant biopolymer of race A (PRB-A) from very long chain fatty acid derivatives (Templier et al., 1992). Templier et al. proposed in their work that the formation of botryals could occur via reduction of VLCFAs into aldehydes, followed by head to head condensation to form botryals. These botryals further gave rise to monoepoxybotryals, which were detected in the race A strain (Metzger and Casadevall, 1989). These monoepoxybotryals probably further go through condensation to form PRB-A (Templier et al., 1992), however, this is an proposed assumption and needs to be proven.

The FAME profile of non-polar lipids for both race A strains was different with respect to the presence of hydrocarbons and decreasing oleic acid abundance (Fig.13B and 13D). The hydrocarbons cannot be derivatized, therefore, the non-polar fraction even after transesterification process showed the presence of hydrocarbons. The FAME fraction derived from non-polar lipids of CCALA 778 showed oleic acid as the most abundant fatty acid present during different stages of growth along with very low abundance of hydrocarbons such as C₂₇H₅₂ (Heptacosadiene) and C₂₇H₅₀ (Heptacosatriene) (Fig.13B). However, CCAP 807/2 had a different profile compared to CCALA 778. A reduction in the abundance of total fatty acid methyl esters (FAME) present in the non-polar fraction was observed with a significant increase in the proportion of hydrocarbons at the later stages of cultivation (Fig. 13D).

The dynamics of hydrocarbons and oleic acid abundance at the different stages of growth is important, since oleic acid is the main precursor for hydrocarbon biosynthesis in race A strains (Templier et al., 1984). The present study has shown a significant decrease in the relative abundance of oleic acid in the FAME profile of non-polar lipids for CCAP 807/2 strain from linear phase (phase II) to mid-stationary (phase III) and late-stationary phase (phase IV) (Fig.13D), however, at the same time a significant increase in the abundance of hydrocarbons was observed. The inversely proportional relation between the oleic acid vs. hydrocarbon suggests a product-precursor relationship where the amount of oleic acid decreases with increasing hydrocarbon production during the late linear phase and early stationary phase. Thus, the results in the present study are in line with the earlier observations (Templier et al., 1984). Furthermore, the results also showed that the hydrocarbon biosynthesis occurs during the linear growth stages, however it was promoted only during late linear (phase II) and early stationary phase (phase III) owing to nutrient depletion. The effect of nutrient depletion on increased hydrocarbon amount was reported earlier by Cheng and co-workers (Cheng et al., 2013; Singh and Kumar, 1992).

Together, the discrepancy in the presence of hydrocarbons between race A strains grown under similar conditions indicates that the product formation abilities of this alga can be strain-specific rather than race-specific. It can be speculated that with the lower total lipid content and only detectable amounts of hydrocarbons in FAME profile of non-polar fraction of CCALA 778 strain, other carbon containing compounds such as exo-polysaccharides (carbohydrates) may have been produced instead. This is further supported by studies showing that *B. braunii* strains producing higher amount of exo-polysaccharides are poor hydrocarbon producer (Fernandes et al., 1989).

5.3 Comparison between extracellular product formation of race A and race B

Extracellular product formation of *B. braunii* includes hydrocarbons and polysaccharides (carbohydrates). The total amount of carbohydrates measured in whole culture (containing cells and supernatant media) and

only in the supernatant (without cells) showed differences among race A strains and race B strain. The race B strain AC 761 produced 36.4% of total carbohydrates in the whole culture against 51.5% in race A strain CCAP 807/2 and 62.3% in CCALA 778 at the end of cultivation. Besides, the total carbohydrates measured in only the supernatant (without cells) was 23.8 % for race B strain. From these results, it was evident that the race B strain produces lower amounts of intercellular carbohydrates. These results were in line with the primary metabolome analysis of race A and race B strains where metabolites related to carbohydrate metabolism were less abundant in race B strain than in race A strains. Besides, as mentioned earlier, race B also produces more lipids than race A strains therefore, it could be possible that the carbon flux is more towards lipid biosynthesis. Moreover, the present study also suggests that the increase in the total carbohydrates may be linked directly to the physiological/growth stages of the alga. The total carbohydrates increased gradually in race B strain until the cells were in the linear growth stage (phase II, day 21) and thereafter, it decreased slowly. This decrease in total carbohydrates could be due to the release of polysaccharide hydrolyzing enzymes which are released because of cell lysis during long term cultivation (Lupi et al., 1994; Banerjee et al., 2002). The growth dependent polysaccharide production has been also reported (Díaz Bayona and Garcés, 2014). Additionally, the potential reason for growth dependent polysaccharide production can be attributed to the production of EPS as a secondary metabolite for cell attachment during colony formation (Zhang and Kojima, 1998).

