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Kinetic study of biomass and hydrocarbon production in chemostat cultures of the microalga *Botryococcus braunii* : a physiological approach

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Résumé

Contexte: une microalgue productrice d'hydrocarbures, Botryococcus braunii

La production d'ingrédients pour les secteurs industriels associés à la pétrochimie à partir de ressources renouvelables est l'un des défis des sociétés contemporaines face à l'épuisement des réserves pétrolières. Peu d'organismes ont la capacité à surproduire naturellement des hydrocarbures potentiellement utilisables pour la production de BioJetFuel ou pour des voies de synthèse chimique pour, par exemple, la fabrication de matières plastiques.

Parmi les espèces photoautotrophes, la microalgue *Botryococcus braunii* (Trebouxiophyceae, Chlorophytes) a suscité un intérêt particulier en raison de sa capacité à produire dans une matrice extracellulaire de grandes quantités d'hydrocarbures à longues chaînes. Plus de soixante souches de *B. braunii* ont déjà été isolées. L'analyse phylogénétique des séquences de gènes 18S a confirmé que toutes ces souches appartiennent à la même espèce, *B. braunii.* A cette même espèce est associée une très grande chimiodiversité, en particulier d'hydrocarbures. Pour cette catégorie de molécules, les souches sont classées en quatre races. Les souches de race A produisent des alcadiènes et alkatrienes, les souches de race B produisent des hydrocarbures triterpénoïdes et celles de race L produisent un seul hydrocarbure tetraterpénoïde. Récemment, une nouvelle race S a été proposée, qui peut produire des chaînes de carbone beaucoup plus courtes que la race A et des atomes de carbone paires. Dans le milieu naturel, les souches de race A et de race B sont les plus abondantes et présentent généralement des teneurs en hydrocarbures plus élevées que les autres races. Elles ont donc fait l'objet d'un nombre plus élevé d'études. L'analyse des données bibliographiques sur *B. braunii* a permis de souligner les caractéristiques particulières des souches de race A et de race B, non seulement la structure de leurs hydrocarbures respectifs, mais aussi les voies biosynthétiques impliquées, leur localisation intracellulaire, les mécanismes de sécrétion et d'accumulation des hydrocarbures. Pour les souches de race A, la biosynthèse des alcadiènes et des alcatriènes implique une élongation de l'acide gras acide oléique par voie enzymatique pour former grâce au pouvoir réducteur de la cellule des acides gras à très longue chaîne, qui sont ensuite convertis par décarbonatation en hydrocarbures par l'intermédiaire de très longues aldéhydes gras à chaîne. Pour les souches de la race B, le squalène et les botryococcènes à plus de 30 carbones sont produits par la voie de synthèse des triterpènes. Que ce soit pour les souches de race A ou celles de race B, les

hydrocarbures sont probablement dérivés de lipides produits dans les chloroplastes, excrétés de ces organites sous forme de globules lipidiques, puis sécrétés et accumulés dans la matrice extracellulaire. Le processus de sécrétion semble impliquer une opération de transport vésiculaire de type exocytose. Le potentiel de production des hydrocarbures extracellulaires semblerait plus fort dans des cultures en croissance active, probablement du fait d'une meilleure disponibilité en énergie (ATP) et en pouvoir réducteur (NADPH). Ce micro-organisme photosynthétique accumule des concentrations d'hydrocarbures élevées, qui sont piégées dans une matrice extracellulaire, composée de polysaccharides et d'hydrocarbures polymérisés.

Si la biologie de l'espèce *B. braunii* a fait l'objet de nombreux travaux, ce n'est que récemment que les cinétiques de production de cette microalgue ont commencé à être étudiées. En effet cette microalgue était réputée pour avoir des temps de génération pouvant dépasser les 72 h. En dépit de leurs faibles taux de croissance, certaines de ces souches de microalgues ont été cultivées de façon relativement efficace dans des photobioréacteurs. Certaines études ont également mis l'accent sur les moyens d'améliorer la productivité soit par un procédé d'extraction dans un système diphasique, qui a été nommé milking car il permet d'extraire en maintenant la viabilité cellulaire et donc de séparer la production de biomasse de celle des hydrocarbures ou par des procédés de co-valorisation des résidus de biomasse selon les approches de bio-raffinerie pour produire des composés d'intérêt industriel, de caroténoïdes à squalène, et même gaz de synthèse.

Objectif de l'étude

La production de biomasse et d'hydrocarbures par des cultures de *B. braunii* est censée dépendre, comme pour d'autres productions de lipides par des microalgues comme *Chlorella* sp, de différents facteurs incluant des facteurs nutritionnels et des facteurs environnementaux. Chaque dispositif de culture, peut être associé à des indicateurs d'efficacité incluant les rendements de conversion, les concentrations maximales et les productivités. Ces dernières peuvent concerner soit la formation de biomasse soit celle d'hydrocarbures. La culture dans un photobioréacteur contrôlé doit permettre d'établir des relations entre ces variables opératoires et ces indicateurs de performance.

Les productivités,

$$P_X = f(nutriments, pH, lumière, température \dots)$$

$$P_{HC} = f'(nutriments, pH, lumière, température \dots)$$

où X représente la biomasse de *B. braunii* et HC les hydrocarbures.

Plusieurs études ont déjà porté sur l'amélioration des vitesses de production de biomasse et d'hydrocarbures en fonction de quelques facteurs environnementaux, tels que la température, la lumière ou les éléments nutritifs. Même si ces travaux ont permis de contribuer à améliorer les connaissances sur les cinétiques de réactions physiologiques associées à la production de biomasses enrichies en hydrocarbures, il reste encore une grande marge de progression pour accroitre les productivités de cultures de *B. braunii* avant d'envisager de satisfaire aux exigences pour une application industrielle.

Il est à noter que la plupart des études antérieures sur *B. braunii* ont été réalisées dans des cultures discontinues, de type batch, principalement sans contrôle du pH. Dans ce mode de culture, les conditions environnementales changent constamment. Un tel système à l'état instable pourrait masquer des différences et des tendances physiologiques subtiles. En effet les variations continues des paramètres environnementaux, tels que le pH ou la fourniture de lumière spécifique, pourraient ne pas permettre de mettre en évidence d'éventuelles relations, par exemple, entre les taux de croissance spécifiques et la production d'hydrocarbures. Des cultures en mode continu de type chemostat avec des régimes stationnaires successifs permettent de surmonter ces inconvénients et donc d'accroître la fiabilité des données expérimentales. En culture continue, la concentration de la biomasse dans le photobioréacteur, le taux spécifique de croissance (μ) de microalgues et le taux de dilution sont liés par l'équation:

$$\frac{dX}{dt} = \mu \cdot X - D \cdot X$$

où μ est le taux de croissance spécifique de *B. braunii*, D est le taux de dilution.
Sachant qu'un régime stationnaire correspond à un état d'équilibre dynamique où :

$$\mu = D \text{ sous réserve que } D < \mu_{max}$$

où μ_{max} est le taux de croissance spécifique maximal de la culture dans des conditions opératoires données.

Ce type de culture en chemostat se révèle être un outil performant pour analyser les cinétiques de réactions physiologiques associées à la production de biomasse cellulaire et/ou la production de métabolites. Ainsi il est possible d'analyser les modifications biochimiques et physiologiques du nouvel état stationnaire induit par la modification d'un des paramètres opératoires, température ou pH par exemples. Nous avons donc utilisé des cultures en mode chemostat pour étudier le comportement de cultures de *B. braunii* en fonction du taux de dilution, de la consigne de pH et des flux lumineux incidents au photobioréacteur.

En effet, l'analyse bibliographique avait montré qu'une seule étude avait porté sur des cultures en mode continu de la souche *B. braunii* race A souche CCC 807/1; ces expériences avaient été réalisées sans contrôle de pH et à seulement deux taux de dilution (0,25 j^{-1} et 0,30 j^{-1}) par Casadevall et al., 1985. Un autre travail sur *B. braunii* race B Showa avait permis d'obtenir des états pseudo-stables dans une culture en mode semi-continu également sans contrôle du pH du milieu de culture (Khatri et al., 2014).

Résultats et discussion

Les principaux objectifs de cette étude étaient d'étudier les effets environnementaux et nutritionnels sur les cinétiques de production de biomasse et d'hydrocarbure de deux souches de *B. braunii* en utilisant la technique des cultures continues en photobioréacteur contrôlée, ainsi que les réponses physiologiques à ces facteurs, en particulier en termes de disponibilité des éléments nutritifs (carbone inorganique dissous, l'azote et le phosphore), le pH et l'intensité lumineuse. Une étude comparative de la race A et la souche race B a également été réalisée. Ces résultats sont analysés en vue de chercher à apporter des éléments de compréhension sur les processus qui conduisent cette espèce microalgale à accumuler des hydrocarbures extracellulaires.

Productivités de la souche SAG 30.81 en cultures continues en fonction du pH et des teneurs en nutriments.

Dans un premier temps, les effets du pH du milieu de culture sur les productivités en biomasse et hydrocarbures ont été étudiés dans des cultures photoautotrophes de *B. braunii* SAG 30.81 (race A). Des états stables successifs de culture, donc en régime permanent, ont été

utilisées pour étudier ces effets sur les cinétiques de production de biomasse et d'hydrocarbures dans des cultures où l'accroissement du pH dans un photobioréacteur clos est contrôlé par des apports automatiques de dioxyde de carbone. L'étude a montré qu'à un taux de dilution fixe de 0,2 j^{-1}, la productivité des hydrocarbures est pH-dépendante entre pH 5,5 et pH 8,0, avec un optimum à pH 6,5. Par contraste, la productivité en biomasse, les rendements de conversion des éléments nutritifs, les rendements photosynthétiques et les ratios des pigments photosynthétiques ont été peu affectés par ces variations de pH de 6,0 à 8,0. En revanche, un pH de 5,5 induit une légère diminution des productivités en biomasse et hydrocarbures. Des mesures physiologiques confirment que les cellules microalgales ont été stressées par des conditions acides à pH 5,5. L'effet du pH est maintenu même après un accroissement des concentrations en nitrates et phosphates dans le milieu de culture d'alimentation. Des productivités en biomasse et hydrocarbures jusqu'à 8,9 g m^{-2} j^{-1} et 0,5 g m^{-2} j^{-1} respectivement ont été obtenues dans ces conditions. Les dosages des concentrations résiduelles en carbone inorganique dissous permettent d'établir une relation directe entre la productivité en hydrocarbures et les teneurs en CO_2, et non en bicarbonate (HCO_3^-). Ainsi une teneur en hydrocarbures maximale de 5,7% de la masse sèche a été atteint au-delà de 1 mmol L^{-1} CO_2 dissous avec une relation linéaire entre la production d'hydrocarbures et la teneur en CO_2 jusqu'à 2,5 mmol L^{-1}, quel que soit l'enrichissement en N et P du milieu de culture AF-6.

Taux de dilution et productivités de la souche SAG 30.81 en cultures continues

Les productions d'hydrocarbures extracellulaires et de lipides intracellulaires ont été déterminées à l'état d'équilibre pour six taux de dilution de 0,12 j^{-1} à 0,36 j^{-1} dans des cultures continues de *B. braunii* SAG 30.81. Nous avons montré que les teneurs en hydrocarbures peuvent être corrélées aux teneurs intracellulaires en lipides, en conditions limitantes ou non en azote. Par ailleurs, les productivités en hydrocarbures sont maximales à des taux de dilution compris entre 0,18 j^{-1} à 0,27 j^{-1}, soit en termes de limitation de l'empreinte hydrique, une valeur de D_{opt} de 0,18 j^{-1}. Une relation linéaire est mise en évidence entre les vitesses spécifiques de production d'hydrocarbures et les taux de dilution, donc les vitesses spécifiques de croissance des cultures. Ces données répondent bien au modèle de Luedeking-Piret (Thierie, 2015), montrant que la production d'hydrocarbures extracellulaires est essentiellement associée dans nos conditions

expérimentales à la croissance des cultures. L'étude révèle également qu'en absence de limitation azotée, cette microalgue suraccumule des lipides intracellulaires, alors que les teneurs en hydrocarbures restent relativement constantes. Cette caractéristique différencie *B. braunii* des autres espèces oléagineuses accumulant des triglycérides lorsqu'elles sont placées en conditions de limitation azotée, donc avec une croissance limitée des cultures.

Intensité des flux lumineux et productivités de la souche SAG 30.81 en cultures continues

Dans un premier temps, la densité spécifique de flux de photons actifs pour la photosynthèse (ou specific light supply SLSR en mol quanta g X^{-1} j^{-1}) a été déterminée pour évaluer pour chaque concentration en biomasse l'intensité lumineuse disponible pour la production de biomasse et d'hydrocarbures à différents taux de dilution, en lien avec quelques réponses physiologiques dans un photobioréacteur exposé à un flux lumineux constant. Seule la variation de la concentration en biomasse détermine les flux de photons disponibles pour chaque unité de biomasse. La gamme optimale de SLSR a été déterminée entre 0,28 quanta mol g X^{-1} j^{-1} et 0,42 mol quanta g X^{-1} j^{-1}. Au-delà de cette dernière valeur, un accroissement des SLSR ne permet pas d'améliorer les vitesses spécifiques de production d'hydrocarbures. Ce résultat suggère l'utilisation d'un turbidostat en vue de maintenir les valeurs du SLSR dans la gamme de 0,28 quanta mol g X^{-1} j^{-1} à 0,42 mol quanta g X^{-1} j^{-1} pour optimiser la production de biomasse enrichie en hydrocarbures. Le lien entre une surproduction de lipides intracellulaires obtenue sous SLSR élevés est interprété dans l'optique de la production et accumulation des précurseurs lipidiques des hydrocarbures (acides gras à très longues chaines) sous forme de globules lipidiques qui ont été visualisés par analyse cytochimique en épifluorescence.

