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Review

Botryococcus braunii lipid production pathways and biorefinery potential

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https://doi.org/10.1016/j.isci.2025.114534

SUMMARY

Botryococcus braunii **is a colonial microalga recognized for its ability to produce and secrete long-chain hydrocarbons, positioning it as a promising feedstock for biofuel and bioproduct applications. This review synthesizes current knowledge on race-specific hydrocarbon profiles, genetic markers for classification, and the biochemical pathways underlying lipid biosynthesis. It evaluates cultivation strategies alongside stress-based approaches to enhance productivity. Lipid recovery technologies are discussed with emphasis on sustainable, non-destructive methods such as milking and switchable solvents, which reduce energy demands and preserve cell viability. The integration of these processes within biorefinery frameworks highlights opportunities for the co-production of fuels and high-value compounds. By linking molecular insights with process engineering, this work underscores the potential of** ***B. braunii*** **to contribute to sustainable energy systems.**

INTRODUCTION

Microalgae are photosynthetic, unicellular or colonial microorganisms capable of converting solar energy and carbon dioxide into a wide spectrum of valuable biochemical compounds, including lipids, proteins, carbohydrates, carotenoids, vitamins, and peptides. With an estimated 40,000 species currently identified, microalgae represent one of the most diverse groups of organisms on Earth.[1] This evolutionary diversity, shaped over more than 650 million years, has enabled microalgae to adapt to a broad range of ecological niches and survive under extreme conditions, such as nutrient deprivation, salinity shifts, high or low temperatures, and pH fluctuations. Although primarily found in aquatic ecosystems (freshwater, marine, and wastewater), microalgae are also capable of colonizing terrestrial environments such as moist soils and snowbanks.

In light of growing environmental concerns and increasing global energy demands, especially following the 2015 Paris Agreement targeting net-zero carbon emissions by 2050, microalgae have emerged as a key focus in renewable energy research. Despite progress in clean energy technologies, fossil fuels still account for 64.4% of the world's energy supply. Among bioenergy alternatives, microalgal lipids offer significant promise due to their high productivity, diverse chemical composition, and compatibility with current fuel infrastructure.[2] These lipids are primarily categorized into two types: fatty acid-based compounds such as triacylglycerides (TAGs), and liquid hydrocarbons. TAGs, which many microalgae produce in response to stress conditions such as nutrient deprivation, fluctuations in temperature, pH, and salinity, can be readily converted into biodiesel via transesterification, forming fatty acid methyl esters.[3]

The disadvantage of fatty acid methyl esters as fuel is, however, that they contain oxygen atoms, so they have a low combustion energy per unit weight. Therefore, currently, hydrocarbon fuels are required for aviation fuel. In relation to this, a wide variety of microalgal species are currently investigated for hydrocarbon production, *Chlorella protothecoides, Desmodesmus* sp., *Scenedesmus* sp., and *Tribonema minus* possess an average lipid content between 47.4 and 64.1%.[4] There is, however, an exceptional algal species that can achieve up to 75% of lipid content, *Botryococcus braunii*.[5,6] While most microalgal species store TAGs and hydrocarbons intracellularly, *Botryococcus braunii* has the ability to synthesize and secrete a substantial portion of its hydrocarbons into the extracellular matrix, giving it a unique morphological structure.[7–9]

B. braunii is classified into three confirmed chemical races, namely, A, B, and L, each producing distinct hydrocarbon types.[10–12] It is worth noting that a fourth race S is referenced within the literature, the existence of this isolated race is still disputed among researchers because data availability on the produced hydrocarbon is limited.[13]

In addition to hydrocarbons, this alga also produces other high-value compounds such as exopolysaccharides and proteins[14,15] that may have value in pharmaceutical and nutraceutical applications, making it a viable candidate for biorefinery approaches.[8,10,13] The unique feature of lipid secretion into the extracellular space not only reduces the energy cost associated with cell disruption but also preserves cell viability, allowing for repeated extraction and continued cultivation. Furthermore,

B. braunii synthesizes significant amounts of lipids, which are typically between 30% and 70% of its dry biomass and can accumulate botryococcenes alone up to 76% of its organic content.[16]

Despite these promising attributes, large-scale commercialization of *B. braunii*-based biofuels is hindered not only by its relatively slow growth rate but also by high production costs associated with cultivation, harvesting, extraction, and downstream purification. These economic challenges remain the primary barrier to making algal biofuels competitive with conventional fossil fuels. Productivity typically ranges from 0.1 to 0.2 g L^{-1} d^{-1}, which is substantially lower than species such as *Chlorella*, which can reach up to 0.538 g L^{-1} d^{-1}.[17–19] This limitation is attributed to the organism's high metabolic investment in hydrocarbon biosynthesis.[20,21] Current research aims to improve this through strategies such as enhancing photosynthetic efficiency, applying stress-based induction, and optimizing cultivation conditions, including temperature, light, pH, and nutrient availability.[7,22,23] Both suspended and attached systems are being explored for their potential to improve biomass productivity while minimizing energy costs.

While some reviews on *Botryococcus braunii* exist, most focus on either hydrocarbon biosynthesis or cultivation strategies in isolation. Our review is distinct in providing a comprehensive synthesis that combines taxonomic classification, race-specific hydrocarbon biosynthetic pathways, cultivation systems, and advanced lipid recovery technologies such as switchable solvents. Furthermore, we highlight recent genomic insights and their implications for species reclassification, which have not been covered in earlier reviews. This integrated perspective aims to bridge gaps between fundamental biology and applied bioprocessing, offering a roadmap for commercial viability.

RACE CLASSIFICATION

The species *Botryococcus braunii* is an alga found mainly in freshwater but sometimes in brackish lakes, pools, and ponds.[24] Furthermore, they form unique colonies made up of individual cells, held together by an extracellular matrix made of polymerized polyaldehydes derived from fatty acids.[25,26] Unlike most microalgal species, which produce and accumulate lipids only intracellularly, the major part of hydrocarbons produced by *B. braunii* can be extracted from dried algal samples without breaking cells, but by only soaking into an organic solvent such as hexane that does not permeate into the cell wall. Then sequent extractions with another type of organic solvent, such as a mixture of methanol and chloroform, which has more cell permeabilities allows an additional recovery of small amounts of hydrocarbons.[27] This means the alga accumulates both intracellular and extracellular hydrocarbons. While it is not known exactly why this species has evolved to form colonies in this manner, according to Russell et al., a possible explanation can be related to the algae's slow growth rate.[28] As an evolutionary response to this slow growth, the algae evolved to form dense colonies that trap extracellular oil, allowing the algae to float to the surface, providing a greater surface area for sunlight exposure, which subsequently increases the rate of photosynthesis.[28] Generally, the colonial structure of *B. braunii* is described as non-flagellated cells in a "wax-like" substance. Each single cell forming a colony is usually green with the color of chlorophylls *a* and *b*, while an entire colony sometimes shows orange or brownish color because the color of chlorophylls can be masked by orange-red carotenoids that accumulate in the extracellular matrix.

In relation to the color of colonies, it was said that there was a correlation between the color of the colony and the type of hydrocarbons produced in the early days of research on this alga.[29] At first, two unsaturated isomeric hydrocarbons of formula $C_{34}H_{58}$ were isolated from orange algal sample collected in the field and named as botryococcene and isobotryococcene.[16] Next, *n*-alkenes, namely hepatacosa-1,18-diene, nonacosa-1,20-diene, and hentriaconta-1,22-diene were characterized from a strain of the Cambridge culture collection (strain No. 207/1B) showing green color.[30] Based on these findings, it was proposed that there were two reversible physiological states in which the types of hydrocarbons were different.[29] Namely, green and actively growing colonies produce a mixture of *n*-alkenes of the general formula C_nH_{2n-2} and $C_nH_{2n-4,}$ while brown colonies at the resting state contain triterpene hydrocarbons.

In 1983, a triterpene hydrocarbon named "darwinene" with the formula $C_{36}H_{62}$ was isolated from the alga with green color and its structure was reported.[31] At the same time, various new strains of *B. braunii* that can be cultured in laboratories were isolated from the field and their hydrocarbon compositions began to be characterized. As the result, it was found that one strain constantly produces only one type of hydrocarbons and then the concept of "chemical races" was established for *B. braunii*.[8] The chemical race producing a mixture of *n*-alkenes of the general formula C_nH_{2n-2} (n is odd number from 23 to 31) and C_nH_{2n-4} (n is 27, 29 or 31) is called "race A" while the one producing triterpene hydrocarbon homologues of the general formula C_nH_{2n-10} (n is from 30 to 37) is "race B." Shortly after the discovery of these two chemical races, the third race so called "race L" was discovered.[8,10] The race L mainly produces a tetratepene hydrocarbon, lycopadiene.

Since then, many strains belonging to either of three chemical races were isolated, and hydrocarbon compositions of those cultured in the laboratory were characterised.[8] From the literature, it is widely known that races A and B produce larger amounts of hydrocarbons compared to race L. Especially, the highest hydrocarbon content reported in the literature, 61% of the algal dry weight, was obtained from race A, the Chaumecon strain.[8] In contrast, one strain of the race A, Overjuro strain 7, accumulates very few hydrocarbons, only 0.4% of the algal dry weight.[32] Hydrocarbons produced by the Race A are derived from fatty acids.[8,33,34]

On the other hand, Race B produces two types of triterpenoids, namely C_{30}-C_{37} botryococcenes as the major component, and C_{31}-C_{34} methylated squalenes as the minor one. Its hydrocarbon contents are accounting from 10 to 76% of the dry weight.[16,22,33] Race L produces a specific tetraterpenoid of C_{40} known as lycopadiene, accounting for only 0.1 to 8% of the dry weight.[12,35] According to Thapa et al., this race also contains lycopatetraene, lycopapentaene, lycopatriene, and lycopahexaene that account for 5% of the total hydrocarbon fraction.[36] Besides those three chemical races, some strains accumulating eicosane (C_{20} *n*-alkane) or

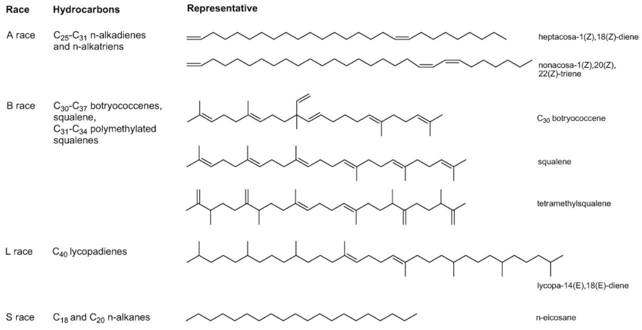

Figure 1. Hydrocarbon production by different races of *B. braunii*[37]

n-octadecanepoxide were isolated and tentatively assigned as "race S" because of the relatively "short" carbon chain length of their products.[13] While there is information on their putative molecular weights obtained by GC-EIMS, no more information, such as retention time on GC, mass spectra showing fragmentation pattern, or NMR data to support structures of those compounds, is available. Thus, the identification of those compounds is still ambiguous because it will be biosynthetically difficult to accumulate only an *n*-alkane with an even-numbered carbon chain as a major component if those compounds are derived from fatty acids. Figure 1 illustrates a summary of the various hydrocarbons produced by each race.[37]

In addition to the classical identification by types of hydrocarbon, molecular phylogeny has been introduced to classify strains of *B. braunii*. The first ambitious attempt of molecular phylogeny based on small subunit ribosomal RNA sequence was carried out for the Berkeley strain (that is nowadays known as the Showa strain in the literature) belonging to the race B.[38] As the result, the alga was classified into Chlorophyceae in this study but it was unfortunately concluded later that the obtained nucleotide sequence was from some green alga that was contaminating in the culture. Next, a phylogenetic analysis based on the 18S rRNA gene was done for four strains of *B. braunii*, CCAP 807/1 (race A), Titicaca (race A), Ayamé (race B), and Songkla Nakarin (race L).[39] Phylogenetic trees made in this study showed that the four strains of *B. braunii* form a monophyletic group in the Trebouxiophyceae. On the other hand, algal strains belonging to race A producing *n*-alkenes were separated from races B and L within the same clade.