The presence of hydrocarbons in the extracellular matrix is one of the main features of *B.braunii* (Banerjee et al., 2002). These hydrocarbons are easily extractable via short exposure to non-polar solvent like hexane (Metzger et al., 1985; Casadevall et al., 1985; Metzger and Casadevall, 1983). In this study, similar approach was carried out to determine the amount of extracellular hydrocarbons at each time-point during 30 days of cultivation for race A (CCAP 807/2) and race B (AC 761) strains. The difference was very evident as race B strain produced maximum. of 57.1% of hydrocarbons against 24% of CCAP 807/2 on day 24. The overall hydrocarbon production at the end of the cultivation was 43.7 % for race B strain AC 761 and 8.7 % for race A strain CCAP 807/2. The variation in hydrocarbon production abilities of both the race A and race B strains could be due to the presence of different biosynthetic pathways and their differential regulation. Race A hydrocarbons are derived via fatty acid elongation-decarboxylation pathway where oleic acid serve as the main precursor (Templier et al., 1991), however, race B hydrocarbons are triterpene derivative derived from MEP pathway (Ioki et al., 2012a). Besides, the race A strains in the present study have shown to produce more carbohydrates than race B which indicates that these strains prefer to store carbon in the form of polysaccharides instead of hydrocarbons.

Importantly, the race A and race B strains differed in their duration of synthesis of hydrocarbons which is further reflected in their extracellular hydrocarbon accumulation. In race A strain CCAP 807/2, the

hydrocarbon biosynthesis was promoted during late linear and early stationary phase and continued to with nutrient depletion. However, for race B strain, the hydrocarbon biosynthesis was continuously increasing from the first day of cultivation. The possible reason for this different behaviour could be the different regulatory pathways involved in the hydrocarbon biosynthesis in race A and race B, whereby the former uses fatty acid elongation-decarboxylation pathway and the latter uses MEP pathway (Templier et al., 1984; Ioki et al., 2012a; Molnár et al., 2012; Niehaus et al., 2011). In green microalgae, nitrogen starvation causes an increase in lipid bodies (Wase et al., 2014b; Ho et al., 2012; Ikarán et al., 2015). Among these lipid bodies, oleic acid was shown to be the most abundant fatty acid (Wase et al., 2014b). The present study has shown oleic acid (C18:1n9c) to be the major fatty acid in *B. braunii* as well. Therefore, it is possible that nutrient starvation (especially nitrogen) may have triggered the carbon flux towards the fatty acid synthesis and subsequently contributed to hydrocarbon formation. However, the results in the present study revealed the hydrocarbon biosynthesis in race B to initiate at the start of the cultivation and reach the maximum hydrocarbon productivity at the linear phase. Earlier studies have shown that gene expression and enzyme activity involved in the botryococcene synthesis was higher in the early phase of cultivation (Okada et al., 2004, 2000; Matsushima et al., 2012). The activity of botryococcene synthase, an enzyme responsible for synthesis of botryococenes in *B. braunii* race B strain was shown to be increased during the initial days of cultivation by 10-fold and then to slow down by the end of the cultivation (Okada et al., 2004). Strengthening this view is the transcriptome analysis of race B strain Showa where samples were harvested from first eight days of cultivation (Molnár et al., 2012) pertaining to reasons mentioned above. In addition, the first reaction of MEP pathway is the formation of 1-deoxy-d-xylulose 5-phosphate (DXP), catalysed by 1-deoxy-d-xylulose 5-phosphate synthase (DXS) (Zhao et al., 2013). DXS enzyme is a rate limiting enzyme in MEP pathway for both bacteria and plants (Nishizaki et al., 2007; Estévez et al., 2001) and has only a single copy gene in the unicellular microalgae (Lohr et al., 2012). Interestingly, race B strain of *B. braunii* have shown to possess three distinct functional DXS proteins (Matsushima et al., 2012). These 3 DXS genes are expressed simultaneously and the accumulation of transcripts and expression level were highest during the initial period of cultivation (Matsushima et al., 2012). Therefore, it may be possible in this study as well that the presence of 3 DXS genes and their expression during the initial days of cultivation contributed for more carbon flux towards the MEP pathway. This subsequently in turn may have resulted in the formation of more isoprene units resulting ultimately in the biosynthesis of hydrocarbons in AC 761 (race B) at the start of the cultivation. Recent study on transcriptome of race A strain CCALA 779 revealed differences in the transcriptome of race A and race B strains studied until now, which includes (Bot-88, CCALA 779- race A and Showa, Bot-22, Bot-70- race B) (Fang et al., 2015; Baba et al., 2012; Ioki et al., 2012a, 2012b; Molnár et al., 2012). These differences indicate that different races of *B. braunii* may belong to different sub-species.