Dans un deuxième temps, les cinétiques de production des hydrocarbures et de lipides ont été étudiées par l'effet d'une exposition du photobioréacteur à des flux lumineux de 150 µmol quanta m^{-2} s^{-1} et 900 µmol quanta m^{-2} s^{-1} sur une culture continue de la souche SAG 30.81 à taux de dilution constant. L'objectif était d'analyser un éventuel processus de photoadaptation. L'accroissement d'un facteur 6 du flux lumineux incident a permis d'accroître la concentration en biomasse pour atteindre une valeur maximale atteignant 63,3 g m^{-2}. Par contre les teneurs en hydrocarbures n'ont pas varié, restant de l'ordre de 5 % la biomasse sèche ; cette donnée expérimentale confirme l'existence d'une forme de saturation de la réponse entre teneurs en

hydrocarbures et flux lumineux. Nous observons que la teneur en lipides intracellulaires passe de 32,2 % à 40,4 %. Le fort éclairement des cultures permet d'accroître significativement les productivités en biomasse, hydrocarbures extracellulaires et lipides intracellulaires. L'analyse des concentrations résiduelles en azote et en phosphore dans les surnageants de culture indique que ces deux éléments sont potentiellement des facteurs de limitation pour une étude future visant à maximiser la production de biomasse enrichie en hydrocarbures. L'existence d'un processus de photoadaptation est mise en évidence par une réduction transitoire et rapide du rendement quantique photochimique maximale du système de la photosynthèse et des teneurs en chlorophylles avant de se stabiliser à des valeurs plus élevées. L'analyse des acides gras de la souche 30.81 montre que l'acide oléique est le principal composant des acides gras totaux, qui comprennent également les acides gras suivants : C13: 0, C16: 0, C16: 1, C18: 1, C18: 2 (n-6), C18: 3 (n-3), C22: 0 et C24: 1. Le passage à 900 µmol photon m^{-2} s^{-1} induit une augmentation de la teneur totale en acides gras mais plus particulièrement en acide oléique. Ces dernières données sont interprétées en relation avec d'une part la composition connue des membranes de globules lipidiques et d'autre part du rôle précurseur de l'acide oléique pour la biosynthèse des hydrocarbures dans les souches de race A.

Etude comparative des souches race A SAG 30.81 et race B BOT 22

La souche SAG 30.81 race A et la souche BOT 22 race B ont été cultivées en mode chemostat à un taux de dilution constant de 0,23 j^{-1}. Les deux souches diffèrent par leur composition biochimique. Les hydrocarbures prédominants dans la souche SAG 30.81 ont été déterminées comme étant nonacosadiene ($C_{29}H_{56}$) et hentriacontadiene ($C_{31}H_{60}$) par GC-FID et GC-MS. La souche BOT 22 est caractérisée par la présence dominante d'un seul hydrocarbure $C_{34}H_{58}$ de type botryococcène.

La souche BOT 22 a des teneurs en hydrocarbures 4 fois plus élevées que la souche SAG 30.81. Ainsi en termes de productivités en conditions standard de culture, la souche de race B présente des valeurs plus élevées en termes de production de biomasse, hydrocarbures et lipides. Le fait que cette plus forte productivité en hydrocarbures puisse être associée à une structure différente des matrices des colonies est discuté. La souche de race B présente un comportement similaire à l'autre souche vis-à-vis des réponses aux accroissements des flux lumineux incidents

et disponibles par unité de biomasse ainsi que vis-à-vis des rendements de conversion de l'azote et du phosphore. Seules des teneurs plus élevées en chlorophylles dans la souche de race B permet de différencier les deux souches en termes de pigments et activités photosynthétiques. Par contre les teneurs en acides gras totaux et acide oléique sont significativement plus élevées dans la biomasse de la souche de race A que dans celle de la souche race B. Cette dernière caractéristique est analysée sur la base des éléments de connaissance disponibles sur voies de biosynthèse des hydrocarbures respectifs de ces deux races de *B. braunii.*

Conclusion et perspectives

Conclusion

Le principal objectif de cette étude était de caractériser les relations entre des modifications de facteurs environnementaux et les cinétiques de production de biomasse et d'hydrocarbures par une microalgue *B. braunii.* Pour cela nous avons utilisé la technique des cultures continues en photobioréacteur contrôlé, qui permet d'obtenir des régimes stationnaires successifs correspondant à des états d'équilibres dynamiques en fonction de modifications de variables opératoires. Cette méthodologie a permis d'identifier des relations entre des réponses physiologiques et des facteurs de culture tels que le pH, l'intensité des flux lumineux et la disponibilité en certains nutriments, tels que le carbone inorganique dissous, l'azote et le phosphore. Les principaux résultats obtenus sont :

1. Les productivités en biomasse et en hydrocarbures de *B. braunii* SAG 30,81 sont dépendantes du pH dans la gamme 5,5 et 8,0. A pH 5,5, l'inhibition de l'activité de PSII pourrait être liée à une réduction de la production d'hydrocarbures. Une valeur optimale du pH de 6.5 correspond aux productivités maximales en hydrocarbures.

2. L'effet du pH sur la production d'hydrocarbures montre que cette microalgue utiliserait préférentiellement du CO_2 (aq) et non du bicarbonate, comme l'indique la relation directe entre teneur en CO_2 (aq) du milieu de culture et productivité en hydrocarbures ; par ailleurs une approche stœchiométrique souligne que cette espèce de microalgue nécessite environ 30 % de plus de carbone que les autres espèces oléagineuses à triglycérides comme *Chlorella*.

3. La souche *B. braunii* SAG 30,81 a une capacité à la photo-adaptation lui permettant d'être cultivée en régime permanent sous flux lumineux continu à forte intensité jusqu'à 900 µmol quanta m^{-2} s^{-1} après une courte période de photoacclimation.

4. Sous forts flux lumineux incidents au photobioréacteur, la croissance de *B. braunii* SAG 30,81 est stimulée permettant ainsi d'améliorer les productivités en biomasse, hydrocarbures extracellulaires et lipides intracellulaires, mais aussi les teneurs en acides gras totaux et acide oléique.

5. La surproduction de lipides intracellulaires n'est pas influencée par une limitation azotée, mais par la densité spécifique de flux de photons actifs pour la photosynthèse. Une des interprétations possibles est que l'accumulation des hydrocarbures serait une stratégie pour stocker des excédents d'énergie lumineuse afin d'éviter les processus de photo-oxydation induits par des photon excédentaires.

6. Pour faire produire des hydrocarbures par la microalgue *B. braunii* SAG 30.81, il n'est pas nécessaire de la placer en limitation nutritionnelle, généralement azotée, comme la plupart des microalgues oléagineuse accumulant des triacylglycérols. Les teneurs en hydrocarbures de la souche étudiée sont relativement invariantes en fonction des taux de croissance et des niveaux d'alimentation azotée testés.

7. La productivité en hydrocarbures est corrélée linéairement à la productivité en biomasse. La vitesse spécifique de production spécifique d'hydrocarbures est corrélée au taux de croissance spécifique de *B. braunii* SAG 30.81. La confirmation d'une relation entre croissance des cultures et production d'hydrocarbures semble être un des facteurs déterminants pour une éventuelle future production industrielle d'hydrocarbures à partir de cultures de *B. braunii.*

8. Malgré un accroissement d'un facteur 6 de l'intensité des flux lumineux, nous n'observons pas de différence importante sur la composition pigmentaire et l'activité photosynthétique entre *B. braunii* la race A SAG 30.81 et la souche de race B BOT 22, sauf la teneur en chlorophylle dans la race B légèrement supérieure à celle dans la race A.

9. Les hydrocarbures prédominants dans la race A souche SAG 30.81 sont le nonacosadiene ($C_{29}H_{56}$) et le hentriacontadiene ($C_{31}H_{60}$) ; leurs structures chimiques sont comparées à celle du botryococcène $C_{34}H_{58}$ dominant dans la biomasse de la souche BOT 22.

10. La souche de *B. braunii* de race B BOT 22 permet d'atteindre des productivités plus élevées en biomasse, hydrocarbures et lipides intracellulaires que la souche SAG 30,81. Cette

différence prévaut également lorsque ces souches sont cultivées sous des flux lumineux plus intenses, 900 µmol quanta m^{-2} s^{-1}. Ces observations confirment que la sélection de souches est déterminante pour espérer améliorer significativement les performances de production d'hydrocarbures par *B. braunii.*

11. Les acides gras totaux et les teneurs en acide oléique sont significativement plus élevés dans la souche de race A que dans celle de race B ; ces caractéristiques sont cohérentes avec leurs voies respectives de biosynthèse des hydrocarbures.

Perspectives

Dans cette thèse, nous avons tenté de mettre en évidence les effets de trois facteurs environnementaux sur les cinétiques de production de biomasse et d'hydrocarbures et sur les réponses physiologiques des deux souches de *B. braunii.* Nous avons confirmé l'intérêt de l'utilisation des cultures en mode chemostat pour ce type d'approche physiologique. Les données acquises montrent que de nombreux aspects restent encore à étudier avant d'envisager une étude de faisabilité pour une production à l'échelle industrielle. Notre travail permet d'ouvrir quelques pistes de réflexion sur les possibilités d'améliorer les performances de production de cette espèce.

1. La culture continue pour les applications omiques

Dans ce travail, nous constatons que le pH pourrait avoir un effet sur la biosynthèse des hydrocarbures, que la teneur en hydrocarbures est supérieure à un pH de 6,5 à celle à pH 7,5. Ensuite, d'autres travaux pourraient maintenir un état stable à un pH de 6,5 et un pH de 7,5 changeant de HC pour identifier l'étape limitante pour la biosynthèse des hydrocarbures. Tels que l'aide de l'analyse des écotechnologies pour identifier quels gènes sont surproduit à un pH de 6,5 ou de comprendre quels intermédiaires d'hydrocarbures sont modifiés par l'analyse des flux de carbone.

2. Détermination des conditions photo-limitantes et photo-inhibitrices

Il est bien connu que le transfert de photons à l'intérieur d'une suspension cellulaire est le principal facteur limitant dans des cultures de microalgues. Nous avons mis en évidence que les souches *B. braunii* SAG 30.81 et BOT 22 ont une forte capacité à la photo-acclimation en se maintenant en régime stationnaire sous 900 µmol quanta m^{-2} s^{-1}. L'existence ou non d'une relation entre excès de photons pour la photosynthèse et mise en place d'un processus

photoinhibiteur reste à élucider. Une première approche basée sur l'estimation de la densité spécifique de flux de photons actifs pour la photosynthèse et sa relation à la productivité en hydrocarbures confirme qu'un rapport surface sur volume optimal du photobioréacteur doit être déterminé pour maximiser la production d'hydrocarbures. Par ailleurs l'impact des alternances jour/nuit sur les performances de *B. braunii* n'a pas encore été étudié.

3. Influence de la température

La température est un des facteurs importants pour la croissance et les réponses des flux métaboliques de cellules de microalgues. L'impact de ce paramètre sur les performances des souches de *B. braunii* reste à déterminer.

4. L'accumulation différentielle des lipides précurseurs à la synthèse des hydrocarbures

Nous avons mis en évidence que les cinétiques d'accumulation des lipides intracellulaires peuvent être associées à celles des hydrocarbures. Il semble essentiel d'améliorer les connaissances sur l'évolution de la distribution des catégories de lipides intracellulaires lorsque les cultures cellulaires passent d'une faible à une forte productivité, en particulier les cinétiques différentielles de quelques précurseurs critiques pour la biosynthèse des hydrocarbures

5. Modélisation de cultures de colonies cellulaires

De nombreuses études ont été publiées concernant des simulations et modélisation de cultures de microalgues, très peu sur l'espèce *Botryococcus*. En fait, une des principales caractéristiques de ce modèle biologique est son organisation structurale en colonies au sein d'une matrice dont la composition biochimique est encore loin d'avoir été élucidée. Ses propriétés physico-chimiques restent à élucider, en particulier celles faisant que cette matrice semble avoir, selon la souche, des capacités maximales de stockage d'hydrocarbures, avec un relargage relativement négligeable dans le milieu de culture.

6. Caractérisation de la matrice extracellulaire

Selon la caractéristique fondamentale de la production extracellulaire hydrocarbures, comment briser le mur de soutènement, mais l'extraction d'hydrocarbures non-cellulaire destructive est significative. En général, le traitement chimique peut être toxique pour les cellules. Ensuite, la contrainte de cisaillement physique pourrait être le deuxième choix.

Cependant, ce type de contrainte de cisaillement physique est adapté à ce microalgues avec adaptation physiologique: aucune diminution Fv / Fm et de la capacité de production d'hydrocarbures sont nécessaires pour étudier. Dans un autre aspect, le métabolisme de la manipulation par le contrôle de la matrice associée à l'expression des gènes peut être réalisé. Peut-être dans un jour, nous pourrions changer la production d'hydrocarbures de croissance associé orgianal pour mélanger la croissance associée ou non-croissance associée. La croissance associée ou non croissance associée: telle est la question.

General introduction

Background and aim of this study

Bioproduction of hydrocarbons is one of the challenges facing post-peak oil production in the future. A credible option for the production of sustainable biodiesel or biojetfuels, but also other refined products for industrial applications, could be microalgae oils. The species *Botryococcus braunii* has attracted particular interest due to its ability to accumulate large amounts of long-chain hydrocarbons in an extracellular matrix.

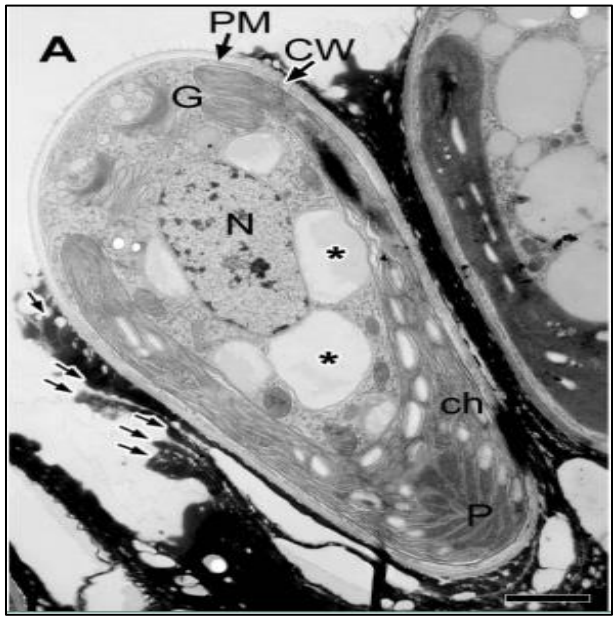


Figure 0-1. Electron microscopy analysis of longitudinal section of *B. braunii* interphase cell; scale bar, 1µm; picture is adapted from Suzuki et al., 2013.

B. braunii is a kind of fresh and brackish water alga, first described by Kützing (Kützing, 1849). **Figure 0-1** shows that there are chloroplast, pyrenoid, nucleus, Golgi apparatus, endoplasmic reticulum, lipid body in the cell, but also a complex extracellular matrix, which composed of polymerized hydrocarbons and polysaccharides. Most of hydrocarbon produced by *B. braunii* actually stored in the extracellular matrix (Suzuki et al., 2013).