After this work, Kawachi et al. made a comprehensive 18S rRNA phylogenetic tree using 31 strains isolated in Japan to see the relationship between hydrocarbons and the molecular phylogeny of *B. braunii,* as illustrated in Figure 2.[13] As a result, classification of strains by the types of accumulated hydrocarbons showed good agreement with molecular phylogeny based on 18S rRNA nucleotide sequences. As for strains assigned to the race S that produce eicosane or *n*-octadecanepoxide, they made a subclade together in a clade composed of strains belonging to race L producing lycopadiene.

To date, the most extensive phylogenetic tree has been produced by Hirano et al., seen in Figure 3.[40] In agreement with previous data, sequences of the 18S rRNA gene show race-specific clades, with the four major races apparent. Race B has also been shown to exhibit variability within the sequence, and as such, two distinct B clusters, B_1 and B_2, have been described.

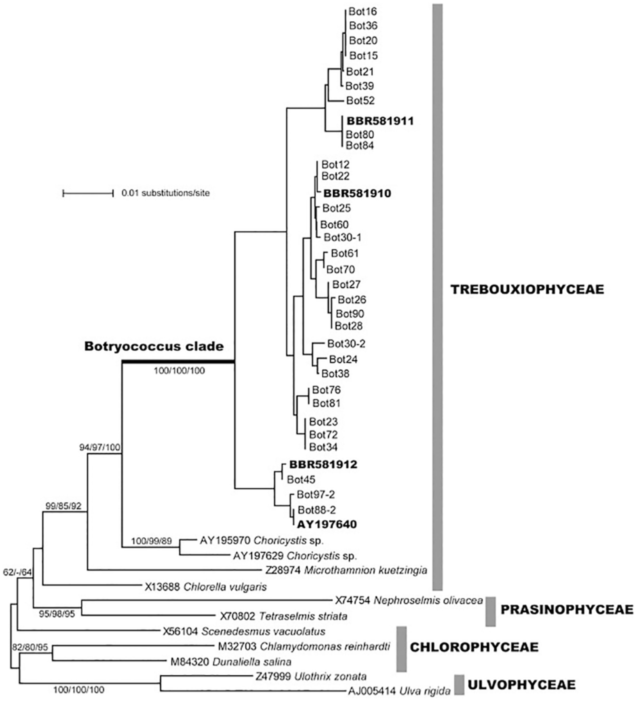

Figure 2. Phylogenetic tree of 18S rRNA gene sequences of *Botryococcus*[13]

Increased sequence availability and improved understanding of the biosynthetic pathways of hydrocarbons in *B. braunii* has allowed not only for more accurate phylogeny to be conducted but have also been used to identify improved techniques for rapid detection and, more recently, distinction between races. The hydrocarbon biosynthetic gene in Race B, *squalene synthase-like protein 3* (*SSL-3*) has been shown to accurately detect and quantify algal abundance in natural environments.[33] The *SSL-3* gene, when co-expressed alongside *squalene synthase-like protein 1* (*SSL-1*) gene, encodes the enzyme responsible for botryococcene biosynthesis, and its presence within samples provides an effective way to quantify *B. braunii*, particularly those belonging to race B.[41,42] Indeed, Hirano et al. demonstrated this by designing species specific primers for the gene, which could reliably detect and quantify race B strains of *B. braunii* under various conditions. More recently, however, Kawamura and colleagues have developed a PCR based approach to distinguish races based on 18S rRNA polymorphisms.[8] The authors utilize a PCR-cleaved amplified polymorphic sequence (PCR-CAPS) technique (referred to as BoCAPS), exploiting the *Hae*III restriction site on the 18S rRNA gene, which varies across the races. Restriction enzymes cut the PCR product at the *Hae*III site where possible, resulting in a race-specific pattern forming. The distinction between race L and race S, however, requires an additional step. The authors acknowledge that further data are required to confidently determine whether this approach can

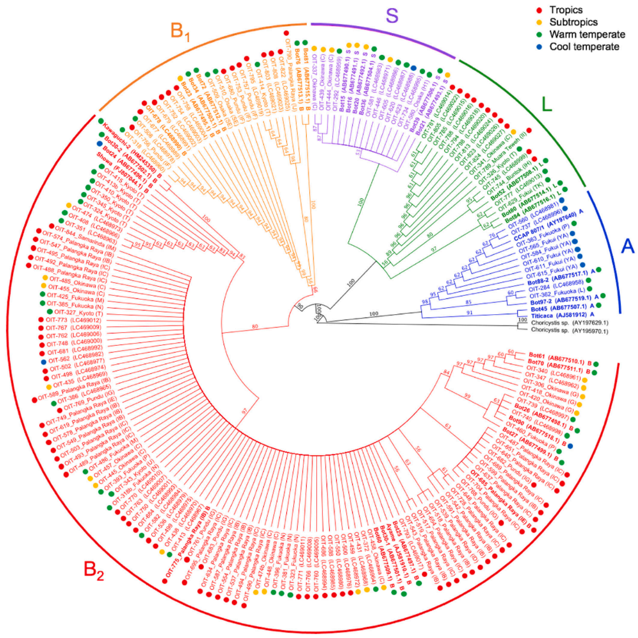

Figure 3. Molecular phylogenetic tree of wild *B. braunii* strains[40]

be applied to further geographic locations beyond Indonesia and Japan; despite this, BoCAPS appears to provide a relatively inexpensive method for initial differentiation between races.[40]

Historically, classification of *Botryococcus* by morphological analyses has also carried out. One example is the study by Komárek and Marvan.[43] In this study, *Botryococcus*-like algae were classified into thirteen species, including five new species: *B. comperei, B. australis, B. fernandoi, B. terribilis,* and *B. pila*. It is, however, difficult to classify Botryococcus-like algae by morphology because even if it is the same strain, it changes its morphology under different culture conditions.[44,45] For example, there is a study in which eight Botryococcus-like algal strains, AICB 413, 414, 416, 418, 440, 442, 870, and 872, were identified as B. terribilis by the morphological classification followed by Komárek and Marvan.[43] Then a phylogenetic tree was made for these eight strains together with other strains identified as *B. braunii* based on the 18S rRNA gene. As a result, all of the strains morphologically identified as *B. terribilis* were completely included in a clade of race B of *B. braunii*. Therefore, algal strains identified as *B. terribilis* in this study should not be classified as a distinct species from *B. braunii* in terms of molecular phylogeny. As this example shows, classification of *Botryococcus*-like algae by morphology is not as precise as molecular phylogeny by 18S rRNA gene or classification by hydrocarbon profiles. Thus, it will be safer not to use the species name for Botryococcus-like algae classified by morphology only.

In relation to this, *B braunii* has been recognized as a single species with three chemical races. A recent study, however, proposed that races A, B, and L of *B. braunii* can be classified into three distinct species.[46] Genome sequences of three strains belonging to race A, B, and L, respectively, were analyzed and compared with those from other *Chlorophyta* algae. It was found that three chemical races of *B. braunii* have sufficiently different genomes as three distinct species. For example, orthogroup analysis revealed that three races of *B. braunii* displayed more gene orthogroup diversity than three related but distinct species of *Chlamydomonas*. Therefore, new species names, *Botryococcus alkenealis* and *Botryococcus lycopadienor*, were proposed to races A and L, respectively, while the species name for the B race remained as *B. braunii*. Within strains classified as race B or L so far, clear branches can be seen in a phylogenetic tree based on 18S rRNA genes. Therefore, as genome analysis of each species progresses, the algal species that have been defined as *B. braunii* may be further subdivided into other species.

BIOCHEMICAL SYNTHESIS OF *B. BRAUNII* HYDROCARBONS

From Figure 1 each race is characterized by its own unique hydrocarbon production. The wide variety of strains within each race of *B. braunii* makes the biosynthetic process and hydrocarbon classification/identification extremely complex. Other than genetic identification, analysis of the hydrocarbons produced is the most accurate method for formally identifying the race and specific strain. It is well established that the race A produces odd-carbon number *n*-alkenes ranging from C_{23} to C_{33}, whereas the race B produces botryococcenes and methylsqualenes that are derived from C_{30} parental compounds, respectively. The race L produces the largest hydrocarbons, C_{40} of carbon atom numbers, most commonly lycopene. Despite these classifications, it is almost impossible to fully profile the entire composition of hydrocarbons produced by each race. This is largely due to the vast variations of each hydrocarbon, making complete isolation of each for analysis virtually impossible. The unusually high levels of hydrocarbons produced by *B. braunii* can be attributed to two distinct sites for hydrocarbon accumulation.[47] An internal pool within the cytoplasm is believed to produce the hydrocarbons, which are then excreted into the external pool, which exists in the trilaminar outer walls. Actually, the composition of hydrocarbons produced by race B is different between the internal pool and the external pool.[48] The internal hydrocarbon pool was composed of less methylated triterpene hydrocarbons compared with the external hydrocarbon pool. Moreover, pulse-chase experiments with radiolabeled precursors were carried out for race B, and shifts of radioactivity were observed from the internal hydrocarbon pool to the external one.[48,49] On the other hand, the same type of experiments conducted for race A did not show such shifts of radioactivity.[47] That means there are two independent sites for hydrocarbon production in race A.