The obtained hydrocarbon quantity in this study was higher in AC 761 than previously demonstrated for race B strains N-836, Berkeley, Yayoi, Dawin, and Showa (Ranga Rao et al., 2012; Okada et al., 1995; Yoshimura et al., 2013). The culture conditions play an important role in hydrocarbon production in *B.braunii* contributing to the variations across strains of same races (Metzger and Largeau, 2005). The effect of culture conditions such as irradiance, percentage of CO₂, pH was studied for *B.braunii* (Yoshimura et al., 2013; Ge et al., 2011). These studies described that the specific growth rate was maximal for race B strain Showa when optimal conditions such as light intensity of 850 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 14:10 light:dark cycle were used and 39 % of hydrocarbons in the Showa strain using 398 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light intensity with 14h of light and 10% of CO₂ (Yoshimura et al., 2013). Besides, elevated CO₂ levels formed larger colonies and increased hydrocarbon production (Ge et al., 2011). A similar effect was also reported for race A strain LB 572 (Rao et al., 2007). Therefore, it seems plausible that CO₂ concentration (6%) and light intensity (350-400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) used in the present study could have contributed to the observed high percentage of hydrocarbons in race B strain.

5.3.1 Comparison of extracellular product formation in race A strains CCALA 778 and CCAP 807/2

Besides having differences in race A and race B, the present study also reports differences in the extracellular product formation in both race A strains. The analysis of carbohydrates in the whole culture (cells + supernatant) and only in the supernatant revealed that CCALA 778 and CCAP 807/2 differ significantly as CCALA 778 produced maximum. ~ 78% of its biomass as carbohydrates against 51% in CCAP 807/2 (Fig 15A and Fig.15B). The measurement of carbohydrates only in the supernatant (without cells) showed that half of the total carbohydrates produced by CCALA 778 were present in the supernatant fraction. While for CCAP 807/2, only 20% of the carbohydrates from 51% were in the supernatant. Moreover, the carbohydrates measured in the supernatant showed a continuous increase in CCALA 778, however, in CCAP 807/2, the increased in total carbohydrates was observed only until the stationary phase (day 15). However, the difference in the carbohydrate content of two race A strains, Austin and Coat ar herno was reported earlier as well (Allard and Casadevall, 1990).

One of the common features of both race A strains used in this study was the sustained increase in their total carbohydrate content in whole culture (cells + supernatant media). This linear increase was independent of the different growth stages, although the possibility of rate of carbohydrate synthesis per cell could be different during the different growth stages (Lupi et al., 1994). However, it is impossible to study this rate given the fact that it is a colony forming microalga. The influence of nitrogen source in the formation of internal as well as external (referred here as supernatant) carbohydrates was reported in many