As any microorganism cultivated within bioreactors, biomass and hydrocarbon productions of *B. braunii* are expected to depend on environmental factors. *There are already* a significant *number of studies on* the effect of the environmental factors, such as temperature (Lupi et al., 1991; Kalacheva et al., 2002; Qin and Li, 2006; Yoshimura et al., 2013; Demura et al., 2014), light supply as measured by the photon flux density (PFD) (Zhang and Kojima, 1998; Baba et al., 2012b; Ruangsomboon, 2012; Sakamoto et al., 2012) or nutrients (Zhila et al., 2005; Dayananda et al., 2005, 2006, 2007b; Ge et al., 2011; Tanoi et al., 2011; Yeesang and Cheirsilp, 2011; Ruangsomboon, 2012; Tanoi et al., 2014) on biomass and hydrocarbons productivities in *B. braunii* cultures. Despite such contributions on growth conditions and hydrocarbons recovery, there is still plenty of scope for further improvement in hydrocarbons productivity from *B. braunii* cultures to meet the requirements for industrial application (Jin et al., 2015).

Then the impact of these factors on the kinetics of biomass and product formations could be described by phenomelogical relations such as:

$$P_X = f(\text{Nutrients}, \text{pH}, \text{PFD}, \text{T} \dots)$$
$$P_{HC} = f'(\text{Nutrients}, \text{pH}, \text{PFD}, \text{T} \dots)$$

It is worth mentioning that most of previous studies on *B. braunii* were carried out in batch cultures, mainly without pH control. In this mode of culture, the environmental conditions are continuously changing. Such an unsteady-state system could mask subtile physiological differences and trends (Hoskisson, 2005). Indeed continuous variations of environmental parameters, such as pH or specific light supply, could confuse the relations between specific growth rates and hydrocarbon production. To overcome such drawbacks, it is a well-known fact that more reliable data could be obtained from continuous cultures at successive steady−states (Hoskisson, 2005; Tang et al., 2012).

However, only one study reported data obtained from continuous cultures of the *B. braunii* race A strain CCC 807/1; those experiments were done without pH control and at only two dilution rates (0.25 d^{-1} and 0.30 d^{-1}) (Casadevall et al., 1985). Another work on the *B.*

braunii race B strain Showa tried to achieve pseudo-steady−states by semi−continuous culture and also without pH control (Khatri et al., 2014).

Then in the present study, we aimed at investigating the effect of some environmental effects on biomass and hydrocarbon production of *B. braunii* strains by using the continuous cultures technique in controlled photobioreactor, as well as the physiological responses to these environmental factors. From the expected experimental data, we aim to open possible avenues for further optimization of hydrocarbon productivity within *B. braunii* cultures.

Organization of the thesis

Following this introduction, this Ph.D report is mainly organized according to six chapters.

Chapter I is a literature review, including the characteristics of extracellular hydrocarbon-rich microalga *B. braunii* for biofuels production and recent insights into biosynthetic mechanisms for producing extracellular hydrocarbons in *B. braunii*. This bibliographic analysis has highlighted the need for *kinetics analysis* of growth and hydrocarbons *productions* in well-controlled cultures of this green microalga.

Chapter II is the presentation of the materials and methods. We described the equipments, their calibrations, and the detailed methods used for this study. This chapter includes a critical analysis of hydrocarbon determination. The treatments of experimental data obtained from steady states cultures are particularly described.

Chapter III presents the effects of pH and nutrients supply on the efficiency of *B. braunii* SAG 30.81 in continuous cultures. In this chapter, startying from batch cultures, the main work is an investigation of a continuous culture at successive steady-states. The effects of environmental pH on biomass and hydrocarbon productivities are studied in photoautotrophic cultures of *B. braunii* SAG 30.81 (race A) fed by two nutrients enriched AF-6 culture medium. Successive steady-states of continuous cultures are used for studying the effects of pH controlled through

carbon dioxide feeding in a closed photobioreactor. The analysis of dissolved inorganic carbon related to hydrocarbon production is also carried out.

Chapter IV presents the effects of dilution rate on *B. braunii* SAG 30.81 in continuous cultures. In this chapter, hydrocarbon and lipid production are determined at steady-state corresponding to six dilution rates from 0.12 to 0.36 d^{-1} in continuous cultures of *B. braunii* SAG 30.81. Relation between the specific production rate of extracellular hydrocarbon and cell specific growth rate is analyzed according to the Luedeking-Piret model. Moreover, the effect of changes in the specific light supply rate, as induced by the dilution rate at fixed incident photon flux density, on biomass and hydrocarbon productions is analyzed in relation to some photosynthetic physiological responses.

Chapter V presents the results of a study dealing with the effects of incident light intensity on *B. braunii* SAG 30.81 in continuous cultures. The kinetics of hydrocarbon and lipid productions are investigated in continuous culture of *B. braunii* SAG 30.81 by exposure to 150 μmol photon m^{-2} s^{-1} and 900 μmol photon m^{-2} s^{-1}. The impact of the specific light supply rate is related as well as to photoadaptation process than to fatty acids profiles.

Chapter VI deals with a comparative study of *B. braunii* race A SAG 30.81and race B BOT 22 in continuous cultures. Chemical analysis confirms that these two strains have different hydrocarbon composition. Their cultivation at steady-state in a chemostat photobioreactor at a fixed dilution rate 0.23 d^{-1} allows to compare their relative efficiency for biomass, hydrocarbon and lipid production.

The aim of this thesis is to achieve a better understanding of the basic physiology of *Botryococcus braunii*. In addition, this investigation is motivated by the potential of this species for future production of hydrocarbons, both the C_{29} and C_{31} n-alkadiene hydrocarbons and long chan.botryococcene ($C_{34}H_{58}$). The main results of this study underline the usefulness of chemostat cultures in photobioreactors for physiological studies, to characterize the environmental factors, including different pH, irradiance and medium conditions. Through the kinetics data in continuous culture from steady states and transient states, some answers are

provided to the questions on the relations of biomass, lipid and hydrocarbon productions. In addition, some biological features of this colonial microalge are considered. For instance, the importance of the colony's matrix is discussed as a potential way for controlling the amounts of hydrocarbon accumulation. From this general conclusion, several perspectives to this work are envisaged.

CHAPTER I: Literature review

1.1. Characteristics of extracellular hydrocarbon-rich microalga *B. braunii* for biofuels production

1.1.1. Introduction

1.1.1.1. Bio-hydrocarbons for fuels: a necessity

The world total petroleum consumption and price are increasing in recent years (**Figure 1-1**). Facing the challenge of fossil hydrocarbon fuels replacing with more sustainable liquid fuels produced from renewable resources, biofuels attract more and more attention.

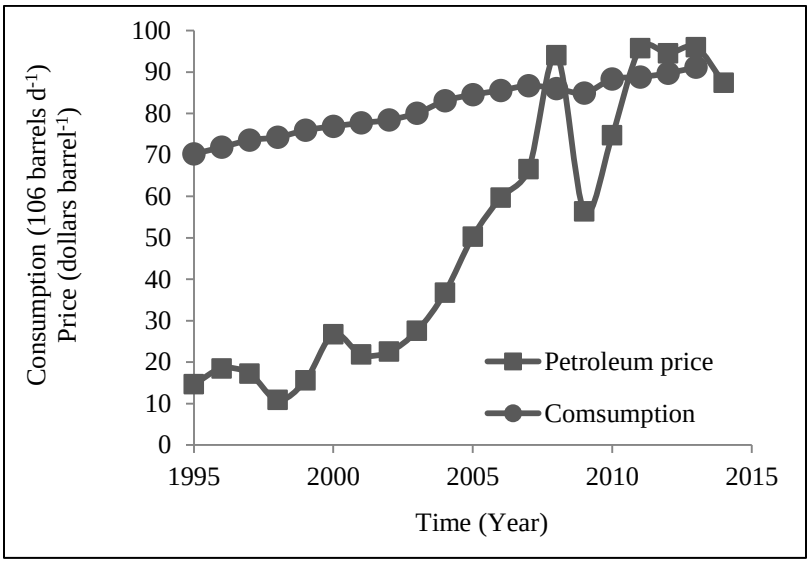


Figure 1-1. World total petroleum consumption and price as a function of time; data obtained from U.S Energy information administration.

At present, biofuels, namely methane, ethanol and diesel, are characterized by a lower energy content than conventional fossil fuels and higher oxygen content, detrimental to combustion engine (Nagano et al., 2012). The hydrocarbon biofuels would be essentially the same as those currently obtained from petroleum; it would not be necessary to modify engines, pumps, or distribution networks to accommodate the new renewable liquids in the transportation sector (Serrano-Ruiz and Dumesic, 2011). Therefore, drop-in jet fuels need hydrocarbons similar to those obtained naturally from the fractional distillation of crude oil, characterized by unoxygenated structures and high energy per volume. The need for hydrocarbon bioproduction

has also been linked to the use of an eco-friendly process, for example, by combining it with carbon dioxide (CO_2) capture system (Wang et al., 2008).

Hydrocarbon-producing crops have been studied by the Nobel laureate Melvin Calvin on *Euphorbia lathyris* (Buchanan et al., 1978) some decades ago. However, energy acquired from land-based biomass productions could compete for agricultural productions. By contrast, microalgae, which may be a source of water and nutrients, can be cultivated on non arable lands and adapted for using wastewater (Aksu, 2005).

1.1.1.2. Hydrocarbons bioproduction: from hypo to hyperproducers

Hydrocarbons are widely distributed in all organisms, including animals, plants, bacteria, and fungi. The share of hydrocarbons in the body of an organism is generally <3% of the dry weight (Jones, 1969; Ladygina et al., 2006; Kunst and Samuels, 2009; Gołębiowski et al., 2011; Huang et al., 2011), as shown in **Table 1-1**.

Table 1-1
General contents of hydrocarbon produced in hypo-producer organism.

Organism	Content (%)	Reference
Animal	0.0064-0.01	Gołębiowski et al., 2011
Plant	0.001-0.35	Huang et al., 2011
Bacteria	0.008-2.7	Jones, 1969
Fungi	0.004-0.7	Ladygina et al., 2006

The hydrocarbon fractions are composed of short-chain hydrocarbons, often semi-volatile organic compounds; medium-chain hydrocarbons, generally in liquid form; and long-chain hydrocarbons, the waxy fractions. These last ones are often linked to cuticle components to protect against predation or viral attacks. Whatever the organisms are, contents of these solid

hydrocarbons are generally in the range of 0.001–0.4% of the dry weight. Microorganisms such as bacteria or fungi are generally hydrocarbon hypo-producers. Low contents of hydrocarbons are also found in microalgae, such as *Chlorella vulgaris* with 0.045% (Afi et al., 1996).

Barely few organisms produce high concentrations of liquid hydrocarbons (**Table 1-2**). Among heterotroph organisms, the halotolerant bacteria *Vibrio furnissii* produce up to 60% of dry biomass as extracellular hydrocarbons, mainly alkanes from $C_{15}H_{32}$ to $C_{24}H_{50}$ (Park et al., 2005). This biosynthesis was shown to be associated with the culture growth (Park et al., 2005). However, the species potential toxicity hampers industrial exploitation (Derber et al., 2011). In addition, *Aurantiochytrium* sp. strain 18W-13a, the eukaryotic heterotrophy microorganism which belongs to the family Thraustochytriaceae of the class Labyrinthulomycetes, accumulates squalene up to 19.8% of dry biomass (Kaya et al., 2011; Nakazawa et al., 2012).

Table 1-2
Maximal contents of hydrocarbon produced in hyper- producer organisms.

Organism	Content (%)	Type	Reference
Aurantiochytrium sp.	19	Heterotroph	Nakazawa et al., 2012
Vibrio furnissii	60	Heterotroph	Park et al., 2005
Dunaliella salina	14	Autotroph	Ribeiro et al., 2011
Botryococcus braunii	61	Autotroph	Metzger and Largeau, 2005

However, the optimized medium of the strain 18W-13a contains peptone, yeast extract, and glucose (Nakazawa et al., 2012), and therefore is very expensive to produce the squalene commercially (Yoshida et al., 2012). The hyperhalophilic microalga *Dunaliella salina* with its high β-carotene content, up to 14% dry weight (Ribeiro et al., 2011), could be looked upon as a hydrocarbon source; however, the high melting point (183 ºC), unless an appropriate chemical

hydrolysis, and the high value added for nutraceutical applications of this carotenoid do not argue for jetfuel applications.

Another well-known hydrocarbon-rich microorganism is the microalga *Botryococcus braunii*, in which the hydrocarbon content could exceptionally reach up to 61 % of dry biomass or more (Brown et al., 1969; Metzger et al., 1985). However we must note that such high contents seem limited only to some papers, when main scientific reports deal with values lower than 50 % dry weight. To the best of our knowledge, humans do not face risk of disease, although there might be some indirect toxic effect on fish and zooplankton in the proximity of *B. braunii* blooms, mainly associated with either the well-known cytotoxic effects of free fatty acids or likewise with toxic effect induced by a decrease in dissolved oxygen (Papa et al., 2008). Then, as shown by Mendes and Vermelho (Mendes and Vermelho, 2013), improvement of *B. braunii* cultures could take advantage of this potential allelopathy. Majority of the hydrocarbons produced by *B. braunii* are accumulated in an extracellular matrix but not in the culture medium. At present, this characteristic is studied to improve hydrocarbon productivity. The extracellular location of the hydrocarbons could support a milking-like process and then the recycling of the microalgal biomass, an advantage in comparison to other microalgae that accumulate triacyglycerol in intracellular lipid body (Moheimani et al., 2013, 2014). In addition, recent studies have shown that the biomass residues, obtained after hydrocarbons extraction, are compatible with co-valorization processes for either residual energy recovery or production of different kinds of valuable bioproducts according *B. braunii* strains (Buono et al., 2012; Watanabe et al., 2014, 2015). Therefore, *B. braunii* is regarded as one of the most outstanding candidate microalgae for biohydrocarbon production. Here, the main characteristics of the microalga used or could be used to improve the efficiency of the hydrocarbon bioproduction are reviewed.

1.1.2. Liquid hydrocarbon fraction from *B. braunii* biomass

Pressure-released extracellular *oil droplets* (Moheimani et al., 2013) can be observed easily under the microscope. These hydrocarbon droplets are excreted from the matrix, where

cells of *B. braunii* are embedded. The medium-chain hydrocarbons constitute this liquid fraction of the *B. braunii* hydrocarbons (Weiss et al., 2012).

1.1.2.1. Hydrocarbon biodiversity and chemiodiversity in B. braunii

The classification is as follows: Empire: Eukaryota; Kingdom: Plantae; Phylum: Chlorophyta; Class: Trebouxiophyceae; Order: Trebouxiales; Family: Botryococcaceae; Genus: *Botryococcus*; Species: *braunii*.

There are more than sixty strains of *B. braunii* being isolated (Metzger and Largeau, 2005), and phylogenetic analysis of 18S rRNA gene sequences has confirmed that *B. braunii* belongs to Trebouxiophyceae, Chlorophyta (Kawachi et al., 2012), as shown in **Figure 1-2**.