Biosynthesis of n-alkadienes and n-alkatrienes by race A

It is well reported that the hydrocarbons found in race A are alkadienes and alkatrienes. These are straight chained hydrocarbons with chains of C_{23}-C_{31} for alkadienes, and C_{27}-C_{31} for alkatrienes.[8] carbon chain elongation of fatty acids such as oleic acid to form very long chain fatty acids from which the carboxyl carbon is removed, probably by decarboxylation to make a one carbon number reduced hydrocarbon backbone with a terminal double bond.[34,50–53] Very effective incorporation of radiolabeled oleic acid into *n*-alkadienes/trienes was experimentally confirmed, suggesting that the elongation of the carbon chain is carried out by adding acetate units from malonyl CoA.[34] The inhibition of the biosynthesis of *n*-alkenes and very long chain fatty acids in race A with trichloroacetic acid, an inhibitor of the elongation process, supports a similar mechanism to the chain elongation of fatty acids.[54] The chain elongation of fatty acids in plants is carried out by a four-step process catalyzed by four proteins, 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD), and *trans*-2,3-enoyl-CoA reductase (ECR). Genome analyses for three chemical races of *B. braunii* (or three species, namely *B. alkenealis*, *B. brunii,* and *B. lycopadienor*) showed the presence of multiple homologous genes encoding for those four proteins.[46] For example, sixteen genes encoding for the plant specific KCS were detected in race A, while twenty and twenty-five were found in race B and race L, respectively. There were also multiple candidates of genes encoding enzymes involved in other steps of chain elongation of fatty acid (KCR, HCD, and ECR). Therefore, the specific enzymes responsible for the elongation of carbon chains to form *n*-alkenes in race A have not been characterized yet. On the other hand, the final step to form one carbon number reduced *n*-alkenes with a terminal double bond from corresponding fatty acids has not been fully understood, though the inhibition of alkene biosynthesis by dithioerythritol, which is an inhibitor of decarboxylation, strongly suggests the involvement of decarboxylation.[34] In relation to this, an enzyme named fatty acid photodecarboxylase (FAP) was first discovered from microalgae such as *Chlorella variabilis* or *Chlamydomonas reinhardtii,* and the wide distribution of genes encoding for the enzyme through various algae, including *B. braunii,* was confirmed.[55–57] This enzyme directly eliminates the carboxyl group of a fatty acid using light energy and generates *n*-alkane or *n*-alkene with one less carbon than saturated or unsaturated fatty acid as its substrate, respectively. The hydrocarbons produced by FAP, however, do not possess the terminal double bond. Therefore, *n*-alkenes present in large amounts in race A do not seem to be the products of FAP. There was a report on the formation of *n*-alkane from fatty aldehydes by *B. braunii* using a cobalt-porphyrin through decarbonylation.[52] It is, however, worth noting that this mechanism is also not applicable to *n*-alkenes with a terminal double bond generally found in the race A.

In terms of the biosynthesis of *n*-alkatrienes with a double bond at the ω-7 position, radiolabeled putative precursors such as *n*-alkadienes or an unsaturated fatty acid, linoleic acid, which possesses two double bonds in its molecule, were incubated with living algal cells, but radioactivity was not effectively incorporated into such *n*-alkatrienes.[50] This suggests that the additional double bond at the ω-7 position in *n*-alkatrienes seems to be formed by the introduction of a double bond directly to the ω-7 position of oleic acid as the precursor, or desaturation at the ω-7 position of very long chain fatty acyl derivatives as an intermediate.

It is widely accepted that hydrocarbon production occurs at different rates at different growth stages. Hirose et al. investigated the synthesis of hydrocarbons using UTEX 2441, in which they successfully mapped the production routes during the cell cycle.[58] The maximum hydrocarbon production occurred just after the septum formation, and lipid accumulation occurred within the cell apex and basolateral region.[58]

Biosynthesis of triterpene hydrocarbons by race B

Botryococcenes, named after the genus name of *Botryococcus braunii,* are specific hydrocarbons comprising of triterpenoids, acyclic, and cyclic compounds that are synthesized only by race B strains. At first, two isomers of molecular formula $C_{34}H_{58}$ were isolated from a wild sample of *B. braunii* with orange color named botryococcene and isobotryococcene, respectively. Due to similar molecular structures of these hydrocarbons, the isolation, identification, and quantification of each compound is significantly difficult to carry out, there are limited data published, which accurately quantifies each hydrocarbon produced by race B. There are 22 botryococcene homologues, of which the structures have been determined. As mentioned above, "botryococcene" was originally the name for a specific C_{34} homologue isolated for the first time, but it became a generic name for homologues of this type of hydrocarbons. In addition to botryococcenes, race B also produces squalene as well as C_{31}–C_{34} methylated squalenes.[59–61]

Hydrocarbons produced by the race B can also be separated into two fractions, namely, intracellular and extracellular fractions, by two-step extraction using *n*-hexane and a mixture of chloroform/methanol. When hydrocarbon composition is analyzed for the two fractions, the intracellular fraction is rich in lower homologues of botryococcenes such as C_{30}-C_{32}, while the extracellular one contains higher botryococcenes such as C_{33}-C_{34}.[62] In relation to this, when radiolabeled farnesol was added to the culture of race B of *B. braunii*, radioactivity was effectively incorporated into botryococcenes and methylsqualenes. Then radioactivity was shifted from the intracellular hydrocarbon fraction to the extracellular one through time. This suggests that the parental C_{30} botryococcene and squalene are synthesized inside a cell and are secreted outside after methylations. Weiss et al. hypothesized based on their ultrastructural observations that the hydrocarbons are transported through the endoplasmic reticulum to the plasmalemma, then subsequently to the exterior of the cell.[33]

The biosynthesis of botryococcenes has been well documented over the past 50 years, with recent work exploring the mechanisms for optimizing and maximizing hydrocarbon synthesis. The large volume of triterpene hydrocarbons produced by race B suggests that there has to be an efficient production/supply of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), as these are the universal precursors in the biosynthesis of terpenes.[22] IPP was considered to be produced by the condensation of acetyl CoA through the mevalonate (MVA) pathway, and many researchers believed this was the route for botryococcene biosynthesis in *B. braunii* race B. Contrary to expectation, though a labeling experiment using acetate was carried out by Casadevall et al., the level of labeling in botryococcenes was considerably low, at around 0.2%.[63] Later, feeding the algae with 1-^{13}C glucose was carried out, and the ^{13}C-labelling pattern indicated that the synthesis of both botryococcenes and methylated squalenes was achieved using the

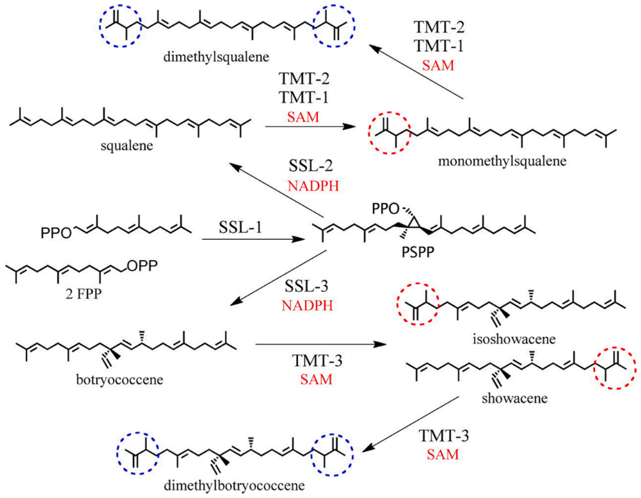

Figure 4. Proposed methylated triterpene synthesis pathway[74]

universal precursors derived from the non-mevalonate pathway, namely, 1-deoxy-D-xylulose-5phosphate (DOXP) pathway.[64]

The biosynthetic pathway to form the precursor for botryococcenes can be divided into two stages. The first step involves the formation of IPP and DMAPP from 1-deoxy-D-xylulose-5 phosphate (DOXP) through the DOXP pathway. There are three DXS isozymes in race B that are believed to help increase the metabolic flow in the production of terpenoids according to Matsushima et al., currently there are no experimental data to support this hypothesis.[65] The DOXP is reduced to produce 2-C-methylerythritol-4-phosphate (MEP), which is then further converted to DMAPP and IPP through five more steps.[66,67] The gene encoding the enzyme, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, that catalyzes the last step of the DOXP pathway, was also identified from race B of the alga.[68]

Squalene is synthesized by the condensation of two molecules of FPP catalyzed by squalene synthase.[69] *B. braunii* also possesses squanele synthase to produce membrane sterols as primary metabolites.[70] On the other hand, botryococcenes were also considered to be produced by the condensation of two molecules of FPP, similarly to squalene, because of its stereochemistry and effective incorporation of labeled farnesol that is a precursor of FPP.[71] Nevertheless, researchers were not able to detect direct incorporation of labeled FPP into botryococcene molecules *in vitro* assay systems for a while. Therefore, Inoue et al. suggested that farnesal or 3-hydroxy-2,3-dihydeofarnesal might be a direct precursor of botryococcenes.[72] Meanwhile, a modified assay system for squalene synthase that can detect the formation of C_{30} botryococcene from FPP was established.[73] By using the assay system, it was suggested that C_{30} botryococcene is biosynthesized from FPP through presqualene pyrophosphate (PSPP), which is the common intermediate to squalene biosynthesis, and distinct enzyme(s) from squalene synthase are concerned with its biosynthesis.[73] Then it was found that the B race of *B. braunii* possesses three squalene synthase such as proteins (SSL-1, 2, and 3) for its unique triterpene hydrocarbon biosynthesis in addition to a conventional squalene synthase that is used for primary metabolism.[70] SSL-1 catalyzes the reaction to generate PSPP from two molecules of FPP. SSL-3 uses not FPP but PSPP as its substrate to form C_{30} botryococcene. SSL-2 also uses PSPP to form squalene. As this system to make a squalene molecule by two enzymes has been found only in the B race of *B. braunii*, squalene molecules derived by SSL-2 seem to be used for the biosynthesis of various squalene derivatives specific to the B race.

Methyltransferases that introduce up to two methyl groups into triterpene hydrocarbons of the B race were also identified.[74] Though methyltransferases that can introduce third or further methyl groups into botryococcene have not been characterized yet. Based on the findings by Niehaus et al., a biosynthetic pathway was proposed, as depicted in Figure 4.

Biosynthesis of race L tetraterpene hydrocarbons

Not like other two strains, the hydrocarbon composition of the L race is rather simple. A triterpenene hydrocarbon, lycopadiene, is always the main component in the hydrocarbon fraction, though very small amounts of homologues of lycopadiene with different numbers of double bonds, such as lyocopatriene or lycopapentaene, could be detected.[39,41] The biosynthesis of lycopadiene is initiated by a squalene synthase-like enzyme, lycopaoctaene synthase, that catalyzes the condensation of two molecules of geranylgeranyl diphosphate (GGPP). The presence of lycopapentaene or lycopatriene in hydrocarbon fractions suggests that the *de novo* synthesized lycopaoctaene seems to be reduced to lycopadiene. Enzymes, however, concerned with such reductions have never been identified, yet.

CULTIVATION SYSTEMS

Like all species of microalgae, *B. braunii* can be cultivated in either an open system (open ponds, raceways, tanks) or a closed system (airlift tubular tanks, flat panels, stirred vessels). Each has its own merits and demerits, which depend on several factors which include, including cost, quantity, location, strain properties, and resource availability. Closed systems offer a more controlled cultivation environment with a reduced capacity compared to open systems, which can be magnitudes of 10 times greater than closed systems. However, the threat of contamination of unwanted chemicals or other biological species competing and inhibiting the growth significantly hinders the efficiency compared to closed systems. This trade-off between volume and controllability is something that must be accounted for when designing the appropriate system for any microalgae.