microalgae (Rossi and Philippis, 2016) including *B.braunii* (Lupi et al., 1994) and *C. maxicana* (Kroen and Rayburn, 1984). The continuous elevated amounts of the total carbohydrates could be due to the intercellular accumulation of sucrose as shown by the intracellular metabolome analysis with simultaneous formation of exo-polysaccharides driven by nutrient depletion (Kroen and Rayburn, 1984; Yang et al., 2010). It is known that following nitrogen starvation microalgae such as *C. reinhardtii*, *Chlorella* and *Scenedesmus sp.* have an increased starch content (Park et al., 2015; Zhu et al., 2013; Yang et al., 2010; Ho et al., 2012). Though, starch content was not determined in this study, the elevated levels of sucrose was measured with progressing cultivation. Additionally, at the same time, EPSs can also be synthesized as a result of stress as they play an important role in protective mechanisms against biotic and abiotic stress (De Philippis et al., 1998; De philippis et al., 1991). Therefore, it can be possible that the observed sustained increase in the whole culture carbohydrate content for both the strains during mid stationary phase (phase III) may be perhaps due to a combined effect of accumulation of sucrose and EPS. However, in the decline phase (after day 21; phase IV) the carbohydrate content of whole culture in CCALA 778 was almost constant against the observed gradual increase in CCAP 807/2 culture. Therefore, it can be speculated that due to high viscosity in the culture of CCALA 778, efficient gas exchange (CO₂) and light exposure was hindered and thus contributed towards inhibiting carbon concentrating mechanism. Another possibility could be the low turnover of enzymes involved in formation of carbohydrates due to prolonged nutrient starvation.

However, strikingly, the total carbohydrate content of only the supernatant fraction was gradually increased for CCALA 778 but was not observed for CCAP 807/2 upon its entry into stationary phase (Fig.15A and 15B). A potential reason for this could be attributed to the presence of different bacterial species for both strains. The association of *Chlamydomonas (ch-10)* and *Streptomyces* has shown that *Streptomyces* was able to digest exo-polysaccharides produced from *Chlamydomonas* (Parker and Bold, 1961). The presence of different bacterial strains with various *B.braunii* strains was reported in some studies (Chirac et al., 1985; Tanabe et al., 2015; Metzger et al., 1985) and these variation were related to the strain origin. Furthermore, the differential regulation of transport mechanism for EPS to extracellular matrix especially following nutrient depletion may have also contributed to these observed differences. Additionally, the influence of polysaccharide hydrolyzing enzyme after the partial cell lysis (Díaz Bayona and Garcés, 2014) in CCAP 807/2 cannot be rule out.

Moreover, the differential product formation features of these strains can also be caused by the presence of different bacterial consortium. Some bacteria can have a positive or negative effect on *B.braunii* depending on the culture condition (Chirac et al., 1985). A recent study on bacterial ectosymbiont of *B.braunii* ,BOTRYCO-2 proposed a potential link between BOTRYCO-2 and its relatives on the algal colonies that

produces specific carbohydrates (Tanabe et al., 2015). Moreover, the study hypothesized that BOTRYCO-2 feeds on the component of extracellular matrix which includes polysaccharides and hydrocarbons (Tanabe et al., 2015). The presence of *Rhizobium sp.* along with *B. braunii* positively influenced *B. braunii* growth, however the exact mechanism of this symbiotic relationship still needs to be elucidated (Rivas et al., 2010). Contrary to this, no significant influence of the bacteria *Aureobacterium barker* accompanying the *B. braunii* UC 58 strain on the yield of algal growth and exo-polysaccharide was reported (Fernandes et al., 1989). These discrepancies in these studies could be due to the different strains used. Therefore, using metagenome approach in order to study *B. braunii* as a consortium accompanied by the endogenous bacterial community may be helpful to understand the potential relationship between algae and bacteria.

The analysis of sugar monomers in this external (supernatant) carbohydrates revealed galactose to be abundantly present followed by rhamnose and uronate in both race A strains and race B strain. A similar finding was reported previously, where galactose was shown as the major component of carbohydrates obtained from race A strains Austin and Coat ar Herno (Allard and Casadevall, 1990). The same study also reported the presence of fucose and 3-O-methyl rhamnose (acofriose) (Allard and Casadevall, 1990) however, the strains used in the present study did not show these sugar monomers. The presence of different monomers except galactose was shown for another race A strain UTEX 572 (Metzger et al., 1990). Recent study on the colony organization of race B strain Showa demonstrated galactose as the backbone of the polysaccharides obtained from shell material (Weiss et al., 2012). Besides, the *B. braunii* UC 58 (race unknown), a high exo-polysaccharide producer strain also described galactose as the main component of EPS (Fernandes et al., 1989). The analysis of monomers in the present work for CCALA 778 strain had glucose in small abundance in their external carbohydrate fraction which was not present in CCAP 807/2 and AC 761.