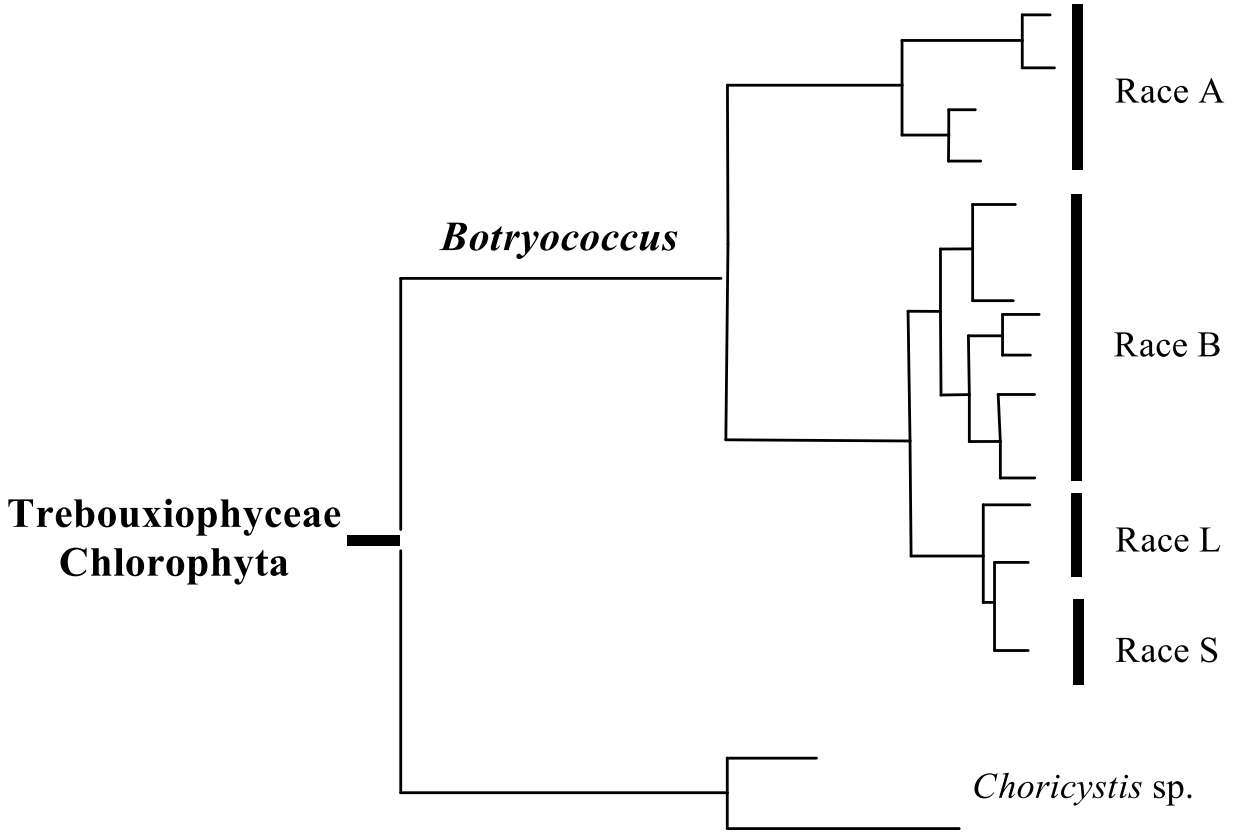
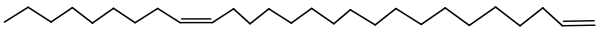
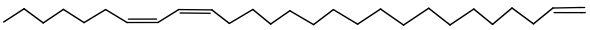
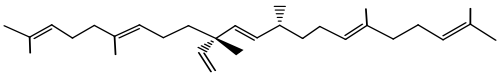
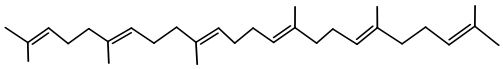
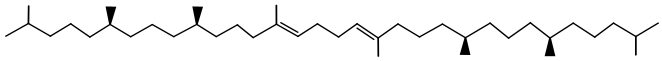


Figure 1-2. Simplified phylogenetic tree showing taxonomic relationships between races A, B, L and S derived from 18S rRNA gene sequences and their close match with the type of hydrocarbon distribution found in *B. braunii* strains. The figure is modified from Kawachi et al., 2012, the original also shows strain names, bootstrap values and other chlorophyte groups.

The hydrocarbons produced are quite different according to strains, although all these strains taxonomically belong to the same species (**Table 1-3**).

Table 1-3
Hydrocarbons produced in different races of *B. braunii*.

Race	Main Type	Formula	Typical Structure	Reference
A	Alka-dienes	C_nH_{2n-2} (n=23, 25, 27, 29, 31, 33)		Metzger and Largeau, 2005
	Alka-trienes	C_nH_{2n-4} (n=29, 31)		
B	Botryococcenes	C_nH_{2n-10} (n=30-37)		Niehaus et al., 2011
	Squalenes	C_nH_{2n-10} (n=30-34)		
L	Licopadiene	$C_{40}H_{78}$		Bertheas et al., 1999
S	Alkane	$C_{20}H_{42}$	 [a]	Kawachi et al., 2012

[a], structure is drawn according to the formula in (Kawachi et al., 2012).

Then considering the hydrocarbon profiles of the liquid fraction, *B. braunii* were classified into race A, race B, race L, and race S. The race A *B. braunii* strains produce alkadiene and alkatriene hydrocarbons, with the odd carbons being numbered from C_{23} to C_{33} and two or three double bonds in the molecules (Metzger et al., 1985, 1997); the race B strains produce triterpenoid hydrocarbons, predominantly C_{30}–C_{37} botryococcenes (Metzger et al., 1985; Okada et al., 1995) and also low amounts of C_{31}–C_{34} methylated squalenes (Huang and Dale Poulter, 1989; Achitouv et al., 2004); the race L strains produce a single C_{40} tetraterpenoid hydrocarbon, lycopadiene (Bertheas et al., 1999). The recently found race S strain produces C_{20} saturated n-alkane (Kawachi et al., 2012).

1.1.2.2. Hydrocarbon contents: methodological considerations

Very high hydrocarbon contents have been reported in some *B. braunii* cultures, with a maximal value of up to 86% of dry biomass (Brown et al., 1969). This particular result was obtained with algae in the resting phase of growth, probably associated with partial lysis or degradation of cell content (Metzger and Largeau, 2005). Some other studies showed hydrocarbon contents at high levels, such as 61% in race A (Metzger et al., 1985) and 64.3% in race B (Xu et al., 2012a). Indeed, a classical protocol for determination of the liquid hydrocarbon content is presented in a simple hexane extraction followed by a gravimetric determination of the dry extract. This extract is generally regarded as the hydrocarbon fraction, considering that the solvent gets into these extracellular metabolites easily (Dayananda et al., 2007b; Furuhashi et al., 2013; Xu et al., 2012a). However, the biosynthesis of ether lipids or extracellular carotenoids is another important characteristic of *B. braunii* in addition to the production of hydrocarbons.

These molecules also could be extracted by hexane in a short time (Metzger et al., 1993), leading to an overestimation of the hydrocarbon content, as shown in **Table 1-4**.The hydrocarbon contents were shown to be lower by applying the method of purification to the hexane extracts on silica gel columns, as well as for race A strains with values <30 or 61% (Metzger et al., 1985) (average value around 11%), 40% (average value around 25%), and 8% (average value around 5.5%) of the dry biomass for race A, race B, and race L race strains, respectively. The hydrocarbon contents could even be lower than 1% in some of the strains (Metzger et al., 1997).

Even if these data are compiled regardless of the culture conditions, the silica gel column chromatography purified values are more realistic than those published based on the raw hexane extracts obtained under different incubation and homogenization conditions of the biomass. Recent data have suggested other more efficient solvents that can supplement hexane to facilitate hydrocarbon extraction, the 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU)/octanol for instance (Samorì et al., 2010). Hydrocarbon composition, generally determined by gas chromatography-mass spectrometry, has been shown to vary according strains of the same race (Okada et al., 1995).

Table 1-4
Purified hydrocarbon content of different *B. braunii* strains.

Race	Content range (% dw)	Strain (content in % dw)	Reference
A	1–10	Portugal Amieiro (0.1); Bolivia Ichu Khota (0.9); Jillamatong 2–4 (5.6–9.5); UTEX 572 (6.7–9.9); Overjuyo 7 (7.8)	Metzger et al., 1997; Eroglu et al., 2011; Li et al., 2013
A	10–20	Lingoult (16.53); UTEX 2441 (11.4–12.8); Yamanaka (14.1–16.1); Jillamatong (15.3–19.3)	Okada et al., 1995; Metzger et al., 1997; Eroglu et al., 2011; Li et al., 2013
A	20–30	Grasmere (27.6)	Metzger et al., 1997
A	>30	Chaumecon (61)	Metzger et al., 1985
B	1–10	ACOI1284 (8.1); Kawaguchi–2 (9.7)	Okada et al., 1995; Li et al., 2013
B	10–20	Kawaguchi–1 (18.8–19.4); La Manzo (16.1); CCAC0121 (13.8)	Okada et al., 1995; Eroglu et al., 2011
B	20–30	Kossou 4 (21.1); Ayame 1 (24.2); Showa (28.9); Overjuyo 3 (24.8); Bot 22 (26.1)	Eroglu et al., 2011; Yonezawa et al., 2012; Li et al., 2013
B	>30	Showa (30–39); Yayoi (33); Darwin (35.2)	Okada et al., 1995, 1997; Yoshimura et al., 2013
L	1–10	Kossou (2–8); Songkla Nakarin (2–8); Madras 3 (7.5); Yamoussoukro 4 (4.1)	Metzger and Casadevall, 1987; Li et al., 2013
S	N/A	Bot 15 (N/A)	Kawachi et al., 2012

N/A, no data available.

Some strains were shown to be enriched in a particular hydrocarbon, such as the BOT 70 or BOT 22 strain chosen for their ability to produce mainly one botryococcene $C_{34}H_{58}$ (Ishimatsu et al., 2012; Yonezawa et al., 2012).

1.1.2.3. Elemental composition and calorific values

The calorific values of microalgae biomass can be measured by calorimetric methods (thermogravimetric analysis, TGA; differential scanning calorimetry, DSC) or chemometrically estimated from their elementary components (Friedl et al., 2005). In addition, changes in lipid content have been linked to modifications in calorific value of biomass of oleaginous microalgae studied for biodiesel production such as *Chlorella vulgaris* under N-limited or N-sufficient conditions (Illman et al., 2000).

Table 1-5
Elemental composition and heating values of some microalgae biomass.

Microalgae biomass	C (%)	H (%)	O (%)	N (%)	Ash (%)	HHV (MJ Kg^{-1})	Reference
B. braunii	66.4 – 77.3	9.7 – 11.3	9.86 – 18.9	1.1 – 3.9	0.7 – 3.11	32.9–54.7	Liu et al., 2012; Talukdar et al., 2014, 2013; Watanabe et al., 2014
Chlorella sp.	53.82	8.48	29.95	7.25	–	26.09	Ehimen et al., 2009
Hapalosiphon sp.	47.94	7.44	37.58	6.45	13.98	14.75	Liu et al., 2012
Dunaliella tertiolecta	43.31	5.96	29.9	4.33	16.5	17.81	Chen et al., 2015
Chlorella vulgaris	52.6	7.1	32.2	8.2	7.0	23.2	Biller et al., 2011
Nannochloropsis sp.	43.32	6.28	28.95	5.69	15.76	19.11	Li et al., 2011
Spirulina sp.	54.4	7.6	26.3	10.9	7.6	21.2	Ross et al., 2010

Table 1-5 shows that the C and H content in the biomass of *B. braunii* are higher in comparison to other microalgae, such as *C. vulgaris* and *Dunaliella tertiolecta*. Whereas, a low N content in *B. braunii*, due to hydrocarbon accumulation. The high heating values (HHV) of *B. braunii* are in the range of 32.9–54.7 MJ kg^{-1}, about twofold higher in comparison to other microalgae (Liu et al., 2012; Talukdar et al., 2013, 2014).

1.1.2.4. Properties of microalgae hydrocarbons for biofuels

Botryococcus cultures have garnered considerable interest in the recent studies for biofuel applications. Indeed, the hydrocarbons obtained from this microalga are close to heavy oil fractions and can be transformed into jet fuels by hydrocracking (Murata et al., 2014).

Early in 1982, after hydrocracking of *B. braunii* oils (Hillen et al., 1982), Hillen et al. acquired a distillate composed of 67% gasoline, 15% aviation turbine fuel, 15% diesel fuel, and 3% residual oil. The physical properties have a direct influence on the combustion of the automotive engine. As shown in **Table 1-6**, the density and surface tension of crude race B B. *braunii* oil have almost the same values than those of the diesel fuel.

Table 1-6
Some properties of *Botryococcus braunii* oil in comparison with other microalgae oils and fuels.

Oil	Density ($kg\ m^{-3}$)	Kinematic viscosity ($mm^2\ s^{-1}$)	Flash point (ºC)	Cetane number	Acid number ($mg\ KOH\ g^{-1}$)	HHV ($MJ\ Kg^{-1}$)	Reference
B. braunii oil	850–853	4.3–5.52	140	55.4	0.49	40.4	Ashokkumar et al., 2014; Yamamoto et al., 2014
Other microalgae oils*	881	4.5	N/A	54.7	0.6	38.4	Mallick et al., 2012
Biodiesel (ASTM)	880	1.9–6.0	100–170	48–65	<0.5	N/A	Amin, 2009; Singh and Singh, 2010
Diesel fuel	850	1.3–4.1	60–80	40–55	<0.5	40–45	Amin, 2009; Singh and Singh, 2010
Jet fuel	780–820	1.3–3.0	41–68	43.4–67.1	0–0.02	43.6–44.5	Cramer et al., 2009; Johnson et al., 2006

*, Oils as biodiesel from *Chlorella vulgaris*; N/A, no data available.

However, the kinematic viscosity is higher in comparison to the diesel fuel (Nagano et al., 2012). A hydrocracking technology was set up for *B. braunii* oil to arrive at properties required for the fuel. A yield of 85% was generated using a simple on-site conversion process; the physical properties of the converted oil was relatively close to the diesel fuel specification (Yamamoto et al., 2014).

1.1.3. Hydrocarbon accumulation in an extracellular matrix

In microbial cultures, lipid secretion is not a common process in the absence of detectable cellular lysis. This attribute is shared by few microorganisms, such as the hydrocarbon bacteria *V. furnissii* or the microalga *B. braunii;* these two species differ in terms of hydrocarbons accumulating in the culture medium or in an extracellular matrix, respectively.