Natural systems

B. braunii can be found in freshwater and brackish ponds, lakes, and rivers around the world. Despite the global reach of this species, the population density is generally low, yet very distinctive, with a thick green layer floating on top of the water.[75] Table 1 summarizes the most common locations around the world where a wide range of *B. braunii* species can be found/obtained, both in nature and within commercial and academic institutions. The key distinction between laboratory and natural culture conditions is that in most cases, laboratory cultures are almost pure cultures

Table 1. *B. braunii* race isolation locations

Culture Location	Race
Yamoussoukro, Ivory Coast, Cote d'Ivoire	Race L
Miyagi, Japan	Race B
Fukui, Japan	Race B
Palangka Raya, Indonesia	Race A/B
Bunto, Indonesia	Race B
Hokkaido, Japan	Race B
Tenggarong, Indonesia	Race A/B
Samarinda, Indonesia	Race A

grown and maintained under a set of precise control conditions either for research purposes or for maintenance. Whereas natural cultures refer to naturally occurring species in freshwater and brackish lakes and ponds that are accompanied by a wide range of other microbial species. It is worth noting that the distribution of *B. braunii* races presented in Table 1 reflects published sampling studies and may not represent the actual global abundance of each race. The entries correspond to specific documented strains and do not imply ecological dominance.

Open systems

Open systems, often referred to as open or raceway ponds, are both subjected to the open environment, with the addition of a paddle wheel in raceway ponds to promote mixing and reduce dead regions within the system.[76] These ponds rely on exposure to large quantities of sunlight in vast open planes. To maximize the efficiency, these ponds typically have large surface areas with depths of between 20 cm and 50 cm.[76,77] The circulation velocity varies depending upon the size of the pond, but it is typically kept between 5 and 30 cm/s.[78] Larger systems that involve fragile cultures will often use multiple paddle wheels to mitigate cultural damage due to turbulent flow. While sunlight can penetrate water to depths of 200 m, the increasing colony density of *B braunii* during the growth phase creates a somewhat self-inhibition. This results in reduced photosynthetic rates for cells at the bottom of the pond, deep-water ponds would decrease the efficiency of the cultivation process.

Williams II et al. describe the development of an open system incorporating robotics to enhance algae cultivation.[79] In large-scale open ponds, one major challenge is maintaining even water distribution and effective harvesting, as poor mixing can create stagnant zones that promote bacterial contamination and hinder algal growth. The robotic system addresses this by adjusting paddlewheel speeds to optimize circulation. Additionally, a suspended wire-based harvesting mechanism enables algae collection without requiring a full system shutdown, supporting a more efficient semi-batch operation. Although promising, the system still requires further refinement alongside advancements in robotic technologies.[79]

Rao et al. investigated the impact of seasonal variation on the growth rate and hydrocarbon production of race A (LB-572) and race B (*N*-836) under open raceway and circular pond cultivation. Their results highlight the differential adaptability of each race to environmental fluctuations.[80] This is an important parameter to consider with open systems, since they are open to the environment, and at the mercy of whatever the weather conditions are, hence why most of these systems exist in tropical and subtropical climates close to the equator, where annual changes in weather are marginal, compared to those closer to the poles. Rao et al. found that race A, using a raceway pond, has a 24% hydrocarbon content, whereas the circular pond only achieved a 19% hydrocarbon content after 18 days of cultivation with a biomass yield of 2 g/L.[80] Race B recorded a hydrocarbon content of 27% after 25 days of cultivation with a biomass yield of only 1 g/L.[80] These findings are concurrent with overall literature observations, illustrating that generally, race B produces larger quantities of hydrocarbons compared to the other races. Ashokkumar et al., reported that the AP103 strain achieved similar levels for lipid yield (17%–19%) and hydrocarbon yield (13%–11%) when cultivated in an open raceway pond compared to laboratory conditions.[81] The cultivation period is highly dependent upon several biotic and abiotic factors and therefore should not be used as a means of evaluation. Furthermore, race B has a significantly lower growth rate compared to race A; therefore, it takes longer for race B to reach a similar biomass level.

Ruangsomboon et al. reported that the KMITL 2 strain achieved a maximum hydrocarbon and lipid content of 58.8% and 74.39%, respectively, with peak productivities observed on day 18. The authors attribute this to nutrient limitation driving enhanced hydrocarbon synthesis.[82] Additionally, outdoor cultivation in a small oval pond produced a 2.04-fold higher biomass yield compared to a large raceway pond; despite a slightly lower hydrocarbon content, the overall hydrocarbon yield was 2.73 times greater, emphasizing the impact of cultivation system on productivity.[82]

A key constraint with open systems is the risk of contamination, both biological and non-biological. A common contaminant is bacteria, whereby, depending upon the aggressiveness of the species, the foreign entity may consume the algae directly, or compete for the same nutrient source. Other means of contamination can be from other algae species or toxic compounds from the surrounding area. For example, agricultural runoff can introduce high levels of nitrates and nitrites, whereas industrial waste in all states can introduce toxic chemicals, directly or indirectly, i.e., gas absorption.

Given the persistent issue of contamination during *Botryococcus braunii* cultivation in wastewater, various strategies have been explored to enhance culture resilience and maintain productivity. One effective approach, highlighted by Song et al., involves initiating cultivation with a higher algal cell density, particularly in mixed cultures, to establish dominance over competing organisms.[83] Interestingly, the authors found that higher density cultures of *C. vulgaris* improved the growth of *B. braunii* by supplying additional chlorellin containing C18 fatty acids.[83] To further limit microbial interference, other researchers have proposed cultivating *B. braunii* under extreme physicochemical conditions, such as elevated temperatures or unbalanced pH levels, which are unfavorable to most contaminants.

The use of co-culture systems involving either beneficial bacteria or other algal species has also gained attention. These systems can enhance resistance to biological threats such as grazers or parasitic species, potentially increasing culture stability.[56,84] However, a major drawback of this method lies in the downstream processing phase: since different species contribute distinct metabolic outputs, their co-existence complicates product purification and limits biomass valorization. Interestingly, some studies have noted that co-cultivation with bacteria or fungi may actually simplify biomass harvesting by promoting aggregation or floc formation, thereby improving separation efficiency post-cultivation.

Closed systems

Open systems are unsuitable for research that aims to investigate the effect/influence of different abiotic factors on parameters such as growth rate and biomass productivity. To achieve a controlled cultivating environment the use of a closed system is required. Closed systems are more commonly known as photobioreactors (PBRs), and can be in several different configurations, depending on the requirements of the research being undertaken. Typical examples of PBRs include tubular, column, bag, and flat plate. These can be further categorized into either suspended or immobilized systems, depending on the requirements of the algae, or the nature of the investigation. Regardless of the different configurations, the general principles of closed systems remain the same, i.e., a transparent plastic/glass tube or cylinder with varying diameters and heights to maximize the surface area to volume ratio, for maximum photosynthesis. Russell et al. discuss the characteristics and features of several PBRs and highlight that a key issue with closed systems is the accumulation of dissolved oxygen.[2] The authors go on to explain that high levels of dissolved oxygen inhibit the photosynthetic rate of microalgae. The volume of accumulated dissolved oxygen varies depending on the reactor configuration.

A study by Gouveia et al. investigated the use of a bubble column photobioreactor using seven *B. braunii* strains, namely, AC755, AC759, AC761, CCALA777, CCALA778, CCAP807/2, and Showa.[18] The Showa strain achieved the highest hydrocarbon content at 42% of dry biomass, followed by AC761 with 39%. In contrast, CCALA777 accumulated the highest carbohydrate content, reaching 76% of dry weight, while its hydrocarbon content remained low at 4%. Strains AC755 and AC759 exhibited moderate performances, with hydrocarbon contents of 21% and 23%, and carbohydrate levels of 39% and 44%, respectively. CCALA778 accumulated 36% carbohydrates with low hydrocarbon levels (8%) and showed elevated fucose concentrations. CCAP807/2 displayed intermediate values, with a hydrocarbon content of 19% and carbohydrate content of 38%.

In the study by Xu et al., a fed-batch cultivation strategy using an airlift photobioreactor was employed to enhance the biomass and lipid productivity of *Botryococcus braunii*.[85] The reactor's design enabled gentle mixing with minimal shear stress, which preserved the delicate colonial morphology of *B. braunii* while ensuring efficient gas exchange and homogenous light distribution throughout the culture. Over a 19-day cultivation period, biomass concentrations were substantially improved, increasing from an initial 1.82 g/L after 15 days to 2.87 g/L by day 19 through optimized operational strategies.[85] The vertical circulation characteristic of the airlift system maintained the cells in suspension while promoting effective carbon dioxide utilization and limiting sedimentation, thereby supporting sustained algal growth. This study demonstrated that airlift photobioreactors offer a robust and scalable closed cultivation platform for *B. braunii*, achieving higher biomass yields compared to traditional batch systems while maintaining culture integrity essential for long-term productivity.[85]

Attached systems

Attached microalgal cultivation systems have gained increasing attention as a sustainable alternative to traditional suspended cultures, offering significant advantages in terms of resource efficiency and process scalability.[86–88] Unlike conventional systems, where algae are suspended in large volumes of water, attached systems facilitate growth on solid substrates such as membranes, films, or panels. This configuration drastically reduces the volume of water required for cultivation and simplifies downstream harvesting. Studies have demonstrated that water use in attached cultures can be nearly halved compared to suspended systems, making them highly attractive for large-scale deployment in water-limited regions.[89]

A key benefit of this system lies in the ease of biomass recovery. Since the algae grow adhered to surfaces, harvesting can be accomplished using basic mechanical methods such as scraping, which negates the need for energy-intensive processes such as centrifugation or filtration.[90] Reported data suggest that this approach can reduce energy consumption associated with harvesting by over 99%, representing a significant step toward energy-positive biofuel production.[89,91]

In terms of productivity, attached cultures are capable of supporting much higher cell densities. For example, *B. braunii* NIES836 has achieved volumetric biomass concentrations exceeding 30.73 kg m^{-3} under attached conditions, nearly 60 times greater than what is typically achieved in open ponds and around five times that of closed photobioreactors.[92] The dense growth not only enhances productivity per unit volume but also supports continuous or semi-continuous biomass collection without severely disrupting the culture system. Moreover, the compact nature of attached systems allows for efficient space utilization. Comparative analyses have shown that these systems may require up to five times less land and roughly 4.5 times lower capital investment than suspended cultures to achieve similar yields.[93] These features make them particularly suitable for urban or industrial settings where land and utility access may be constrained.

Attached cultivation systems have also shown promise in diverse applications such as enhanced lipid extraction, nutrient removal in wastewater treatment, and hybrid integration with other cultivation systems.[94–96] This versatility, coupled with their operational efficiency, positions attached systems as a compelling strategy in the development of next-generation algal bioprocessing technologies.