Taken altogether, CCALA 778 produces carbohydrates with only detectable amount of hydrocarbons in intracellular FAME fraction derived from non-polar lipids, however CCAP 807/2 can produce both hydrocarbons and carbohydrates although, the latter being higher. The possible reason for these variations in the product formation may be due to the differential gene expression which greatly depends on strain origin and bacterial influence. The lower lipid content along with very low (< 1%) of hydrocarbons for CCALA 778 may also suggest more carbon flux towards the carbohydrates (exo-polysaccharides) production. Therefore, the results from this work are in accordance with the previously described reports that demonstrated some *B. braunii* strains to be poor hydrocarbon producer while producing higher exo-polysaccharides amounts (Fernandes et al., 1989).

5.3.2 The potential role of EPS in *B. braunii*

Exo-polysaccharides in microalgae can have protective role against oxidative stress because of their ability to scavenge ROS (Tannin-Spitz et al., 2005). This antioxidant mechanism of EPS is attributed to the supply of hydrogen from these polysaccharides, which form stable products after combining with radicals so as to eliminate radical chain reaction (El-Sheekh et al., 2012; Rossi and Philippis, 2016). It was proposed that this scavenging mechanism is intracellular and initiates during the sugar assembly prior to the extracellular excretion of the sugar polymers out of the cells or into the surrounding medium (De Philippis et al., 1998). Apart from this, EPS also plays an important role in desiccation tolerance in *B. braunii* as their amphiphilic nature allows them to trap the water molecules, in addition to the control of the uptake and loss of water molecules (Demura et al., 2014; Pereira et al., 2009). Some studies also reported that algal-bacterial co-cultures can enhance the accumulation of EPS due to stressful environment (Angelis et al., 2012). This stressful environment can be either due to nutrient competition or possible toxic compounds produced by some of the members of consortium (which includes bacteria, viruses etc.), thus, EPS are used to overcome these constraints (Bell, 1983). Given the fact that, the *B. braunii* strains used in the present study are non-axenic, it is possible that an algal-bacterial symbiotic interaction may have prompted the *B. braunii* strains to produce as to counteract competition or for better survival. Apart from this theory, the effect of the oxidative stress, nutrient depletion, and increased osmotic pressure due to dissolved CO₂ can also enhance the EPS production in this alga.

5.3.3 Hexane extractable extracellular crude hydrocarbons from race A strains

This study has shown that the presence of these extracellular hydrocarbons only in the CCAP 807/2 while only detectable amounts of hydrocarbon were present in the external pool (ECM) of CCALA 778. Furthermore, these results complemented the findings of FAME profile derived from non-polar lipids. Together, these results clearly indicate that all the tested strains behave differently in terms of product formation. In race A strains, the maximum detected amount of hydrocarbons was 23.6% in CCAP 807/2 on day 24 (phase IV) (Fig.18). Besides, the amount of extracellular hydrocarbons increased during the late linear (phase II) and early stationary stage (phase III). Thus, it was evident from this result that the hydrocarbon production was promoted following nutrient depletion conditions. These findings also support the fact that physiological condition of *B. braunii* cells plays an important role in the hydrocarbon production (Brown et al., 1969; Casadevall et al., 1985).