They are also found in intracellular lipid bodies of the microalga, which have no storage function in contrast to triacylglycerol bodies of other microalgae (Largeau et al., 1980; Suzuki et al., 2013; Taylor L Weiss et al., 2010). In general, there is no significant diffusion of liquid hydrocarbons in the *B. braunii* culture medium, although a simple physical pressure is sufficient for inducing the release of hydrocarbon droplets in the culture medium without significant effect on the microalgal physiology (Moheimani et al., 2013).

All *B. braunii* strains are morphologically characterized by a complex organization of colonies, where cells are radially organized within an extracellular matrix (ECM) (Weiss et al., 2012). However, the size distribution of the colonies seems to be not only race and strain dependent but also a function of the hydrodynamic conditions in the photobioreactor (PBR) (Zhang and Kojima, 1998). The size of the colony is generally higher in the race B strain in comparison to the race A strains (Hou et al., 2014). In some *B. braunii* cultures, single cells are spontaneously released from colonies without the loss of viability, a feature of some race A strains (Hou et al., 2014). The size of the colonies depends on the culture conditions, such as irradiance, nutrient availability, or dilution rate (Khatri et al., 2014; Tanoi et al., 2014; Zhang and Kojima, 1998); the variations are in the range of 90–340 µm (Khatri et al., 2014).

The ECM is a complex structure made up of solid hydrocarbon polymers acting as a backbone for protecting the colony and trapping the liquid hydrocarbon fraction.. A polysaccharide layer draped with a fibrillar sheath encloses the ECM (Uno et al., 2015; Weiss et al., 2012). The extracellular hexane-insoluble polymers are also classified as algaenans, an operational term for aliphatic, non-hydrolyzable biopolymers that have similar chemical features (Kodner et al., 2009). They are more abundant in the *Botryococcus* strains, ranging from 6 to 33% dry weight (Largeau, 1995), in comparison to other microalgae, such as *Chlorella emersonii* or *Chlorella minutissima,* where their highest content is found to be 3 and 6% dry weight, respectively (Allard and Templier, 2000; Gelin et al., 1999; Versteegh and Blokker, 2004). Studies suggested that all races contain condensed long-chain polyaldehydes backbone, and race B acetalized with triterpenoid diols (Metzger et al., 2007, 1993), while race L bearing a C_{40} tetraterpenoid side chain is substituted by oxygenated functional groups (Bertheas et al., 1999). Although some progress was made, the distribution of these reticulated polymers and their putative physiological and protective roles have not been studied in detail. They seem to account for the occurrence of well-preserved *B. braunii* colonies in sedimentary rocks but mainly for their ability for storing liquid hydrocarbons (Volkman, 2014).

1.1.4. Biomass and hydrocarbon productivities

Recent studies are opposed to the fact that *B. braunii* is a very slow-growing microalga (Melis, 2012). Indeed, some strains were shown to grow at specific rates up to 0.5 d^{-1} (Yoshimura et al., 2013). With biomass concentrations of up to 20 g L^{-1}, these experiments have demonstrated that this hydrocarbon-rich microalgae could be moderately cultivated in semi-continuous PBR efficiently (Khatri et al., 2014). But these performances could still be improved to a great extent as regards those obtained with other microalgae.

The high generation times could be related to the colonial state of the cell suspensions, and the potential nutrients, CO_2 and/or light transfer limitations within the extracellular matrix.The biosynthesis of high hydrocarbon quantities, as either liquid or solid extracellular fractions, is believed to be completed at the expense of cell division (Melis, 2012). Indeed, it has been reported that the photosynthetic carbon is directed towards either terpenoids or fatty acids

biosynthetic pathway, respectively, for race B strains (botryococcene synthesis) and race A (alkadienes and alkatrienes synthesis) (Melis, 2012). While other unstressed oleaginous microalgae were suggested to partition about 80% of the photosynthetic carbon towards sugar biosynthesis, this feature was very specific to the *B. braunii* species (Lindberg et al., 2010).While maximal growth rate of 0.5 d^{-1} is achieved in test tubes, the specific growth rates of *B. braunii* cultures are usually in the range 0.1–0.3 d^{-1} (Yoshimura et al., 2013). Stoichiometry equations relative to *B. braunii* could be deduced (**Table 1-7**) on the basis of biomass elemental composition.

These equations substantiate that biomass production of this hydrocarbon hyperproducer needs about 30% more CO_2 in comparison to *Chlorella* sp. or *Spirulina* sp. and about 25% less nitrogen, calculated as NO_3^- (Ranga Rao et al., 2007).

Table 1-7
Stoichiometric equations and theoretical conversion yields for biomass production in *B. braunii* and other microalgae cultures.

Species	Stoichiometric equations	$Y_{X/CO2}$ ($g\ g^{-1}$)	$Y_{X/NO3}$ ($g\ g^{-1}$)	Reference
*B. braunii**	$CO_2 + 0.03\ NO_3 + 0.88\ H_2O = CH_{1,75}O_{0,15}N_{0,03} + 1.41\ O_2$	0.38	8.91	Watanabe et al., 2014
Chlorella sp.	$CO_2 + 0.12\ NO_3 + 0.95\ H_2O = CH_{1.89}O_{0.42}N_{0.12} + 1.44\ O_2$	0.51	3.00	Biller et al., 2011
Spirulina sp.	$CO_2 + 0.17\ NO_3 + 0.84\ H_2O = CH_{1.68}O_{0.36}N_{0.17} + 1.50\ O_2$	0.50	2.07	Ross et al., 2010

*, average values of the elemental composition given by Watanabe et al., 2014.

The volumetric productivities, as illustrated in **Table 1-8**, of *B. braunii* cultures in general are relatively low, even in controlled PBR, where the maximal biomass productivity was about 240 mg $L^{-1}\ d^{-1}$ in comparison to the twofold values obtained in *Chlorella pyrenoidosa* cultures (Wang et al., 2014). However, since *B. braunii* cultures are tolerant towards desiccation and heat stress, their growth is r*obust* in open ponds *under natural* conditions (Demura et al., 2014). From data obtained from cultures of race B *B. braunii* strains under natural light regimes and in raceways

with working volumes in the range of 1–2 m^3, a very optimistic estimation could be extrapolated to an areal biomass productivity in the range of 90– 100 t ha^{-1} y^{-1} (Ashokkumar and Rengasamy, 2012). However, we cannot exclude the detrimental effect of some microbial contamination (Masters, 1971).

Table 1-8
Current biomass and hydrocarbon oil productivities of *B. braunii* in different culture systems in comparison to *Chlorella pyrenoidosa.*

Algae species	Culture systems		Productivities (mg L^{-1} d^{-1})		Reference
	Type	Volume or area	Biomass	Hydrocarbon oil	
B. braunii GUBIOTJTBB1	Flat reactor	3 L	13.0	6.8 [a]	Talukdar et al., 2013
B. braunii 765	Column PBR	3 L	92.4	22.6[a]	Ge et al., 2011
B. braunii SAG 30.81	BioFlo PBR	8 L	207.3	68.4 [b]	Sydney et al., 2010a
B. braunii	Air lift PBR	10 L	239.5 [d]	71.1 [a]	H. Zhang et al., 2011
B. braunii UTEX 572	Circular pond	50 L	77.8	13.2 [a]	Ranga Rao et al., 2012
B. braunii N–836	Open raceway pond	80 L	40.0	10.8 [a]	Ranga Rao et al., 2012
B. braunii LB572	Panel reactors	1000 L	15.0	2.4 [b]	Bazaes et al., 2012
B. braunii AP103	Open raceway pond	1800 L	114.0	12.5 [a]	Ashokkumar and Rengasamy, 2012
B. braunii AP102	Open raceway	1800 L	121.0	29.3[b]	Ashokkumar et al., 2014
B. braunii FACHB 357	Attached PBR	0.08 m^{-2}	5.5 [f]	1.06 [c f]	Cheng et al., 2013
B. braunii TN101	Open raceway	25 m^{-2} (5000 L)	33.8 [f]	8.2–13 [b f]	Ashokkumar et al., 2014
Chlorella pyrenoidosa	Flat–plate PBR	3 L	365–444	113–134 [b]	Wang et al., 2014

a, hexane extract as crude hydrocarbon; b, chloroform and methanol extract as total lipids; c, purified hydrocarbon; d, mixotrophic cultures; f, g m^{-2} d^{-1}.

Biomass concentrations of up to 20g L^{-1} were obtained in a semi-continuous trickle-film PBR, corresponding to dilution rates of 0.05 d^{-1}, in a stoichiometrically balanced growth medium with 5–7% CO_2 in air (Khatri et al., 2014). These performances were achieved by preparatory cultures at initially high biomass concentrations of 15 g L^{-1} dry weight, in a short light-path-length PBR, 5% CO_2-enriched air, and under high light intensities (average of 282 µmol m^{-2} s^{-1}). *B. braunii* has the capacity to metabolize some organic compounds, such as acetate or glucose, under photoheterotrophic conditions as other microalgae species (Tanoi et al., 2011).

Table 1-9
Hydrocarbon production of *B. braunii* strains under different growth stages.

Strain	Race	Cultivation mode	Growth stage	Content (% dw)	Productivity (mg L^{-1} d^{-1})	Reference
CCC 807/1	A	Batch	Linear phase	44.2	265.0	Casadevall et al., 1985
			Stationary phase	23.3	20.0	
Yayoi	B	Batch	Linear phase	33.3 *	19.2 *	Okada et al., 1997
			Stationary phase	31.9 *	14.2 *	
CCC 807/1	A	Continuous	High dilution rate	27.0	121	Casadevall et al., 1985
			Low dilution rate	26.1	97	
Showa	B	Continuous	High dilution rate	26.8 *	300 *	Khatri et al., 2014
			Low dilution rate	22.3 *	250 *	

* Calculated from authors data.

This property could be used by growing this microalga in acetate-enriched medium, such as the wastewaters from paper mill wasters, in order to compensate the biomass loss during the night (Kong WeiBao et al., 2012; Liu et al., 2013).

For the majority of oleaginous microalgae, unfavorable conditions with limited cell growth, such as nitrogen limitation, have been shown to trigger lipid biosynthesis and accumulation (Peccia et al., 2013). By contrast, hydrocarbon contents and productivities were found to be growth associated and appeared to follow a clear positive correlation with the specific growth rate (Niitsu et al., 2012; Yoshimura et al., 2013). The hydrocarbon production of cells in linear phase of discontinuous cultures, or at high dilution rates of continuous cultures, is higher in comparison to those in stationary phase or low dilution rate. Supplying additional CO_2 to the culture is considered necessary for their growth (Ranga Rao et al., 2007; Ge et al., 2011; Yoshimura et al., 2013). Hydrocarbon content increased with the increase of CO_2 concentration (Ge et al., 2011). While maximal hydrocarbon productivities are around 300 mg L^{-1} d^{-1} in continuous cultures within classical PBR (**Table 1-9**), they are higher in the trickle-film PBR (Khatri et al., 2014). A conceptually designed oil-producing plant has been used for hydrocarbon-enriched biomass productivity in line with the productivity level of the data for microalgal triglycerides productivities (Chisti, 2007; Shiho et al., 2012).

1.1.5. Characteristics for improving production and reducing cost

Recent studies have highlighted some techniques to use the *B. braunii*-specific characteristics for trying to accomplish a cost-effective bioproduction of hydrocarbons; indeed, one of the main bottlenecks of applying microalgae for producing the biofuel is the low productivities of the cultures (Khan et al., 2014).

1.1.5.1. Controlling reactions involved in hydrocarbon biosynthesis and secretion

It is critical to determine the factors controlling the hydrocarbon overproduction in *B. braunii* colonies and its secretion and accumulation in the ECM. Some transcriptome studies have been performed by de novo assembly of pyrosequencing cDNA reads from both race A and race B strains, such as BOT 22, BOT 70, and BOT 88-2 (Baba et al., 2012a; Ioki et al., 2012b, 2012c). Hydrocarbon biosynthesis modes of different races have been developed based on comparative gene expression analysis (Ioki et al., 2012a). Some biosynthesis reactions could be the limiting step, for example, the entry point into the very long chain fatty acid elongation

pathway in race A and the mevalonate-independent pathway in race B (Ioki et al., 2012a). Recent studies suggested, during cell division (Hirose et al., 2013; Uno et al., 2015), that adenosine triphosphate (ATP)-binding cassette transporters were secreted along with hydrocarbons(Molnár et al., 2012). There is a need to further elucidate on the secretion of hydrocarbons and formation of ECM. It is important to uncover this process as it is unclear whether or not it is enzyme mediated; this can be traced via the DNA that codes for the proteins involved.

To improve the productivity of hydrocarbons, the genes acquired from the limiting reaction method could be the potential targets of genetic engineering. In addition, since some genes of botryococcene biosynthesis enzymes have successfully coexpressed in yeast, an approach could be inserting hydrocarbon-related genes into other fast-growing microorganisms (Niehaus et al., 2011, 2012). For a new cell factory, seeking for and controlling the critical factors to enhance the hydrocarbon secretion and recovering ratio could also be substantial (Guarnieri and Pienkos, 2014).

Typically, comparative proteomics are used to mine data on the molecular mechanisms involved by changes in culture conditions (Garnier et al., 2014). Optimization of microalgal metabolism toward improved hydrocarbon production is still a challenge that requires the use of metabolic network models. However, to date, few reconstructed algal networks are available, not involving *Botryococcus* (Koussa et al., 2014). In addition, in contrast to bacterial aggregations *B. braunii* colony sizes are in the range of the aggregates found in some in vitro plant cell cultures where their occurrence occurs under nutrient-sufficient conditions as well (Tanaka, 1982). We can consider that the colonies constitute a solid phase, which could confer resistance to substrate and light diffusion, but the condition of the colonies could also be a means to improve the efficiency of the metabolic reaction as in plant cell aggregates (Hsu et al., 1993), maybe by a cell-density-dependent signaling mechanism which could control the hydrocarbon excretion rates (Gallio et al., 2002). We cannot exclude the existence of a mechanism linked to the microenvironment inside the colonies, which could control the kinetics of hydrocarbon secretion and accumulation.

1.1.5.2 Optimizing the culture conditions

Although nutrient requirements, such as carbon (Tanoi et al., 2011) or nitrogen (Cheng et al., 2014), and environmental conditions, such as light (Sakamoto et al., 2012) and temperature (Yoshimura et al., 2013), have been well identified, their transfer rates to and inside the microalgal colonies could still be one of the limiting steps for improving productivities (Stewart, 1998). Some productivity gains could be achieved in laboratory PBR by using hollow fibers or microfluidic microbubbles for gas transfer inside the culture systems (Ying et al., 2013), considering the high CO_2 demand of *B. braunii* cultures no matter what the feasibility is to scale up such technological solutions.