CULTIVATION CONDITIONS

Like all microalgae species, the cultivation conditions are fundamental to increasing the biomass productivity and high-value compound synthesis. *B. braunii* cultivation has historically been more difficult to cultivate than other species, such as

Chlorella vulgaris, Haematococcus pluvialis, and *Scenedesmus obliquus*. This is well documented within the literature, but appears to be more directed at race B and L, rather than A. While there is no general conclusion as to why this is the case, one possible explanation is centered on their morphology, since race B forms dense colonies that are fragile to the molecular diffusion of compounds that may break the intramolecular bonds holding the colonies together. Despite the fragile nature of the colonies, a recent study by Murayama et al. developed a simple method for the regeneration of strain NIES836 colonies from a single cell.[97] Optimal cultivation conditions of *B. braunii* are generally similar to those of most microalgae species. It is reported within the literature that *B. braunii* often requires higher cultivation temperatures; however, it is worth noting that most commonly used strains originate from subtropical or temperate regions and therefore do not *B. braunii* strains require elevated temperatures.

To increase the biomass yield and high-value compound biosynthesis, investigation into the effects and responses to different operating conditions, such as light intensity, temperature, pH, salinity, CO_2, and nutrients, can be simulated in an almost limitless number of configurations. Researchers usually employ the use of design of experiment (DoE) to get a more profound understanding of the most influential operating conditions and ranges that promote the highest quantity of high-value compounds and biomass yield.

Temperature response

The optimal temperature for *B. braunii* is strain-specific, along with the other factors being changed simultaneously. According to Garcia-Cubero et al., the optimal temperature range for the strain CCALA 778 ranges from 23°C to 26°C and is tolerant to temperatures as low as 4°C.[98] There are little to no further data that suggests that any strain of *B. braunii* can tolerate such low temperatures, and therefore, tolerance does not imply sustainable growth. Yoshimura et al. showed that the Showa strain did not grow at all at 5°C.[99] The authors simulated summer and winter conditions by changing the light irradiance and temperature. They found that under winter conditions of 825 μmol $m^{-2}s^{-1}$ and 23°C yielded a maximum biomass of 1.4 gL^{-1}, whereas under summer conditions of 2000 μmol $m^{-2}s^{-1}$ and 26°C yielded a maximum biomass of 1.3 gL^{-1}.

Yoshimura et al. investigated the growth response of the Showa strain using nine temperature levels (5, 15, 20, 25, 27.5, 30, 35, 38, and 45°C), combined with six to ten photosynthetically active radiation (PAR) levels between 0 and 2000 μmol $m^{-2}s^{-1}$ for each temperature.[99] The authors found that the algae did not grow at the extreme temperatures of 5°C and 45°C, whereas at 35°C and 38°C, initial growth was detected just after inoculation, growth decreased significantly. It was concluded that the optimal temperature range for the Showa strains is between 15°C and 30°C, with a maximum specific growth rate of 0.496 $days^{-1}$ occurring at 30°C.[99] This is surprisingly higher than what most researchers consider the optimal, as research by Li et al. compared the growth of three strains of *B. braunii*, one from China, Japan, and the UK.[100] Li et al. examined the growth and lipid content at 20, 25°C, and 30°C intervals over a 30-day period. The authors found that the Chinese strain grew best at 20°C–25°C, whereas the UK and Japanese strains showed the highest growth at 25°C.[100] These findings support the work done previously by Casadavall et al. and Fernandes et al., respectively.[21,101]

A article by Kalacheva et al., investigated the effect changing temperatures has on both the intracellular and extracellular lipids in the *B. braunii* strain known as Kutz No LB 807/1 Droop 1950 H-252 obtained from Cambridge University, UK.[102] The authors set three temperature levels, namely, 18°C (suboptimal), 25°C (optimal), and 32°C (supraoptimal). Cells were cultivated for 14 days, with the highest biomass being recorded at the supraoptimal of 0.7 g/L, followed by 0.44 g/L at the optimal, and 0.43 g/L at the suboptimal level.[102] Kalacheva et al., confirmed they could not reveal any significant statistical differences in the volume of intracellular lipids produced volume of intracellular lipid production, between suboptimal and optimal conditions. They recorded a decrease in lipid content from 22 to 5 dry wt % after 13 days in the supraoptimal region. In regard to extracellular lipid production, the authors also stated that there was no viable statistical data regarding the change in cultivation temperature and extracellular lipid production, although the authors do note that there was a considerable variation in the extracellular lipid concentration, ranging from 2 to 15 mg/L.[102] The relationship between cultivation temperature and intracellular lipid production is not surprising, since the biosynthetic pools that produce intracellular lipids are mainly for cell metabolism.

Al-Hothaly et al. conducted a 60-day cultivation study on two *B. braunii* strains, Kossou-4 and Overjuyo-3, across three different temperature settings. Their investigation revealed that 25 °C was the optimal temperature for maximizing both biomass accumulation and oil production.[103] Upon further refinement of additional growth parameters such as light intensity, nitrogen concentration, and iron availability, they achieved substantial enhancements in productivity. Biomass yields for the Kossou-4 and Overjuyo-3 strains increased markedly from 16.47 g/L and 31.37 g/L to final concentrations of 173.90 g/L and 217.21 g/L, respectively. Likewise, oil content rose from 3.24 g/L and 2.11 g/L to 26.42 g/L and 22.06 g/L for the two strains.[103] This work is concurrent with the findings of Kalacheva et al., who used the LB 807/1 Droop 1950 H-252 strain and found that although overall biomass production peaked at 33 °C, the highest accumulation of both intracellular and extracellular lipids occurred at 25 °C.[102]

Temperature regulation becomes even more critical in attached culture systems, where the reduced water volume can lead to greater fluctuations in thermal conditions. Murphy et al. noted that increasing the water layer's thickness in these systems can help buffer against temperature swings, improving cultivation stability.[104] However, this advantage comes at the cost of elevated water loss through evaporation, which accounts for up to 95% of total water consumption in attached systems. The large surface area typical of attached photobioreactors intensifies this evaporative effect, presenting both a design challenge and an opportunity for optimization in large-scale operations.[89]

Cultivation media

The selection of the appropriate media for cultivation is critically important when aiming to optimize the growth and biomass yield, as different culture media contain varying salt compounds, in varying quantities, as well as changes to the trace elements. The selection of the appropriate culture media for *B. braunii* is highly dependent upon the race and sub-race species. From the literature, the most commonly used culture media for *B. braunii* is a modified Chu13. There are, however, several variations to the modified Chu13 recipe, which are used by individual research groups; all, however, stemming from the original. The original Chu13 media contained citrate and Fe-citrate salts, which were later modified by French research groups, whereby they replaced the Fe-citrate with Fe-EDTA.[105] These changes aim to either stabilize the microalgae growth, or induce stress conditions, to promote the production of biochemical compounds. A study by Yoshimura et al. used selenium in the form of disodium selenite to investigate the effect this new modification has on the growth rate of the Showa strain. They found that in systems where the medium lacks sufficient micronutrients, the additional vitamins and selenium can influence the growth.[99] It is worth noting that this recipe is not widely used among the niche research communities of *B. braunii*.

The optimal salt concentration is also highly dependent upon what parameter is being measured. Research concerning the species LB572, which is one of race A, found that a modification to the concentration of potassium phosphate and magnesium sulfate had the most impactful influence on both biomass productivity and lipid production.[106] The optimal concentration of potassium phosphate and magnesium sulfate based on biomass productivity was found to be 0.058 g/L and 0.09 g/L, respectively. Furthermore, the optimal concentration of potassium phosphate and magnesium sulfate based on lipid productivity was found to be 0.083 g/L and 0.100 g/L, respectively.[106] A study by Dayananda et al. cultivated the SAG 30.81 and LB-527 strains using four different growth media (BBM, BBM with ammonium, BG11, and modified Chu 13) and found that BG11 was the best for optimal growth and maximizing hydrocarbon production.[107] Despite this, many other researchers claim that the modified Chu 13 is the most effective.

Nutrient deprivation

Nutrient deprivation, particularly nitrogen limitation, is widely applied as a deliberate cultivation strategy to trigger metabolic responses and enhance hydrocarbon accumulation, rather than being part of the standard growth environment. This approach is often integrated with medium composition adjustments or productivity optimization protocols to balance biomass growth and lipid synthesis. Al-Hothaly et al. found that a reduction in nitrogen concentration increased both the biomass and oil production of the strains Kossou-4 (Ko4) and Overjuyo-3 (Ov3) using a modified BG11 medium that contained half the quantity of nitrogen.[103] The original BG11 contained 1500 mg L^{-1} which yielded 32.4 mg L^{-1} and 21.1 mg L^{-1} of oil for Ko4 and Ov3, respectively. Whereas at 750 mg L^{-1}, the oil production achieved was 254.9 mg L^{-1} and 214.3 mg L^{-1}, respectively.[103]

The strain diversification of *B. braunii* causes conflicting findings within the literature, suggesting that the response to nitrogen and indeed, phosphorus is highly dependent on the strain itself. This is evident when searching for an *optimal* growth medium, as generally it varies between BG11, BBM, and a modified Chu 13 medium. A article by Cheng et al. reported that a modified Chu 13 medium was optimal for the SAG 807-1 strain obtained from the SAG culture collection.[108] The same authors also varied the concentration of sodium nitrate and potassium nitrate present in the medium, using urea and ammonia as a means of comparison on the biomass productivity.[108] The authors found that nitrogen was essential for growth and determined that nitrogen in the form of nitrates was the most favorable for biomass yield. On the contrary to this, Nakamura et al. found that the Showa strain had the highest growth rate using ammonium, in the presence of the buffer N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), as this mitigates the toxicity effects of ammonium.[109] Of all 6 samples, Cheng et al. found that potassium nitrate had the highest biomass productivity at 6.81 $gm^{-2}d^{-1}$, followed by sodium nitrate at 6.45 $gm^{-2}d^{-1}$. This coincided with data published by Flynn, whereby the uptake of ammonia nitrates is considerably higher initially; however, as the concentration of ammonia within the cell increases, the product of ammonium assimilation causes rapid inactivation of nitrate transport within the cell's metabolic systems.[110]

Some data suggests that vitamins such as vitamins B and C should be added periodically to the culture in order to promote and sustain a healthy culture.[19] According to Tanabe et al., no algae can synthesize vitamin B_{12}, however, B_{12} functions as a cofactor for several metabolic pathways, suggesting that the algae salvage B_{12} from their natural external environment.[111] It is generally accepted that algae obtain their B_{12} requirements from coexisting bacteria present in the growth medium. The morphological colonial structures are capable of trapping bacteria that might be utilized to achieve their B_{12} requirements, and this could explain why *B. braunii* have highly efficient metabolic pathways and biosynthetic pools for hydrocarbon production.[112]

Xu et al. used the use of a closed airlift cultivation system over a 15-day period during which biomass reached approximately 1.82 g/L, and nutrient feeding was initiated to sustain and enhance further growth.[85] The study identified nitrate as the principal limiting nutrient in the system, while phosphate, even at higher concentrations, did not significantly affect biomass accumulation. An optimized molar ratio of nitrate to phosphate at 34.7:1 was established, suggesting that starting with the full initial nitrate concentration and only one-quarter of the phosphate concentration effectively supported growth without nutrient waste. Upon administering a single nutrient feeding, biomass levels increased to 2.56 g/L after 18 days. Moreover, introducing two feeding events spaced two days apart resulted in a higher biomass concentration of 2.87 g/L by day 19.[85] Importantly, this incremental feeding strategy allowed the culture to avoid nutrient depletion phases that would otherwise induce lipid accumulation through stress responses at the cost of biomass productivity. The fed-batch method preserved active cell division for longer durations, thereby improving the overall volumetric yield compared to batch culture approaches.