The reasons for the variations of extracellular hydrocarbons in one strain CCAP 807/2 and in other CCALA 778 could be attributed to the genetic background, strain origin, and presence of different bacterial community associated with these strains. Hydrocarbons from race A are derived from fatty acid elongation-

decarboxylation pathway (Templier et al., 1984; Metzger and Largeau, 2005), however CCALA 778 produces very less amount of lipids compares to CCAP 807/2. Therefore, it may be possible that because of differential gene expression in both strains, the carbon sink towards the biosynthesis of fatty acid is lower. This could also be the potential cause for the limited carbon flow towards the biosynthesis of hydrocarbons via elongation-decarboxylation of VLCFAs, in turn resulting in the very low amount of extracellular hydrocarbons in CCALA 778. Transcriptome study on race A strain Bot-88 reported that substrate entry to VLCFAs (which are essential for hydrocarbon biosynthesis in race A strains) may be regulated through glycerolipid metabolism (Ioki et al., 2012a). Considering the fact that the polar lipids, (consisted of glycerolipid and phospholipids) were prominent in CCALA 778 in all the growth stages, the differential regulation of this substrate entry into VLCFAs in CCALA 778 and CCAP 807/2 could be causal for the observed differences in the hydrocarbon production. Besides, the presence of bacterial community can also influence the hydrocarbon production. Numerous studies in the past have shown both negative and positive effects of bacteria on hydrocarbon production (Chirac et al., 1985; Tanabe et al., 2012, 2015; Casadevall et al., 1985; Metzger et al., 1985). Chirac and co-workers first reported about the degradation of hydrocarbons in the outer walls (extracellular matrix) by bacteria like *Pseudomonas sp.* and *Flavobacterium sp.* (Chirac et al., 1985). The same study also reported that *F.aquatile* had a positive effect on hydrocarbon and biomass production of *B.braunii* (Chirac et al., 1985). In contrast, the study from Metzger et al (Metzger et al., 1985) reported the existence of two bacterial species capable of degrading hydrocarbons associated with the alkadienes and alkatrienes producing strain (Morocco [Atlas] strain). Therefore, it is evident that bacterial presence in non-axenic strains of *B.braunii* can have influence on hydrocarbon production, nevertheless this influence varies from strain to strain and also depends on the culture conditions.

In the present study, it was observed that the hexane extractable hydrocarbons in CCAP 807/2 were decreased during prolonged stationary phase (Day 27 and day 30). The observed decrease could be due to the aging of culture which may have eventually affected the preferential location of hydrocarbons. Casadevall and co-workers (Casadevall et al., 1985) showed that the decrease is largely due to the bacterial presence. One possible explanation for this could be the effect of the prolonged nutrient depletion on the transport system (usually transport proteins) for VLCFAs and hydrocarbons from endoplasmic reticulum to extracellular matrix via Golgi apparatus.

The metabolism of alkadienes into the biosynthesis of biopolymer (Templier et al., 1992), which is highly resistant to chemical degradation, could also be one of the reasons for decreased amount of hydrocarbons at the end of cultivation. Although, the presence of hydrocarbon chain in this biopolymer was confirmed

by previous studies (Laureillard et al., 1986; Banerjee et al., 2002), the physiochemical conditions for the biosynthesis of this biopolymer are not well understood.

Conclusively, this present work provides a detailed systematic metabolic characterization of *B.braunii* race A and race B strains by highlighting the shared and distinct characteristics of race A and B strains in terms of growth behavior and product formation. In addition, this study also provides the influence of different physiological state of the cells on the respective overall metabolome of *B.braunii* strains.

6 Perspectives

The ability of *B.braunii* to produce large amounts of hydrocarbons and exo-polysaccharides is widely regarded as potential sources for biofuel and bio-polymer. However, the slow growth rate poses a main challenge for the large scale application of this microalga. The systematic metabolic characterization of different races of *B.braunii* in this work provided evidences for the influence of different physiological state on the product formation and on the overall metabolism of this alga. The results in this study also provide the precise details on the culture duration during which *B.braunii* race A and race B strains begin forming exo-polysaccharides and hydrocarbons. These data will be vital in the selection of the sampling time-points for further transcriptome and proteome studies. A recent study on the transcriptome analysis of race A strain CCALA 779 showed that there are many unique and novel genes in race A strains for hydrocarbon biosynthesis and sequences of these enzymes are still unknown (Fang et al., 2015). Ultimately, for a strong foundation for *B.braunii*, the transcriptome, proteome, metagenome and metabolome based studies are essential. These sequencing projects will also aid in speeding up the isolation of novel genes and their subsequent characterization for further improvements in algae biofuel feedstock.