While the existing PBR has been mainly designed for culturing free cell suspensions (Lee et al., 2014), other suitable PBR for *B. braunii,* such as the trickle-film, allows biomass production up to 20 g L^{-1} (Khatri et al., 2014). The expected biomass concentrations could go up to 96.4 kg m^{-3} for an estimated productivity of 91 t ha^{-1} y^{-1}, with algal biofilm bioreactors (Ozkan et al., 2012).

As maintaining axenic culture is expensive and unrealistic for mass cultures, Some authors suggest using coculture systems with un-detrimental bacterial populations. Studies report that the presence of bacteria seems to have no impact on the hydrocarbon profiles of *B. braunii* (Chirac et al., 1985), or they seem to have a positive effect on hydrocarbon productivity as shown by the *B. braunii* Ba10 strain grown with bacterial ectosymbionts (Tanabe et al., 2012). The coculture of other microalgae, such as *Chlorella vulgaris,* with *Azospirillum brasilense* was shown to supply nitrogen for biomass production (de-Bashan et al., 2005). Bacteria could stimulate algal growth by producing CO_2 or releasing substances such as vitamins or coenzymes. Despite the natural protection offered by its ECM, increasing the robustness of *B. braunii* strains towards bacterial and fungal contamination is of major interest.

1.1.5.3. Recycling fuel gas and reuse waste water with environmental benefits

In an effort to reduce the greenhouse gas emission (Wang et al., 2008), *B. braunii,* as other photosynthetic microalgae, can be used for CO_2 sequestration (from industrial exhaust gases). For the hydrocarbon biosynthesis, *B. braunii* cells have CO_2 fixation rates higher in

comparison to *Arthrospira platensis, D. tertiolecta,* and *C. vulgaris* (Sydney et al., 2010a). Flue gas with high CO_2 content is useful for the hydrocarbon production by this microalga (Yoo et al., 2010). In fact, *B. braunii* has been cultivated in wastewaters, as a means to supply nutrients, mainly nitrogen and phosphorus, and recycle waters for algae cultivation (Aksu, 2005; Yonezawa et al., 2012; Kong WeiBao et al., 2012). For instance, after 12 days of *B. braunii* cultivation, in secondary pretreated piggery wastewaters, 8.5 g L^{-1} dry weight biomass and 0.95 g L^{-1} hydrocarbons were obtained (An et al., 2003). Recently, the strain *B. braunii* 765 was cultivated successfully in aerated swine lagoon wastewaters without sterilization and pH adjustment (Liu et al., 2013). After 14 days of *B. braunii* culture, 79.63% of the nitrogen and phosphorus were removed after wastewater treatment (Sydney et al., 2011). These features confirm the robustness of this microalga, perhaps in relation to its colonial state.

1.1.5.4. "Milking" for nondestructive oil extraction

"Milking process" is defined as the possibility of reusing microbial biomass after in situ metabolite extraction. Since the microalgae have to stay alive after repeated solvent extraction, they mainly depend on the availability of biocompatible solvents, such as dodecane, and the physiological state they are in (Hejazi et al., 2004). It has been applied to nongrowing microalgal cultures, with a residual growth to compensate for cell death resulting from the water–solvent interphase contact. Applications to extracellular products, even trapped within a polymeric matrix, are a priori considered more feasible in comparison to intracellular metabolites, such as β-carotene or triacylglycerol (Hejazi et al., 2004).

In early 1989, hydrocarbons were semi-continuously extracted by biocompatible solvent from free and immobilized *B. braunii* cells (Frenz et al., 1989). Since then, other milking-like treatments have been tested on *Botryococcus* cultures. These chemical protocols were more efficient, ranging from 10 to 50%, in comparison to the physical treatment as shown in **Table 1-10**. Heptane and tetradecane are biocompatible with *B. braunii* cells, which have been extracted nondestructively and repeatedly for up to 70 days (Moheimani et al., 2014). In addition, under several conditions such as incubation in an aqueous-dihexyl ether system, not only were the initial hydrocarbon concentrations recovered but, after some days, there was also some overproduction (Moheimani et al., 2013). This hydrocarbon production was not growth

associated, as no nutrients except CO_2 was supplied to the cultures (Sim et al., 2001). However, few of the algal strains have successfully been adapted to such milking treatments, the output typically being cell culture degeneration after 2 months of successive treatment (Frenz et al., 1989; Sim et al., 2001; Kleinegris et al., 2011; Griehl et al., 2015).

Table 1-10
Physical and chemical hydrocarbons extraction in relation to cell viability of *B. braunii*.

Strain (race)	Method	Duration (d)	Oil extraction Ratio (%)	Cellular effects	Reference
CCAP 807/2 (A)	Physical pressure (flat slide, 8 min)	6	1	No effect on physiology	Moheimani et al., 2013
CCAP 807/2 (A)	Heptane (16.6%, mixing for 20 min)	6	35	No effect on physiology	Moheimani et al., 2013
FACHB 357 (B)	Tetradecane (10%, flow rate 10 mL min^{-1} with microporous membrane)	4	50.15	No solvent toxicity No shearing stress	Zhang et al., 2013
Bot 22 (B)	Heptane (16.6%, mixing for 20 min)	80	10 [a]	Fv/Fm reduced over 30% after 70 days repetitive extract	Moheimani et al., 2014
UTEX 572 (A)	Octane (33%, spouting at 100 mL min^{-1} for 9 h)	66	50.8 [b]	Growth inhibition <20%; cells can be regenerated	Choi et al., 2013

a, calculated from authors data; b, octane extract and then purified using hexane by silica gel chromatography.

Another property of the *Botryococcus* strains is their capacity to separate cells from the ECM. First data confirmed that viable cells are more easily obtained from race A strains in comparison to race B strains (Hou et al., 2014). It is important to acquire single viable cells from colonies considering not only single-cell handling for genetic manipulations but could also be hypothetically considered as a means for recovering both liquid and solid hydrocarbon fractions while recycling viable cells; the last one would not only be efficiently separated from the polymeric matrix but would also have to regenerate a new one.

1.1.5.5. Co-valorization of residues to reduce the cost

After extraction, the biomass residue still has high C and H content and relatively high calorific value, which can be classified as a hydrogen-rich solid fuel comparable to fossil fuel, such as coal, rather than other algal and terrestrial biomass (Watanabe et al., 2014).

After pyrolysis of biomass residues, catalytic gasification is more efficient for *B. braunii* in comparison to other microalgae, such as *Nannochloropsis oculata* (Watanabe et al., 2015). Although the operative parameters for pyrolyzing the residues are not identical, the hydrogen yield for *B. braunii* is three times higher. However, the heat activation is higher for *B. braunii* in comparison to other microalgae such as *Hapalosiphon* sp. (Liu et al., 2012). The apparent pyrolysis activation energy of *B. braunii* biomass residue (coorongite) is much lower in comparison to most kerogens (Garciano et al., 2012). Steam pyrolysis of the oil-extracted residue biomass of the *B. braunii* at 873–973 K can produce highly carbonized char, gases, H_2, and tar including mainly heavy component such as polycyclic aromatic hydrocarbons (Watanabe et al., 2014). The spent biomass of *B. braunii* could also be used for bioethanol production (Kavitha et al., 2014).

In addition, *B. braunii* biomass is a source of valuable chemicals, such as carotenoids, squalene, fatty acids, polysaccharides, sterols, and vitamins (Tanabe et al., 2014). As some of these metabolites, for example, squalene (Huang and Dale Poulter, 1989; Achitouv et al., 2004) and echinone (Matsuura et al., 2011), are accumulated in the ECM, they could be extracted as a milking process. In addition, phenolic extracts of *B. braunii* have high phenolic contents, up to 12.8% of dry biomass, with high antioxidant activities (Rao et al., 2006). *B. braunii* fatty acids have been proposed as enhancers to improve the drug absorption into and across the skin (Fang et al., 2004). Aqueous extracts of *B. braunii* have been shown to reduce skin dehydration, induce synthesis of new collagen, stimulate adipocytes differentiation, and promote antioxidant and anti-inflammatory activities (Buono et al., 2012). Such potential co-valorizations could add to the economic effectiveness of hydrocarbon bioproduction by *B. braunii* cultures.

1.2. Recent insights into biosynthetic mechanisms for producing extracellular hydrocarbons in *B. braunii*

1.2.1. Introduction

Hydrocarbons in *B. braunii* could be secreted out of cell and accumulated in the extracellular matrix, making a non-cell-destructive oils milking extraction possible (Moheimani et al., 2013; Griehl et al., 2015). Hydrocarbons oils in *B. braunii* is similar to those found in fossil petroleum, which made this microalga containing high heating values up to 54.69 KJ g^{-1} (Talukdar et al., 2014) and *B. braunii* hydrocarbons oils could be converted to biodiesel or jet fuels with a high yield of 85% (Yamamoto et al., 2014). In addition, wet biomass of *B. braunii* with moisture content up to 30% could be directly used for the reaction of biodiesel production by *in situ* esterification/transesterification, indicating low cost potential for biofuels production (Hidalgo et al., 2015).

However, *B. braunii* is a slow growing microalga, and there already have been many studies on improving the biomass and hydrocarbons productivities, such as optimizing the culture conditions and culture systems for the growth of *B. braunii* (Ge et al., 2011; Tanoi et al., 2011; Ranga Rao et al., 2012; Ruangsomboon, 2012; Sakamoto et al., 2012; Shimamura et al., 2012; Cheng et al., 2013; Yoshimura et al., 2013; Ashokkumar et al., 2014; Khatri et al., 2014; Procházková et al., 2014; Tanoi et al., 2014; Ruangsomboon, 2015) and attempts on efficient biomass harvesting and hydrocarbons oils extraction (Buono et al., 2012; Zhang et al., 2013; Moheimani et al., 2014; Griehl et al., 2015; Saga et al., 2015; Hidalgo et al., 2016). Even though these works make some contributions to the growth enhancement and hydrocarbons recovery, hydrocarbons productivity from *B. braunii* cultures is still not high enough to meet the requirements for industrial application (Jin et al., 2015).

Therefore, this situation leads us to think about the mechanisms again how *B. braunii* produces hydrocarbons and how can we improve the productivity of hydrocarbons. Since 30 years ago, there have been started some works on the biosynthetic mechanisms of hydrocarbons

in *B. braunii* from chemical structures of the hydrocarbons and feeding experiments using radioactive isotopes. In recent years, a number of new studies were performed.

With the developing of genetic engineering, genome size of race A (166.0 ± 0.4 Mb), race B (166.2 ± 2.2 Mb) and race L strain (211.3 ± 1.7 Mb) are obtained (Taylor L. Weiss et al., 2010; Weiss et al., 2011) as well as mitochondrial genome of a race B strain Showa is completed (Zou and Bi, 2016). Also, the full length enriched complementary DNA library of the *B. braunii* race A and race B strains are constructed and the expression sequence tags (EST) datasets have been deposited in the GenBank database (Baba et al., 2012a; Ioki et al., 2012b, 2012c). Analysis of enzyme-coding genes expression between different races of *B. braunii* were carried out (Baba et al., 2012a; Ioki et al., 2012b, 2012c; Fang et al., 2015; Kumaar and Kumar, 2015). Moreover, new enzymes in biosynthetic pathways are identified by expression of the isolated genes inserted into bacterial and yeast (Niehaus et al., 2011, 2012). These new findings could map the overall biosynthetic pathways. With advanced electron microscopy and fluorescent staining technique, such as quick-freeze deep-etch electron microscopy, confocal Raman microspectroscopy and fluorescent lipophilic dye Nile Red staining technique, new images have probed the ultrastructure of *B. braunii* cells, colonies and hydrocarbons distribution (F. Zhang et al., 2011; Weiss et al., 2012; Suzuki et al., 2013; Hirose et al., 2013; Uno et al., 2015). By these investigations, the presence of N-terminal sequence motifs of the biosynthetic enzymes encoded by genes for their localization was examined and the transformations of lipid bodies related to hydrocarbons production in cell lifecycle stages were observed. Then, new information could predict the hydrocarbons biosynthetic localization and secretion, as well as the growth stages for hydrocarbons producing.

In early 1989, hydrocarbons were semi-continuously extracted by biocompatible solvent from free and immobilized *B. braunii* cells (Frenz et al., 1989). Since then, other milking-like treatments have been tested on *Botryococcus* cultures. These chemical protocols were more efficient, ranging from 10 to 50%, in comparison to the physical treatment as shown in **Table 1-10**. Heptane and tetradecane are biocompatible with *B. braunii* cells, which have been extracted nondestructively and repeatedly for up to 70 days (Moheimani et al., 2014). In addition, under several conditions such as incubation in an aqueous-dihexyl ether system, not only were the initial hydrocarbon concentrations recovered but, after some days, there was also some overproduction (Moheimani et al., 2013). This hydrocarbon production was not growth

associated, as no nutrients except CO_2 was supplied to the cultures (Sim et al., 2001). However, few of the algal strains have successfully been adapted to such milking treatments, the output typically being cell culture degeneration after 2 months of successive treatment (Frenz et al., 1989; Sim et al., 2001; Kleinegris et al., 2011; Griehl et al., 2015).

There are more than sixty strains of *B. braunii* being isolated (Metzger and Largeau, 2005), and classified into 4 races (Kawachi et al., 2012). Among the races of *B. braunii* , lots of studies on the hydrocarbons biosynthesis mechanisms on race A and race B strains because these two races are more abundant in nature and generally exhibit higher hydrocarbons contents than others (Jin et al., 2015). While, there are few reports on biosynthetic mechanisms of concerning *B. braunii* race L and race S, except some studies of their hydrocarbons structure and its biopolymer (Bertheas et al., 1999).

Here, we examined studies on the biosynthesis on *B. braunii* race A and B. Recent opinions on biosynthetic mechanisms basing on new findings were critical reviewed, including the pathways, localization, secretion, accumulation and cellular growth stage for hydrocarbons production. In order to further improving the hydrocarbons productivity in *B. braunii* culture, we highlighted some salient questions and proposed some ideas for further investigation.

1.2.2. Hydrocarbons biosynthetic pathways: diversity between race A and race B

B. braunii race A produces straight-chain hydrocarbons, odd carbon numbered alkadienes and trienes, while race B produces triterpenoid hydrocarbons, called botryococcenes and squalenes. The numbers of carbons and the structures of hydrocarbons produced by race A and race B strains are different due to different biosynthetic pathways.

1.2.2.1. Biosynthesis of hydrocarbons (alkadienes and trienes) in B. braunii race A

In race A, as shown in **Figure 1-3,** hydrocarbons are biosynthesized from VLCF acids via VLCF aldehydes. The VLCF are elongated from oleic acid, which is found to be the precursor of hydrocarbons and is abundant in *B. braunii* (Templier et al., 1984; Choi et al., 2011).