Light wavelength and duration

Light variation, both in wavelength and duration, is one of the most varied and researched growth factors of *B. braunii*. Light is the most important factor for photoautotrophic cultivation, as this provides the essential energy for the synthesis of metabolites. The maximum absorption of light is critical in achieving the maximum yield of biomass and desired metabolites. Like with most factors affecting microalgal growth, the term *optimal* is subjective and highly dependent upon what is being investigated; for example, optimal growth light requirements differ from optimal metabolism light requirements.[113]

Photosynthesis occurs in membrane-bound compartments within the chloroplast known as thylakoids. There are generally three pigment groups required for photosynthesis, namely, chlorophyll, carotenoids, and phycobilins. It is worth noting that chlorophyll is the only one to possess the core antenna for capturing light; however, the presence of carotenoids acts as antioxidants, protecting the thylakoids.[114] Each of these pigments has a wavelength range in. The amount of light absorbed by the intracellular pigment chlorophyll is affected by the shading between the individual cells/colonies, resulting in light attenuation, whereby the light intensity distribution varies significantly.[114]

Investigation by Ruangsomboon et al., on the wild strain KMITL 2, native to Thailand, found that the optimal light intensity for biomass production, lipid content, and lipid yield was 87.5, 538, and 538 μE m^{-2} s^{-1}, respectively.[115] The authors also varied the light durations and found that optimal biomass production was achieved at 24-h light, lipid content, and yield at 16 h light 8 h dark.[115] This highlights that the term *optimal* is subjective to the investigated variable. Several methods have been developed for determining the relationship between biomass growth and metabolite production with changes to light wavelength, duration, and intensity.

Sakamoto et al. used a staggered light varying system to determine the effect of changing the light intensity on the concentration of chlorophyll, dry weight of biomass, lipid, and sugar production over a 20-day period. Each sample was cultivated for 8-day using a light intensity of 40 μmol m^{-2} s^{-1}, after which half the samples were subjected to 200 μmol m^{-2} s^{-1} for the remaining 16 days, while the other half remained under 40 μmol m^{-2} s^{-1}.[116]

The strain Bot-144, which is a constituent of race B, primarily produces triterpenoid hydrocarbons, which when subjected to stress conditions, distinct color changes occur. Baba et al. investigated the effect of red, green, and blue light on the morphology, photosynthetic rate, and hydrocarbon production of the strain.[112] It was concluded that the photosynthetic rate was 1.8-fold greater using blue compared to red, with green having the least influence.[112] A surprising conclusion made by Baba et al. was that the colony shape, color, and rate of photosynthetic CO_2 fixation were influenced by changes in light color, whereas the metabolic pathway for hydrocarbon production was not. Furthermore, while blue light yielded the highest carbon fixation, red light was deemed optimal for the production of hydrocarbons.[112] This suggests that these variables are dependent upon the extreme ranges of the visible light spectrum. These findings were corroborated by Okumura et al., who determined that high intensity blue light was the most efficient for the biomass production of *B. braunii*.[117]

Few studies have considered the light path length as an influential factor in microalgae cultivation, as most consider the wavelength and duration. Wang et al. used a two-factorial design of varying light path length (0 cm–15 cm) against the incident light intensity (0–800 μmol/m^2/s) and found that the shorter the light path length, the greater the biomass concentration of IPE 001 (race B).[113] A maximum biomass concentration of 2.54 g/L was achieved at 690 μmol/m^2/s with a path length of 2.5 cm.[113] Photoinhibition caused negative effects on the growth rate at extremely high light intensities. Optimal hydrocarbon production was influenced by higher light intensity and longer light paths. These findings were contradictory to the work by Yoshimura et al., who found that there was no correlation between light irradiance and hydrocarbon production using the Showa strain.[99] Furthermore, Sakamoto et al. found that higher light intensity was required for optimal biomass compared to hydrocarbon production, using Bot-22.[116] These findings highlight further the diversity within each race of *B. braunii*, as IPE 001, Showa, and Bot-22 are all part of race B.

Agitation/aeration

While there are not any independent studies concerning the effects agitation/aeration has on the growth rate and lipid production, from the literature, it is clear that any disturbances to the structure of the colonies have a detrimental consequence on productivity and growth. This is particularly prominent in race B cultures, as typically the colonies are significantly more dense and larger compared to race A and L. Increased agitation through mechanical mixing or aeration results in more intense eddy formation and turbulence, which can rupture and break the colonies. The increase in kinetic energy can also inhibit the diffusion of vitamins and nutrients required for cellular activity. Liu et al. investigated the *B. braunii* 765 strain using swine lagoon wastewater and found that with aeration assistance, the hydrocarbon accumulation was 23.8% compared with sterile cultivation in BG11 media, achieving only 11.9%.[118]

pH

The response to changes in pH varies significantly, depending on the method of varying the pH. The most common method is varying the CO_2 flow rate, followed by changes to particular salt concentrations containing nitrogen and phosphorus compounds within the modified medium. These again vary depending upon the scope of research. Dayananda et al. investigated the impact varying growth parameters, such as pH has on the biomass yield and hydrocarbon production of the *N*-863 strain, obtained from the National Institute for Environmental Studies, Tsukuba, Japan.[119] The authors concluded that there was no significant effect on both the biomass yield and hydrocarbon production with varying the pH between 6 and 8.5, with the optimal being 7.5 for both biomass and hydrocarbon production.

While it is well established that the hydrocarbon production with *B. braunii* is not pH-dependent, research by Jin et al. confirmed this and determined that biomass productivity was not pH-dependent using the SAG 30.81 strain, which belongs to race A.[120] The authors used a modified AF-6 medium as a standard, plus a 2P-AF-6 (2-fold phosphate) and 3N6P-AF-6 (3-fold sodium nitrate and 6-fold phosphate) to simulate a pH range of 5.5–8.0. Since one of the main precursors for hydrocarbon production in race A is oleic acid, the authors found that when only considering the effect of pH in the range of 6.0–8.0, there was a linear relationship between extracellular and intracellular production ($R^2 = 0.939$).[120] When considering pH levels of 5.5, this relationship is invalid. It was concluded that the optimal pH for hydrocarbon production was 6.5 across all three media. Furthermore, the most influential factor for pH is related to dissolved oxygen when considering hydrocarbon production. Biomass productivity was almost constant in the pH range 6.0–8.0, with a decrease at more acidic conditions at a pH of 5.5.[120] The optimal pH is highly sensitive to the race of *B. braunii* due to their different metabolic pathways for lipid production, both intracellular and extracellular. It is generally observed that *B. braunii* favors slightly acidic cultivation conditions. The true extent of this relationship required more investigation.

Salinity

B. braunii is typically found in freshwater habitats, so it is generally expected to exhibit optimal growth in low-salinity conditions.[121] Nonetheless, this microalga has shown a degree of tolerance to saline environments with some strains surviving in sodium chloride (NaCl) concentrations as high as 3 M.[121],[122] Despite this tolerance, peak growth performance has been observed at a lower NaCl concentration of 0.25 M.[123] It is worth noting that research on how salinity influences both the growth rate and lipid production of *B. braunii* has produced inconsistent results.

The effect of salinity on *B. braunii* is often measured against changes to the lipid profiles. The salinity concentration is manipulated by changing the quantity/concentration of sodium chloride present within the growth media. It is worth noting that increasing the sodium chloride concentration will stimulate changes to the pH, most likely due to the carbonate chemistry occurring within the water. It is therefore considered that the responses observed are independent of changes to the pH, as Yoshimura et al. found that varying the pH to the same range yielded different results.[99] When considering the effect of salinity on *B. braunii* growth rate and lipid productivity, the race has a significant impact on the tolerance to increasing salt concentrations. This can be linked to the colony structure and density.

The Showa strain, which is part of race B, showed a significant decrease in growth with increasing NaCl concentration. Unlike the study by Kalacheva et al., Yoshimura et al. investigated salinity concentrations between 0.4M and 18.1M.[99] The authors reported little change to the growth rate at 0.7M, which supports the work by Kalacheva et al., for an increase in lipids at this concentration.[124] The Showa strain appears to have a relatively low tolerance to changes in salinity, which is why this strain does not grow well.[99] The growth and lipid content of a strain from China (CHN-357), United Kingdom (UK 807.2), and Japan (JAP-836) were investigated using 0.00M, 0.15M, 0.25M, and 0.50M of NaCl by Li et al.[100] The authors observed a decrease in growth rate with increasing NaCl concentration in all three cases. The relative lipid content was not affected by salinity in the CHN-357 strain; however, the JAP-836 strain showed the most significant reduction in lipid content.[100]

Furuhashi et al., first explored the effects of salinity on the growth and hydrocarbon accumulation of the *B. braunii* Showa strain using four different culture media: modified Chu13 (freshwater), natural seawater, artificial seawater, and a NaCl-supplemented medium.[125] To equalize osmotic pressure across treatments, 8.8 g/L of NaCl was added to the modified Chu13 medium, matching that of the artificial seawater. Cultures were maintained under standardized conditions - 50 μmol m^{-2} s^{-1} light intensity, 12:12 light/dark cycle, and 25 °C -for 35 days. The study found that although growth was inhibited under saline conditions, with specific growth rates dropping by around 50%, hydrocarbon content increased significantly. After three culturing cycles in saline media, the hydrocarbon concentration reached 39.6%, compared to 30.7% in the freshwater control.[125]

Building upon this earlier work, the same research group conducted a more detailed investigation in 2016, focusing specifically on hydrocarbon extractability in response to salinity.[126] They reported that culturing the Showa strain in 0.3% artificial seawater (equivalent to ~3 g/L NaCl) maintained biomass productivity (5.7 g/L) comparable to that in freshwater medium (5.5 g/L), while significantly enhancing hydrocarbon recovery from wet biomass. Higher salinities (0.5% and 0.7%) led to reduced biomass yields (4.7 g/L and 3.9 g/L, respectively), but facilitated even greater hydrocarbon extractability - exceeding 90% at 0.7% salinity following extended agitation with *n*-heptane.[126]

Increasing the salinity in the LB-572 species had a positive effect on the biomass productivity according to Rao et al.[122] The authors varied the NaCl concentration between 17 mM and 85 mM and found that maximum biomass productivity was achieved in 17 mM and 34 mM salinity. A similar trend was observed for hydrocarbon production, with maximum content being achieved in 51 mM and 68 mM salinity.[122]