Despite the characterization of enzymes involved in the hydrocarbon biosynthesis of race B (Okada et al., 2000; Niehaus et al., 2011, 2012), not much is known about the genes and enzymes involved in the formation of alkadienes and alkatrienes in race A strains. Thus, the future goals should focus on the strain improvement whereby the role of concerned genes and enzymes in the formation of hydrocarbons in race A needs to be elucidated. The results from the present study may be helpful since it provides the precise details on the dynamics of the fatty acids involved in the biosynthesis of hydrocarbons in race A during the different growth stages.

One of the interesting findings of the present work was the presence of VLCFAs in both the races of *B.braunii*. VLCFAs are involved in the highly resistant polymer produced by *B.braunii* and in hydrocarbon biosynthesis in race A as well (Yong et al., 1986; Laureillard et al., 1986). Therefore, it would be interesting to understand the bottlenecks and their effects on the pathways involved in the biosynthesis of these resistant polymers in race A.

Additionally, a thorough transcriptome and proteome based analysis for the genes and proteins of the carbohydrate and hydrocarbon metabolism can provide insights into how these genes are differentially regulated under varying physiological conditions. Moreover, metabolic flux analysis using radiolabeled carbon and nitrogen can reveal the consumption rate of these elements into various intracellular biochemical pathways.

Algal-bacterial interaction and metagenome approach

Besides intracellular biochemical processes, studies to assess external biotic factors such as bacterial consortium associated with the *B.braunii* are very important. This is because *B.braunii* in nature harbors many different bacteria and the potential symbiotic or parasitic relationship between *B.braunii* and bacteria can have either positive or negative effect on its biomass and hydrocarbon productivity (Chirac et al., 1985; Tanabe et al., 2015). The strains used in the present work were non-axenic and therefore the effect of these consortium on product formation remains to be investigated. The study also shows that the extracellular hydrocarbons were decreased at the end of the cultivation for CCAP 807/2 and this could be the result of degradation of hydrocarbons by the bacterial consortium as earlier reported (Chirac et al., 1985). In order to study this relationship, obtaining an axenic strain is essential. Therefore, in order to elucidate the algal-bacterial relationship and its effects on the products such as extracellular hydrocarbons, further study in this direction can be performed to investigate the relation of *B.braunii* and its ectosymbiont.. This study will essentially include the metagenome sequencing of the whole consortium and identifying the possible algal-bacterial relationship with the help of metabolic approach as earlier reported by (Dittami et al., 2014). This approach involves the isolation of different components from the consortium and their DNA, followed by the sequencing of these components. To remove the known components, the metagenome and metatranscriptome reads can be mapped against algal or isolated bacterial genomes. Sequencing will thus help to reconstruct the individual metabolic networks based on genome scale metabolic modeling and their merging. Finally, this merged network can be used to study acclimation processes in the consortium.

The concept of co-cultivation depending on the algal-bacterial interaction can be beneficial in terms of large scale microalgal cultures. Earlier studies have shown that probiotic bacteria such as *Rhizobium sp.* have significantly encouraged the growth of *B.braunii* (Rivas et al., 2010). An alphaproteobacteria, *Candidatus Phycosocius bacilliformis* (BOTRYCO-2) has been reported to exert positive impacts on growth and hydrocarbon production of *B.braunii* (Tanabe et al., 2015). However, the exact mechanism of this interaction is still enigmatic and hence, it will be important in near future to determine it.

Biotechnological applications for 'milking' of hydrocarbons and exo-polysaccharides

One of the larger goal of this project was to provide the basic essential information regarding the product formation so as to conceive the possibility of milking hydrocarbons and/or exo-polysaccharides. Milking is the process of *in situ* extraction of metabolites with the possibility of reusing the microalgal/microbial biomass (Jin et al., 2015). Applications of these extracellular products is more feasible as compared to the intracellular metabolites such as TAGs and β -carotene. This avoids the destruction of the microalgal biomass and also reduces the use of extraction solvent (Griehl et al., 2014; Moheimani et al., 2013). The