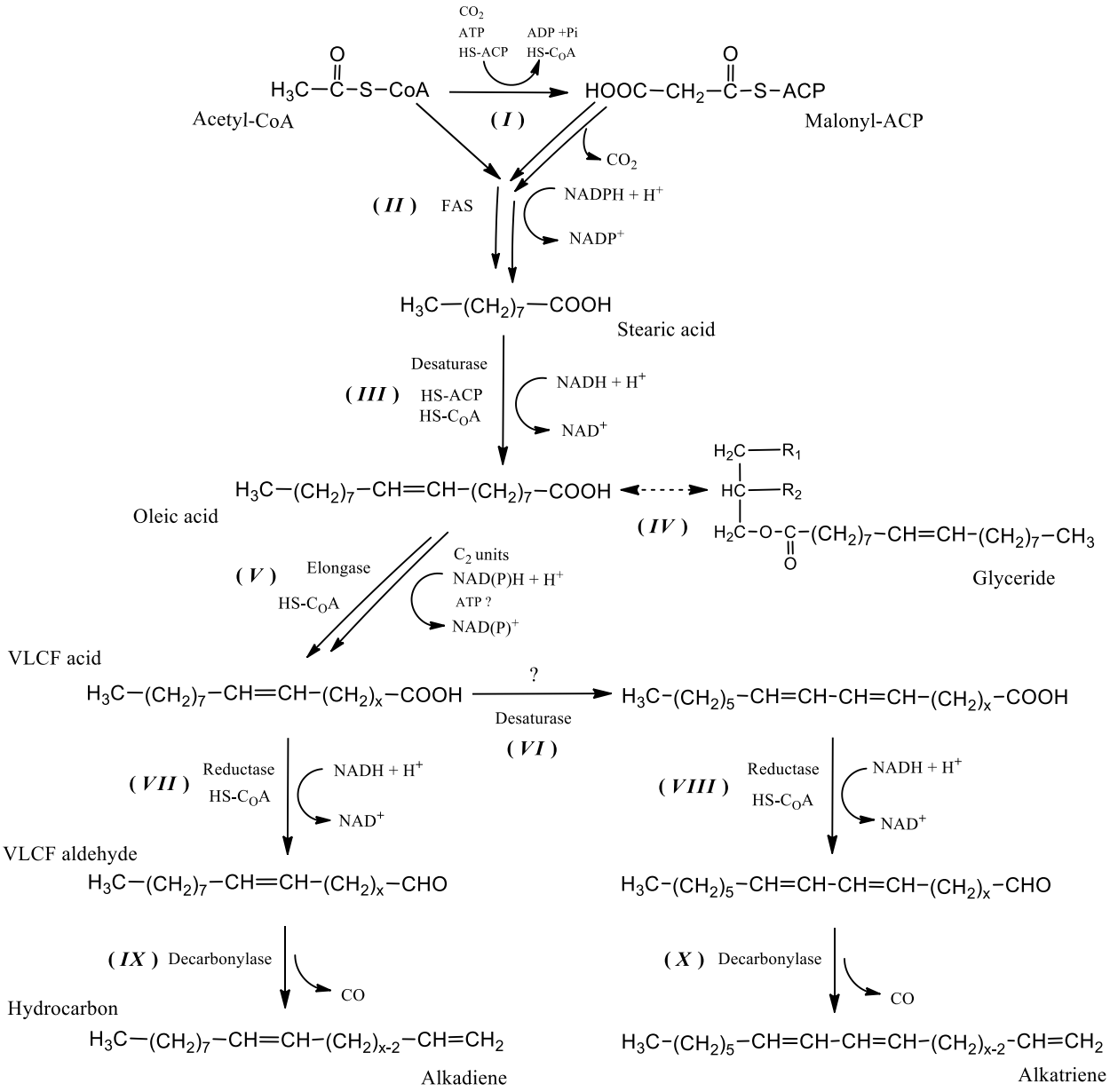


Figure 1-3. Schematic biosynthetic pathways of hydrocarbons in *B. braunii* race A; x=9, 11, 13, 15, 17, 19, 21, 23. The figure is organized according to the references: *I*, (Chan and Vogel, 2010; Joyard et al., 2010); *II*, (Baba et al., 2012a; Hu et al., 2008); *III*, (Pereira et al., 2003; Ioki et al., 2012a); *IV*,(Baba et al., 2012a; Ioki et al., 2012a); V, (Hlousek-Radojcic and Evenson, 1998; Banerjee et al., 2002); *VI*, (Templier and Largeau, 1991; Villarreal-Rosales et al., 1992); *VII and VIII*, (Dennis and Kolattukudy, 1992; Wang and Kolattukudy, 1995); *IX and X*, (Dennis and Kolattukudy, 1991, 1992); ACP, acyl-carrier-protein; ATP, adenosine triphosphate; CO, carbon monoxide; CoA, coenzyme A; FAS, fatty acid synthase; VLCF acids, very long chain fatty acids; VLCF aldehyde, very long chain fatty aldehydes.

1.2.2.1.1. Fatty acid synthesis for oleic acid (step I, II and III)

Oleic acid in *B. braunii* could be synthesized through ACP way as in other microalgae or plants, which begins from the conversion of acetyl-CoA to malonyl-CoA catalyzed by acetyl-CoA carboxylase (Chan and Vogel, 2010; Joyard et al., 2010) with ATP and NADPH as co-factors (Hu et al., 2008), since acetyl-CoA carboxylase and ACP synthase related genes were expressed at high levels in a *B. braunii* race A strain BOT-88-2 (Baba et al., 2012a). The fatty acid desaturation process from stearic acid to oleic acid is considered to be catalyzed not only in the ACP bound form but also in the CoA-bound form, according to the results that EST data set of race A strain contained both stearoyl-CoA 9-desaturase and acyl-ACP desaturase and expressed at high level (Ioki et al., 2012a). From the consideration of cofactors, both these two form desaturations are dependent on NADH (Los and Murata, 1998; Pereira et al., 2003), but why the desaturation process in *B. braunii* in two forms is unknown. Fatty acid desaturation in the CoA-bound form is usually in animals, yeasts and fungi, but not in photosynthetic organisms (Los and Murata, 1998).

1.2.2.1.2. Oleic acid elongation conversion to VLCF acids (step V and VI)

Early in 1984, Templier et al. suggested the biosynthesis of *B. braunii* hydrocarbons probably takes place via an elongation mechanism, relating to what is operating in some higher plants (Templier et al., 1984). In this mechanism, oleic acid elongated by successive addition of C_2 units derived from malonyl CoA (Banerjee et al., 2002). In recent years, candidate genes associated with very long chain fatty (VLCF) acids elongation encoding enzymes, such as enoyl-CoA hydratase and trans-2-enoyl-CoA reductase, have been found in the transcriptome of race A strain BOT-88-2 (Baba et al., 2012a; Kumaar and Kumar, 2015). It needs to be noticed that the step from oleic acid enter to VLCF acids seems to be a rate-limiting step, because the gene expression of the entry point catalyzed by 3-ketoacyl-CoA synthase was very low in race A *B. braunii* (Baba et al., 2012a).

As for trienic hydrocarbons, on the basis of the feeding experiment, it is unlikely that trienes directly derived from linoleic acid or formed via desaturation of dienes (Templier and

Largeau, 1991). The study of trienes distribution during growth confirmed that trienic hydrocarbons are synthesized from diunsaturated VLCF acids (Villarreal-Rosales et al., 1992).

Generally speaking, fatty acid elongation in both plant and animal requires NAD(P)H and uses a variety of cofactors with varied requirements for ATP (Hlousek-Radojcic and Evenson, 1998). To date, it is unclear whether ATP is necessary for the elongation in this green microalga and few reports about the cofactors for the reaction of desaturation of VLCF acids.

1.2.2.1.3. VLCF acids conversion to hydrocarbons via VLCF aldehydes (step V II, VIII, IX and X)

The final step in biosynthesis of hydrocarbons was ever regarded to be via a decarboxylation mechanism (Templier et al., 1984), while later experimental results suggested that synthesis of hydrocarbons from VLCF acids is via a VLCF aldehydes intermediate, which could be the immediate precursor of hydrocarbons (Dennis and Kolattukudy, 1992). Long-chain aldehydes could be produced by catabolism of several lipids (Rizzo, 2014) and the VLCF aldehydes are also considered to be the immediate precursor of hydrocarbons in epidermal cells (Marsh and Waugh, 2013). What's more, VLCF aldehydes-generating fatty acyl-CoA reductase has been found and purified in *B. braunii* race A (Bognar et al., 1984; Wang and Kolattukudy, 1995). This enzyme catalyzes the reduction of fatty acyl-CoA to aldehyde with NADH as reductant. In addition, decarbonylase, another key enzyme for the final step, which contains a Co-porphyrin was also purified (Dennis and Kolattukudy, 1992). Consequently, the final step is a catalyzed conversion of VLCF aldehydes to hydrocarbons and CO via the decarbonylation mechanism.

It is worth to mention that not all enzymes associated with curated ESTs in *B. braunii* are confirmed, although more enzymes were found by new processing of the transcriptome data (Kumaar and Kumar, 2015) or in another race A strain *B. braunii* 779 (Fang et al., 2015). It is possible that we might not currently know the sequences encoding enzymatic activities of hydrocarbon biosynthesis in *B. braunii* race A. This microalga might have a novel pathway for hydrocarbon biosynthesis, since microalgae could have some unconventional enzymes, for

example, a novel fatty acid desaturase was screened in a bloom forming marine phytoplankton *Emiliania huxleyi* rencently (Kotajima et al., 2014).

Interestingly, in *B. braunii* race A, glycerolipid metabolism genes also strikingly strong expressed (Baba et al., 2012a; Ioki et al., 2012a). Glycerolipids are important storage molecules of carbon in various organisms (Yen et al., 2008) which are stored within lipid bodies which could be biosynthesized from oleic acid and catabolized into oleic acid (Ducharme and Bickel, 2008; Wang, 2015). This suggested the active carbon exchanges between hydrocarbons and glycerolipids via oleic acid (*step IV*).

1.2.2.2. Biosynthesis of hydrocarbons (botryococcenes and squalenes) in B. braunii race B

In race B, squalene and botryococcene C_{30} are biosynthesized through triterpene synthesis pathway via MEP, and then, methylated for higher hydrocarbons, as shown in **Figure 1-4**.

1.2.2.2.1. MEP pathway for IPP formation (step I, II and III)

IPP and its isomer DMAPP are the universal precursors of natural products called isoprenoids. *B. braunii* race B biosynthesizes triterpenoid hydrocarbons from IPP and DMAPP (Banerjee et al., 2002; Metzger and Largeau, 2005). In plants, it has been well established that IPP is biosynthesized from the mevalonate or the MEP (also named as non-MVA or MVI) pathways, which are located in cytosol and plastids, respectively (Hampel et al., 2005; Zhao et al., 2014). In *B. braunii*, IPP is known to be biosynthesized through MEP pathway (Sato et al., 2003) and this is confirmed by transcriptome dataset analysis in *B. braunii* race B strains (Ioki et al., 2012b, 2012c).

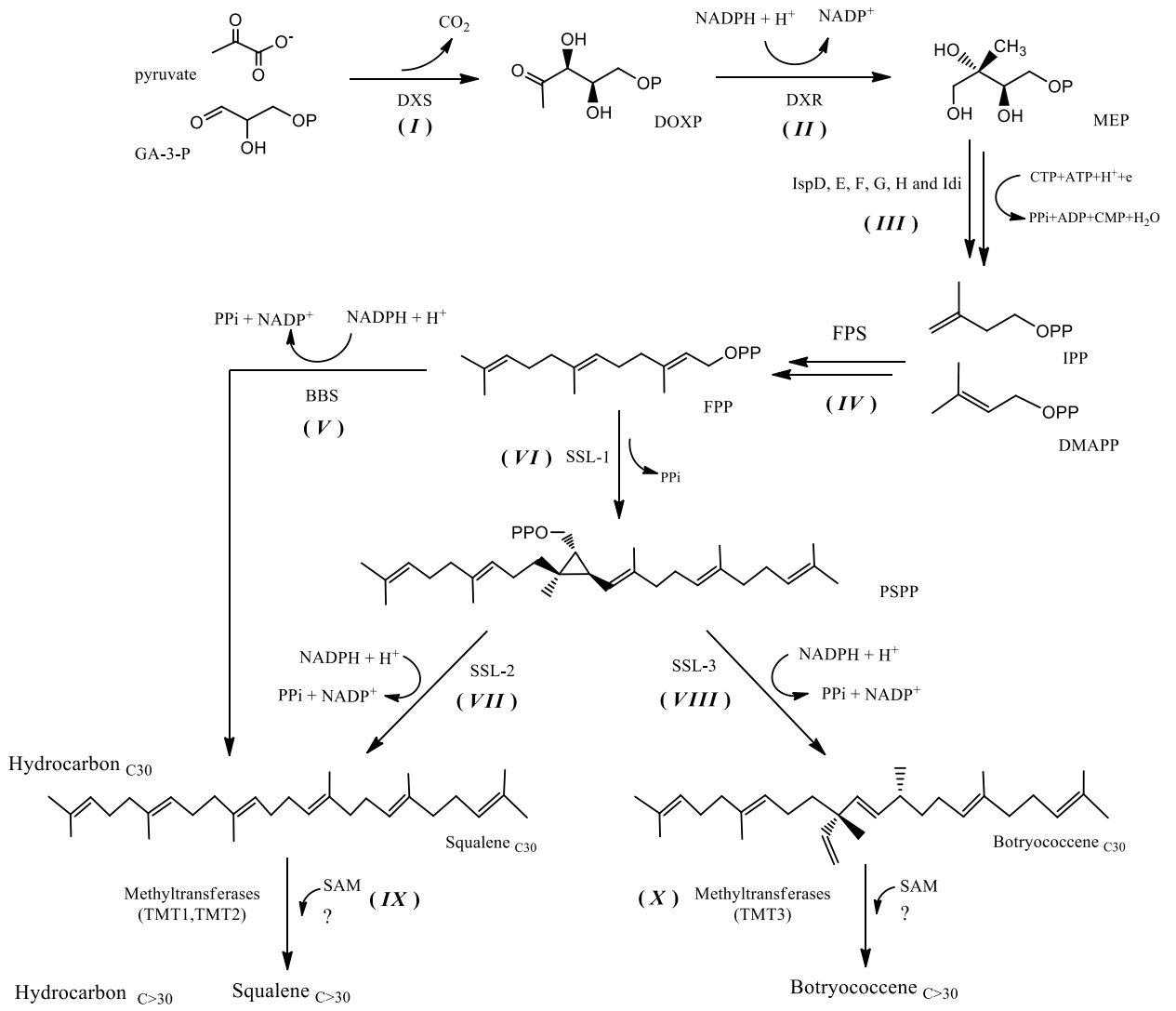


Figure 1-4. Schematic biosynthetic pathways of hydrocarbons in *B. braunii* race B. The figure is organized and drawn according to the references: *I*, *II* and *III*, (Matsushima et al., 2012; Molnár et al., 2012); *IV*, (Bergstrom et al., 2000; Dunford et al., 2001) ; *V*, *VI*, *VII* and *VIII*, (Metzger and Largeau, 2005; Niehaus et al., 2011); *IX* and *X*, (Metzger and Casadevall, 1987; Niehaus et al., 2012). BSS, *Botoryococcus* squalene synthase; CTP, cytidine triphosphate; DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; FPP, farnesyl pyrophosphate; FPS, farnesyl pyrophosphate synthase; GA-3-P, glyceraldehyde 3-phosphate; IDI, isopentenyl-diphosphate isomerase; IPP, isopentenyl pyrophosphate; IspD, 4-diphosphocytidyl-2C-methyl-D-erythritol cytidylyltransferase; IspE, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; IspF, 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; IspG, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate Synthase; IspH , 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase; MEP, 2-C-methyl-D-erythritol-4-phosphate; PSPP, presqualene diphosphate; SSL, squalene synthase-like; SAM, s-adenosyl methionine.