Mixotrophic vs. heterotrophic cultivation conditions

Mixotrophic cultivation of *B. braunii* utilizes both light and organic carbon sources, allowing it to simultaneously perform photosynthesis and assimilate exogenous carbon substrates such as glucose. Zhang et al. demonstrated that under mixotrophic conditions, the growth rate and final biomass concentration of *B. braunii* were significantly enhanced compared to both photoautotrophic and heterotrophic modes.[127] Among the tested carbon substrates, maltose, glucose, sucrose, lactose, glycerol, and starch, glucose was identified as the most efficient for promoting growth, yielding a cell density of 4.55 g L^{-1} and hydrocarbon content of 29.7% in dry biomass when cultivated in a 10 L airlift photobioreactor.[127] Furthermore, the study confirmed that mixotrophy allowed higher growth rates, particularly during the exponential phase, and this effect was maximized under an optimal initial glucose concentration of 2.5 g L^{-1} and KNO_3 concentration of 0.4 g L^{-1}. Importantly, the phototrophic component of this strategy ensures that the energy demands for hydrocarbon biosynthesis are met more effectively than under heterotrophic conditions.[127]

Heterotrophic growth studies of *B. braunii* are rare, with only basic preliminary concept studies being conducted. Weetall et al. examined strains from the Texas Culture Collection facility and found that none of them grew heterotrophically in the dark; however, the growth was enhanced by the addition of several sugars, such as glucose, fructose, sucrose, and mannose as a carbon source.[128] Efforts to scale up production have shown positive results, whereby heterotrophic seed cultures have successfully been maintained and used for photoautotrophic cultivation.[129] Moreover, Tanoi et al. reported higher growth rates of the B70 strain in heterotrophic conditions using glucose, compared with autotrophic conditions where glucose was not present.[44] The findings showed that glucose and mannose were the most effective carbon sources for heterotrophic cultivation, as well as morphological changes to the organelles within the cells. Most notably, the shape of the chloroplast appeared more rectangular when cultivated under heterotrophic conditions.[44] Zhang et al., observed that the strain exhibited poor utilization of organic carbon in the absence of light, and growth rates were even lower than those under photoautotrophic conditions for most carbon substrates tested, with glucose being the only exception that showed modest improvements. This limited metabolic plasticity under heterotrophic conditions constrains the practical applicability of this method for large-scale hydrocarbon production, especially when considering energy and cost efficiency.[127,128]

A study by Khichi et al. provided further insights into substrate uptake kinetics under carbon-limited and nitrogen-limited conditions. Their work emphasized the critical role of the C:N ratio in governing both biomass accumulation and lipid productivity in *B. braunii*. The optimal C:N ratio for maximum biomass and lipid productivity was found to be 29:1, yielding 4.44 g L^{-1} of biomass, 0.390 g L^{-1} d^{-1} of lipid productivity, and a specific growth rate of 0.0873 h^{-1}.[130] At this ratio, glucose and nitrate uptake rates were balanced to support both energy generation and biosynthesis without excess carbon repression or nitrogen limitation. Notably, higher C:N ratios (e.g., 61:1) led to reduced biomass due to carbon excess and nitrogen limitation, despite increased specific nitrate uptake rates, highlighting a threshold beyond which further increases in carbon availability hinder growth.[130] Similarly, in nitrate-limited conditions, high glucose availability could not compensate for reduced nitrogen, resulting in decreased biomass despite elevated lipid accumulation, which highlights the links between carbon and nitrogen metabolism in *B. braunii*.

The findings demonstrate that nitrogen limitation leads to metabolic rerouting from glycolysis to the pentose phosphate pathway (PPP), favoring NADPH generation for lipid biosynthesis but at the cost of reduced glucose assimilation and cell division.[131,132] In contrast, nitrogen-sufficient conditions promote TCA cycle flux and protein synthesis, enhancing growth rates and overall productivity by providing the necessary reducing equivalents and carbon source for anabolic metabolism.[131] This metabolic balancing act highlights the superior performance of mixotrophic and photoheterotrophic systems, where light-driven energy production complements organic carbon assimilation for enhanced yields.[127,130]

Mixotrophic cultivation stands out as the most effective strategy for growing *B. braunii* when aiming for high biomass and hydrocarbon productivity. It leverages the synergistic effects of phototrophic energy generation and heterotrophic nutrient uptake, overcoming the limitations of each method when used in isolation.[127] Heterotrophic cultivation, while potentially simpler in design, remains restricted by poor substrate utilization in the absence of light, and is therefore suboptimal for *B. braunii* under most practical cultivation scenarios, with growth rates often lower than those observed even under photoautotrophic conditions unless glucose is present as the carbon source.[127] The choice of cultivation method should thus be guided by target outputs (e.g., biomass vs. lipid content), availability of light and carbon sources, and economic considerations in scaling.[130]

LIPID EXTRACTION

Like all microalgae, lipid extraction can be carried out in several different ways, ranging from mechanical, chemical, enzymatic, and microwave. Due to the unique lipid production process of *B. braunii,* there is limited published data for mechanical extraction methods, such as ball mills, screw expeller pressing, and high-pressure homogenization. The most common lipid extractions are based on several solvent extraction methods, both destructive and non-destructive. *B. braunii* is the pioneering species for the development of the ‘‘milking’’ process, which aims to repeatedly extract lipids from the same batch of algae.

Solvent lipid extraction can be carried out in two ways. The distinction lies in the presence of water pre-extraction. The ‘‘dry route’’ aims to dewater the algae prior to extraction. This is a highly energy-intensive process, often requiring the use of a centrifuge. Contrary to this, the ‘‘wet route’’ retains the water and exploits the miscibility between the solvent and water. Both routes have their merits and demerits, which Russell et al. discuss in detail.[2]

Destructive solvent extraction

A significant proportion of the available literature on solvent extraction for *B. braunii* is concerned with the ratio of solvent to algae, combined with exposure time. Boni et al. investigated the efficiency of *n*-hexane using the Soxhlet method for lipid extraction.[133] The authors used 175 mL of *n*-hexane with continuous extraction over 96 hours, reporting a modest 24% lipid yield. Similar results were reported by Ryckebosch et al., who used equal parts methanol and chloroform to achieve a 23% lipid yield.[134]

Investigation into the destructive nature of biocompatible solvents such as tetradecane on the FACHB 357 strain was carried out by Zhang et al.[135] Exposure to solvents invokes stress upon the cells and colonies, which have been shown to increase the lipid productivity by up-to three times. The authors observed that when *B. braunii* is exposed to a 10% (v/v) tetradecane solution, the solvent perforates the cell membrane, which stimulates the endocytosis/exocytosis diffusion of intracellular biochemical compounds.[135] Furthermore, the larger lipid bodies over time block and seal the holes formed by the solvent to allow for further lipid extraction, illustrating one possible mechanism for why ‘‘milking’’ is possible. These findings would support claims that limitations on the number of repeated extractions are observed.

Non-destructive solvent extraction

In-situ extraction, sometimes referred to as ‘‘milking,’’ is a non-destructive lipid extraction method that is undoubtedly the most common within the literature. This is thanks to the unique property of extracellular lipid accumulation. Having the ability to extract lipids without compromising the integrity of the cell membrane is critical to repetitive non-destructive extraction. The biocompatibility of the selected solvent is key to the success of this method of extraction. The most influential factor is the partition coefficient *LogP* value of the solvent, which is a measure of the dissociation between non-polar and polar liquids. Generally, the positive values indicate some form of hydrophobic characteristics, with higher values showing a greater hydrophobicity, which is more desirable for the extraction of lipids.[136]

Research by Kleinert et al. investigated the extractability of three biocompatible solvents: *n*-hexane, *n*-heptane, and *n*-octane, using 12 different strains of *B. braunii*, 3-race B, and 9-race A.[37] Unsurprisingly, the race B strains – Showa and Bot22 – showed the highest extracted lipids with all three solvents; however, there was no clear relationship between extractability and carbon chain length. The authors concluded, however, that *n*-octane had the better biocompatibility compared to *n*-heptane and *n*-hexane, due to having the higher *LogP* value of 5.51 compared with 4.50 and 4.00, respectively. This is concurrent with the *LogP* criteria.

A similar study investigated the extractability efficiency of botryococcenes using both heptane and dodecane with three repetitive extractions. Surprisingly, heptane showed the highest lipid recovery with the first being 11.3% compared to dodecane having only 0.8%.[137] While dodecane is more biocompatible for *B. braunii* compared with heptane, the increased hydrophobic nature of dodecane made it almost impossible to penetrate the cell membrane, with Jackson et al. reporting an increase in biomass productivity over the three extractions.[137] This suggests that longer-chained hydrocarbons require a pre-treatment prior to lipid extraction.

Hydrocarbon recovery of the Showa strain using a thermal pre-treatment was investigated by Kita et al., using five temperatures from 75°C to 120°C. The authors reported a 97.8% hydrocarbon recovery at 90°C with hexane.[138] Elemental analysis of the post extraction hexane solution confirmed that microalgae hydrocarbons were indeed extracted. Furthermore, Magota et al. also used thermal pre-treatment on the Showa strain and the Yamanaka and Kawaguchi-1 strains.[139] They reported similar findings of over 90% hydrocarbon recovery at temperatures of 85°C, 60°C, and 75°C, respectively, suggesting that thermal pre-treatment has the potential to reduce the energy consumption of oil recovery processes that use wet *B. braunii* cells.[139] The Showa strain belongs to the B race, which contains long-chain hydrocarbons, which further supports the findings by Jackson et al.[137]

In relation to hydrocarbon recovery with hexane after thermal pre-treatment, Furuhashi et al. found that most of the hydrocarbons can be recovered from a suspension of algal culture without thermal pre-treatment if the alga was cultured with brackish water using the Showa strain belonging to race B as the material.[125] At this moment, the mechanism of such easier extractions of hydrocarbons from algal cells cultured in brackish water has not been uncovered, but the "colony sheath" surrounding the outermost layer of the *Botryococcus* colony was reduced in such algae, similarly to those subjected to thermal pre-treatment.[33,140] Reductions of the colony sheath layer may enable contacts of an extraction solvent to hydrocarbons trapped in the extracellular matrix more easily.

The effect of solvents over prolonged periods of time begins to degrade the cells, which ultimately leads to their destruction. However, the selection of the appropriate solvent can extend the lifetime of the cell's exposure to the solvent. Zhang et al. used tetradecane (1:10 solvent: culture) to achieve a 50% hydrocarbon extraction yield while maintaining a 92% viable culture.[141] Moheimani et al. showed that repetitive hydrocarbon extraction is possible for up to 80 days using *B. braunii*, with no additional nitrogen or phosphorus being added.[142] Extraction was carried out every 11 days, to allow sufficient quantities of lipids to accumulate prior to extraction. This proved a very effective milking arrangement. The exploitation of a biochemical concentration gradient can have either a destructive or non-destructive extraction; this can be achieved through the manipulation of the salt concentration within the solution.