present work provides information on the products formed, their composition in terms of hydrocarbon chain length and sugar monomers in race A and race B strains at different growth phases. Thus, the effects of the different physiological state of the cells can help to determine the harvesting or milking time-point for the extraction of these products when the productivities for these hydrocarbons and polysaccharides are highest. Continuous efforts have been made in this direction to improve *in situ* hydrocarbon extraction process using membrane dispersion methods (Zhang et al., 2013). Besides, sea-water cultured *B.braunii* was used for the improvement in hydrocarbon recovery. No pretreatment of the cells was required making it beneficial for potential reuse of the biomass for milking, however, a reduction in its growth rate was observed (Furuhashi et al., 2013). Owing to its slow growth rate, the efforts for extracting the products and reusing the biomass from *B. braunii* strains will be required to have an ultimate sustainable system.

Furthermore, the studies will be required for the genetic modification of *B.braunii* or genetic engineering of other organisms to produce hydrocarbons such as alkadienes or botryococcenes for the use in biofuel production. However, modification of *B.braunii* will also require a better understanding and basic knowledge of *B.braunii* molecular biology. Because of their ability to produce large amount of hydrocarbons and excretion into extracellular matrix, the studies related to *B.braunii* will likely remain driven by a focus on algae biofuel research in the near future.

Conclusively, the novel findings from this study wherein the link between the cell physiological condition and its subsequent influence on product formation along with a detailed metabolome analysis highlight unique characteristics of *B.braunii* strains providing strong argument to continue the basic biological research of this interesting microalga.

7 References

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9 Curriculum vitae

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Education

Jan 2013 - Present - **PhD**, *University of Bielefeld, Bielefeld, Germany.*

Mar 2010 - May 2012 - **Masters**, *TU Kaiserslautern, Kaiserslautern, Germany (Grade - 1.4).*

June 2005 - Aug 2009 - **Bachelors**, *Dr.D.Y. Patil Bioinformatics and Biotechnology Institute, Pune, India (Grade- 3.34/4).*

Research Experience

Jan 2013 – Present - PhD project, *Systematic metabolic characterization of hydrocarbon and exopolysaccharide producing microalga Botryococcus braunii (EU-SPLASH-7th framework).*

Center for Biotechnology, University of Bielefeld, Bielefeld, Germany.

March 2011 – Aug 2012 – MSc, *Construction and Characterization of E. coli Aspartokinase III Mutants and Recombinant Aspartokinase by Domain Swapping.*

Institute of Bioprocess and Biosystems engineering, TU Hamburg, Hamburg, Germany.

May 2010 – July 2010 – MSc, *Research student, Fermentation of Trichoderma harzianum HA 130-97 and isolation of Gliovirin.*

Institute of Biotechnology and Drug Research, TU Kaiserslautern, Kaiserslautern, Germany.

Dec 2010 – Jan 2011 - MSc, *Research student - Functional protein expression in Xenopus laevis oocytes.*
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Industrial/Work Experience

Aug 2009 – Jan 2010 Technical Sales officer

Lilac Medicare Pvt. Ltd. (now a part of Tosoh corporation), Mumbai, India.

Publications

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Conference presentations/posters

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- 2016 Young Algaeneers Symposium (YAS), Malta.
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- 2014 International Bielefeld-CeBiTec Research Conference: Advances in Industrial Biotechnology: Prospects and challenges for the development of algal biotechnology, Bielefeld, Germany.

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- 2016 Young Algaeneers Symposium (YAS), Malta.
- 2015 4th Annual retreat talk SPLASH project Berlin, Germany (2016).
- 2015 3rd Annual retreat talk SPLASH project University of Ghent, Ghent, Belgium (2015).
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- 2013 1st Annual retreat talk SPLASH project University of Cambridge, Cambridge, UK (2013).

Hobbies/Interests

Travelling, Sports (Cricket, Chess, Badminton), Writing poetry and Reading.

10 Declaration

I hereby declare that I have conducted the work presented here myself and have used only the specified sources and resources. All literature and supplemental sources were cited accordingly. All work from other authors, including images and figures, has been appropriately referenced, or reproduced with permission. I declare that this dissertation has not been submitted in whole or in part, to another faculty with the aim to acquire an academic degree. I hereby apply, for the first time, for the degree of Doctor of Natural Sciences at the University of Bielefeld.

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5th December 2016