Switchable solvent extraction

Switchable solvents, alternatively termed "smart" or "reversible" solvents, constitute a unique class of solvents capable of altering their polarity/hydrophilicity reversibly in response to external inducers such as CO_2 or N_2. This distinctive group is broadly categorized into switchable polarity solvents (SPSs) and switchable hydrophilicity solvents (SHSs). The polarity and hydrophilicity of these solvents undergo a reversible switch upon exposure to an inducer, respectively.[2] Typically, this form of lipid extraction is favored under nitrogen deficient conditions, nitrogen acts as an inducer to switch the solvent back to its hydrophobic form; therefore, a higher nitrogen content suppresses the switchability of the solvents.[143] On the contrary to this, the consumption of large quantities of CO_2 may raise concerns of acidifying the water; however, studies using N, N-cimethyl cyclohexylamine (DMCHA) have indicated that the high basicity of DMCHA neutralizes the hydrogen protonated water.[144]

B. braunii was the first microalgal species to be investigated using such solvents, due to the extracellular lipid matrix. Samorì et al. investigated the possibility of repetitive non-destructive micro-lipid extraction of the race A strain SAG 807-1 using the SPS 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU).[145] The authors found that DBU was more effective at extracting lipids with a yield of 8.2% compared with both octanol 5.6% and hexane 4.4%.[145] It is important to note that on a micro-scale (1-5 mL) this is not a true reflection of the performance of such systems. Unfortunately, at the time of this article, all available literature employs the use of micro-extractions. DMCHA is one of the more common SHS's known and was investigated by Boyd et al., using freeze dried *B. braunii* with varying volumes up-to 10 mL.[146] Boyd et al. used the Bligh and Dyer method, which uses a mixture of chloroform/methanol as a comparison against DMCHA. Twenty-two weight per cent of hydrocarbons was recovered at 80^0C using 10 mL of DMCHA, compared with 52wt % using the Bligh and Dyer method.[146]

A recent experimental article by Russell et al. investigated the extraction efficiency of DMCHA and N, N-diisopropyl ethanolamine (DIPA), using five algal species, which include two *B. braunii* strains, namely, *B. braunii* sp (Race B) and UTEX 2441 (Race A).[6] The authors report an impressive lipid yield recovery of 61.88 wt % ± 10.50 with DIPA and 56.10 wt % ± 6.66

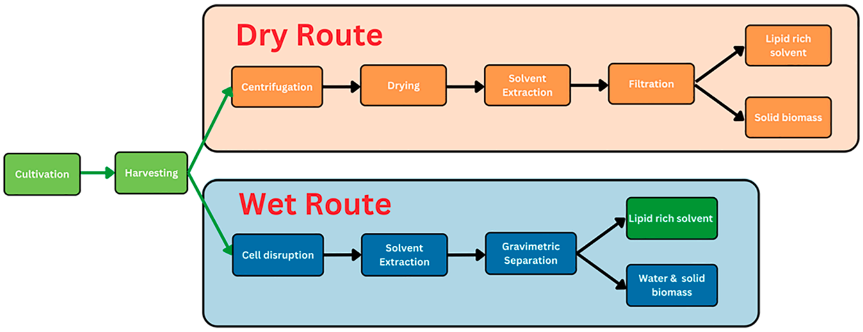

Figure 5. Wet and dry lipid extraction routes

with DMCHA, respectively.[6] This work is concurrent with the literature suggesting that switchable solvents are a suitable alternative to traditional solvent extraction.

A key factor to be considered when using switchable solvents is the extraction route, i.e., wet or dry, as this will have a high influence on the efficiency of both solvent and lipid recovery. Figure 5 illustrates the general distinction between the wet and dry routes.[147] It is worth noting that in order for switchable hydrophilicity solvents to undergo the transition from a hydrophilic to a hydrophobic state, water must be present, and therefore, research involving freeze-dried samples must add sufficient quantities of water to induce this process.[6]

Despite their advantages in tuneable polarity and recyclability, switchable solvents present significant challenges in downstream processing, particularly during product polishing. Complete removal of residual solvent from the extracted hydrocarbons is difficult because of strong interactions between tertiary amines and lipid fractions, which can leave traces that compromise fuel quality and safety standards. This may require additional purification steps such as vacuum stripping, distillation, or adsorption, increasing energy demand and operational costs. Furthermore, solvent recovery efficiency and recyclability must be optimized to minimize environmental impact and ensure economic feasibility. Future research should focus on integrated extraction-purification systems and the development of low-boiling-point or easily phase-separable solvent formulations to overcome these limitations.

Recent developments in bio-based and green solvents have gained attention as sustainable alternatives to conventional petroleum-derived solvents for lipid extraction. These include solvents derived from renewable resources such as bio-alcohols (e.g., ethanol), terpenes (e.g., limonene), and natural deep eutectic solvents (NADESs), which offer reduced toxicity and environmental impact. Ionic liquids and switchable hydrophilicity solvents have also been explored for their tunable polarity and recyclability, enabling efficient extraction while minimizing solvent loss and hazardous waste. Studies have demonstrated that these green solvents can achieve comparable or even superior lipid recovery compared to traditional solvents such as hexane, while aligning with biorefinery principles of sustainability and circular economy. Their integration into non-destructive extraction methods, such as milking, further enhances process efficiency and reduces energy consumption.

BIOREFINERY CONCEPT

The biorefinery concept, derived from the operational model of petroleum refineries, is structured to systematically fractionate biomass into multiple valuable products in a sequential and integrated manner. It offers a holistic approach to biomass utilization by extracting fuels, chemicals, pigments, proteins, and other bioactive compounds from a single feedstock source. In the case of microalgae, this concept is particularly compelling due to their biochemical diversity and rapid adaptability under various cultivation conditions and environments.[147] The process generally encompasses five stages, namely, strain selection, cultivation, harvesting, extraction, and purification, each with defined technological options based on the compound of interest and the biomass composition, as summarized in Table 2.

B. braunii is uniquely positioned as a high-potential candidate for biorefinery applications due to its capacity to synthesize and secrete long-chain hydrocarbons extracellularly, which can be extracted through non-destructive methods such as milking.[129,148] The reduction in energy requirements for cell lysis and dewatering, which are traditionally among the most energy-intensive stages in microalgal processing, aligns well with the core objectives of the biorefinery, namely, process efficiency, resource conservation, and sustainability.[143,149,150] In addition to hydrocarbon production, *B. braunii* biomass contains an array of other valuable compounds such as proteins, polysaccharides, and carotenoids.[151,152] This biochemical versatility makes the species particularly suitable for integrated biorefinery systems aimed at co-producing energy and high-value biochemicals.

Furthermore, advancements in cultivation systems, particularly the development of attached culture technologies, have enhanced the feasibility of large-scale biorefinery implementation. These systems have demonstrated improvements in biomass density, ease of harvesting, and minimization of water usage, thereby reinforcing the alignment of *B. braunii* cultivation with sustainable biorefinery principles. The algae's compatibility with both closed and semi-closed photobioreactor systems, along with its tolerance to environmental fluctuations, adds further operational flexibility in biorefinery design. The biorefinery concept not only enhances the sustainability of *B. braunii*-based fuel production but also unlocks its potential across multiple industries, including nutraceuticals, pharmaceuticals, and cosmetics.[153] The organism's compatibility with non-destructive extraction, its broad metabolic profile, and the synergy with advanced cultivation technologies collectively place *B. braunii* at the forefront of next-generation algal biorefineries.[151,154]

CONCLUSION

Botryococcus braunii remains one of the most promising candidates in the pursuit of sustainable biofuels and bioproducts due

Table 2. Biorefinery aims and technologies

Stage	Strain Selection	Cultivation	Harvesting	Extraction	Purification
Aim	The quantity, quality, and types of lipids required	Combination of biomass production against production and capital costs	Separation of biomass from the growth medium	Cell lysis for lipid recovery (can be integrated with harvesting)	Refine the product to the required purity by removing impurities
Technology/ Technique	–	● Open system ● Closed system	● Sedimentation ● Filtration ● Centrifugation ● Flocculation ● Ultrasonication ● Homogenization	● Bead mills ● Enzymatic ● Chemical ● Solvent ● Osmotic ● Ionic liquids ● Milking	● Chromatography ● Filtration ● Saponification ● Evaporation

to its ability to produce and secrete long-chain hydrocarbons. Its unique colonial morphology, hydrocarbon-rich extracellular matrix, and race-specific metabolic diversity distinguish it from other microalgal species and position it as a frontrunner for integrated biorefinery applications.

This review has illustrated the vast biochemical and phylogenetic diversity of *B. braunii*, highlighting the profound differences between races A, B, and L, with the tentative inclusion of race S. Advances in genetic and molecular tools, such as SSL gene identification and BoCAPS PCR methods, are now enabling more precise race discrimination and monitoring, enhancing our understanding of evolutionary divergence and hydrocarbon biosynthesis pathways. Ensuring the correct genetic identification is essential when investigating this species. When conducting research, with a vast array of conflicting conclusions within the literature, this review serves as a foundational resource in ensuring correct race identification.

Despite these advances, several challenges hinder the commercial viability of *B. braunii*-based biofuel systems. Foremost is the species' inherently slow growth rate, particularly in race B strains, which poses a major bottleneck to biomass production. Innovative cultivation strategies, including mixotrophic and attached culture systems, are demonstrating significant potential to overcome this limitation by improving biomass density, process scalability, and water-use efficiency.

Environmental stressors such as nutrient limitation, light wavelength modulation, temperature shifts, and salinity adjustments have proven to be powerful levers for manipulating metabolic outputs. However, strain-specific responses complicate the generalization of these findings and underscore the need for tailored optimization. Future work should embrace systems biology approaches such as integrating genomics, transcriptomics, and metabolomics to more precisely decode these differential responses and design race-specific cultivation protocols.

Lipid extraction methodologies for *B. braunii* are at a crossroads. While traditional solvent-based techniques remain prevalent, the evolution of non-destructive methods such as "milking," offers a path toward sustainable and energy-efficient hydrocarbon recovery. Scaling up these techniques while maintaining high yields and cell viability will be pivotal in the commercial viability of microalgae-based bioproducts.

The integration of synthetic biology, CRISPR-based genome editing, and bioreactor engineering holds promise for enhancing photosynthetic efficiency, accelerating growth, and increasing hydrocarbon productivity. Moreover, redefining race boundaries at the genomic level may lead to the reclassification of existing strains into novel species, further refining our exploitation of *B. braunii*.

Limitations of the study

This review synthesizes extensive literature on *Botryococcus braunii* taxonomy, cultivation, and lipid recovery, but several limitations should be acknowledged. While race-specific hydrocarbon profiles and genetic markers are discussed, the functional characterization of many biosynthetic enzymes remains incomplete, limiting mechanistic insights. Most cultivation and extraction strategies presented have been evaluated at laboratory or pilot scales, and their scalability and economic feasibility under industrial conditions require further validation. Environmental and geographic variability in strain performance introduces uncertainty in generalizing findings across global contexts. Addressing these gaps will require integrated multi-omics approaches, standardized protocols, and techno-economic assessments to translate laboratory advances into commercially viable bioprocesses.

AUTHOR CONTRIBUTIONS

Conceptualization, analysis, and writing – original draft, C.Ru; writing – review and editing, C.Ro, R.M., and S.O; resources and supervision, C.Ro.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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