The entry route into the MEP pathway seems to be via the formation of 1-deoxy-D-xylulose-5-phosphate, since *B. braunii* Showa possesses three distinct 1-deoxy-d-xylulose 5-phosphate synthase (DXS) (Matsushima et al., 2012), although transcriptome analysis in another strain *B. braunii* BOT-70 implicated there could be an alternative entry route into the MEP pathway via xylulose-5-phosphate (Ioki et al., 2012b). Other substrates, products, enzymes and co-factors in MEP pathway were well summarized by Matsushima et al. (Matsushima et al., 2012) and Molnár et al. (Molnár et al., 2012) for *B. braunii* Showa. Reactions start with condensation of GA-3-P to produce DOXP, catalyzed by DXS. Then, the enzyme DOXP reductoisomerase (DXR) converts DOXP to MEP with NADPH. Subsequently, MEP reacts with CTP, ATP and H^+ to produce IPP and DMAPP, releasing pyrophosphate, ADP and CMP through six reactions catalyzed by a series of enzymes (IspD, IspE, IspF, IspG, IspH and Idi) (Matsushima et al., 2012; Molnár et al., 2012).

1.2.2.2.2. Triterpene synthesis pathways for squalene and botryococcene C_{30} (step IV, V, VI, VII, and VIII)

FPP is the precursor for both squalene and botryococcene C_{30} in *B. braunii*, confirmed by feeding experiments and stereochemical studies (Inoue et al., 1993, 1994, 1995; Okada et al., 2004), which could be formed from IPP and DMAPP catalyzed by FPP synthase (FPS) in absence of NAD(P)H or ATP (Bergstrom et al., 2000; Dunford et al., 2001). Then, two molecules of FPP form a molecule of PSPP which is regarded as the immediate precursor of botryococcene C_{30} and squalene (Huang and Dale Poulter, 1989; White et al., 1992). These kinds of processes have been well reviewed by Metzger (Metzger and Largeau, 2005). Nevertheless, at that time, it was unsure whether only one single enzyme machinery, or two separate enzymes are responsible for the synthesis of squalene and botryococcene. Recent studies clarified the characteristics of other enzymes. Before, only *Botoryococcus* squalene synthase (BSS) was demonstrated for hydrocarbon biosynthesis in race B *B. braunii* (Okada et al., 2000). However, by screening a *B. braunii* cDNA, it was found that there are still three squalene synthase-like (SSL) enzymes besides the typical BSS, catalyzing reactions with NADPH (Niehaus et al., 2011). In addition, expression of the isolated SSL genes inserted into bacterial and yeast revealed that SSL-1 catalyzes PSPP biosynthesis and SSL-2 and SSL-3 respectively catalyze from PSPP to form

botryococcene and squalene. As for the typical BSS, it can catalyze the reaction from FPP to squalene directly (Niehaus et al., 2011).

1.2.2.2.3. Methylating reactions for higher hydrocarbons (step IX and X)

In 1985, the results of ^{14}C pulse-chase experiments demonstrated that the C_{30} hydrocarbon is the precursor of all compounds of higher hydrocarbons in *B. braunii* (R. Wolf et al., 1985). Botryococcene C_{30} is often methylated by using SAM as a methylating agent and each intermediate botryococcene C_{31}-C_{33} are the precursors of their next highest homologue (Metzger et al., 1987). Recently, six of triterpene methyltransferase (TMT) genes were isolated and functionally characterized. Three of these genes were resulted in the accumulation of mono- and dimethylated forms of both triterpene scaffolds when co-expressed in yeast with complementary BBS or SSLs expression cassettes (Niehaus et al., 2012). TMT-1and TMT-2 also exhibit preference for squalene as the methyl acceptor substrate, whereas TMT-3 shows a striking preference for botryococcene as its methyl acceptor substrate (Niehaus et al., 2012).

However, not all the mechanisms responsible for methylation of botryococcene and squalene in *B. braunii* are identified. It still needs to discover additional TMTs or other mechanisms for the complete methylation pattern. Moreover, as in race A, some enzymes associated with curated ESTs in *B. braunii* race B still have not been confirmed until now, although more enzymes were identified later (Kumaar and Kumar, 2015).

Interestingly, 36 out of 100 enzymes in *B. braunii* Showa involved in biosynthesis of botryococcene or squalene were also found in a race A strain *B. braunii* 779 (Fang et al., 2015). Some unknown factors might control the expressing of genes for biosynthesis of which type hydrocarbon in race A and race B, and metabolism in race A cells might can change to be that in race B.

1.2.3. Hydrocarbons biosynthetic localization and secretion: from intracellular lipid bodies to extracellular matrix

B. braunii is a hydrocarbons rich microalga, but, the hydrocarbons proportion in cell is just about 5%, while most of the hydrocarbons are accumulated out of the cell in the extracellular matrix (Largeau et al., 1980). It is interesting to understand how hydrocarbons are secreted from cell to the extracellular matrix. Here, we discuss recent opinions on these issues and summarized as **Figure 1-5** and **Figure 1-6**, although the biosynthetic localization and secretion of hydrocarbons are still not very clear.

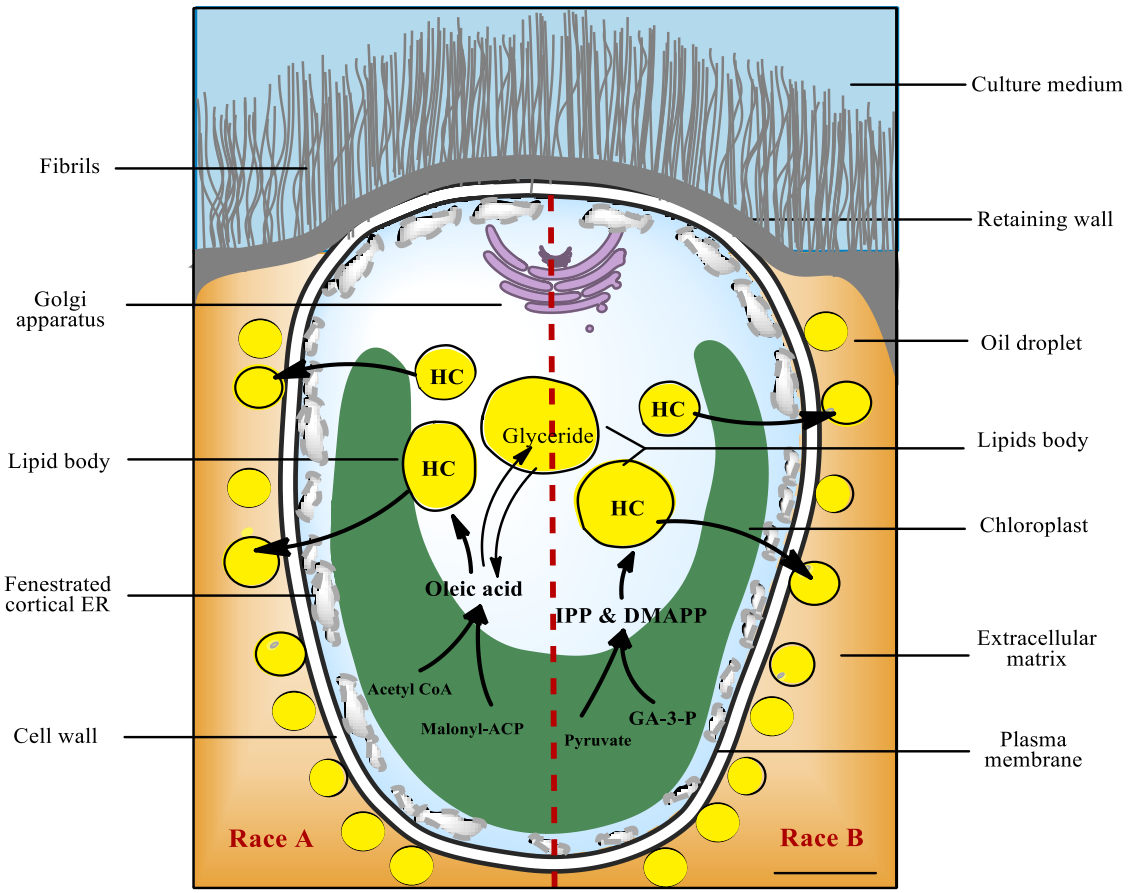


Figure 1-5. Putative hydrocarbons biosynthetic localization and secretion in *B. braunii;* the left of the middle dash line means in race A, the right of the middle dash line in race B; HC in race A mainly includes alkadienes and trienes, or precursors; HC in race B mainly includes botryococcenes and squalenes, or precursors; figure is drawn according to (Weiss et al., 2012; Suzuki et al., 2013; Hirose et al., 2013; Uno et al., 2015). For simplification, not all cellular organelles are shown. Scale bar, 2 μm.

1.2.3.1. Precursors of hydrocarbons are biosynthesized starting from chloroplast

Biosynthesis of hydrocarbons could start from chloroplast in both *B. braunii* race A and race B, as is shown in **Figure 1-5**. In *B. braunii* race A, oleic acid, the precursor of hydrocarbon, is generally assumed to be biosynthesized in chloroplast (Ohlrogge et al., 1979).

Then, the initial steps of biosynthetic pathway for the hydrocarbons produced by the *B. braunii* race A may occur in the chloroplast, in a similar way as that in some plant cells (Kunst and Samuels, 2009). In race B, the biosynthesis starts from the MEP pathway and its precursor could be DOXP. Three distinct DOXP synthases all turned out to possess chloroplast signal sequences at the N-terminal (Matsushima et al., 2012). In addition, gene coding for the enzyme IspF in the MEP pathway cloned from race B BOT 70 contained chloroplast signal sequences at their N-terminal (Ioki et al., 2012b). Thus, the first steps of biosynthesis in race B are likely to occur in the chloroplast as in race A.

1.2.3.2. Hydrocarbons or precursors are biosynthesized in cell and intermediately stored in lipid bodies

However, from which step that the intermediate transfers out of the chloroplast is unsure. In race A, oleic acid is the main component of total fatty acids in *B. braunii* (Thompson, 1996) and generally exported towards other cell compartments (Drapier et al., 1982)(Drapier et al., 1982). While in race B, the enzyme Idi which catalyzes the final reaction of IPP and DMAPP biosynthesis is likely to be directed into the secretory pathway, because a signal peptide directing the protein to the secretory pathway was found in its N-terminal (Ioki et al., 2012b). Moreover, genes encoding key enzymes (SSL-1, 2 and 3) for the final steps of hydrocarbons biosynthesis have no chloroplast signal sequences in race B (Niehaus et al., 2011).

It is generally regarded the site of biosynthesis of hydrocarbons located in cell of *B. braunii*. ER, which is obviously prominent in *B. braunii* and lacks ribosomes, is proposed to be the most likely conduit for the transformation of hydrocarbons or precursors both in these two races (Suzuki et al., 2013, 2015). In plant cuticles, ER is also the cellular organelle for fatty acid elongation to produce precursor for hydrocarbon biosynthesis (Kunst and Samuels, 2009). Lipid

bodies are seemed to be the first destination of transportation, then hydrocarbons or precursors are intermediately stored in them, respect to the results that hydrocarbons were detected in lipid bodies of race A (Largeau et al., 1980) and race B strains by Raman spectroscopy (Taylor L Weiss et al., 2010).

It is worth noting that oleic acid in race A and immature C_{30} hydrocarbon in race B were ever suggested to be excreted to the extracellular matrix (Metzger et al., 1987), and then biosynthesis of hydrocarbons from these precursor took place out of the cells (Largeau et al., 1980; Templier et al., 1984). However, little evidence confirmed that view later. The enzyme of the last step of hydrocarbon biosynthesis, the aldehyde decarbonylase, purified from *B. braunii* race A strain was suggested to be microsome dependent (Dennis and Kolattukudy, 1992). The enzyme TMTs responsible for last steps of hydrocarbon methylating reactions in *B. braunii* race B were also found to be membrane-associated (Niehaus et al., 2012). If the final steps of biosynthesis of hydrocarbons really occur in the extracellular matrix, the membrane systems should be also existed out of the cell, in addition to the intermediates, enzymes and cofactors of the terminal steps. It needs further investigation to confirm that.

1.2.3.3. Hydrocarbons oils are secreted to and accumulated in the extracellular matrix

An interesting aspect of secretion is the question how the hydrocarbons oils are biosynthesized in the cell and then secreted to the extracellular matrix. Basing on electron microscope observation, it was shown that the decrease in the number of lipid bodies is strongly correlated with the accumulation of oil droplets in the extracellular matrix both in *B. braunii* race A and B (Hirose et al., 2013; Suzuki et al., 2013). Hydrocarbons or precursors in lipid bodies probably are to be secreted across cellular membrane by a vesicle-mediated transport as the process of exocytosis (F. Zhang et al., 2011), which is an ATP-dependent process (Burgoyne and Morgan, 2003). In other cells, such as in hepatocytes and enterocytes, lipids are also generally secreted from membrane-bounded compartments by exocytosis (Mather and Keenan, 1998). The unique fenestrated cortical ER, with pores and directly underlying cell membrane, is suggested to be vesicle (Weiss et al., 2012) and illustrated by 3D microscope observation (Suzuki et al., 2015). Plasma membrane could contact with the ER cisterna which containing hydrocarbon oils, and

merge them, then secrete them to the extracellular matrix through the cell wall (Hirose et al., 2013), as the model shown in **Figure 1-6.**

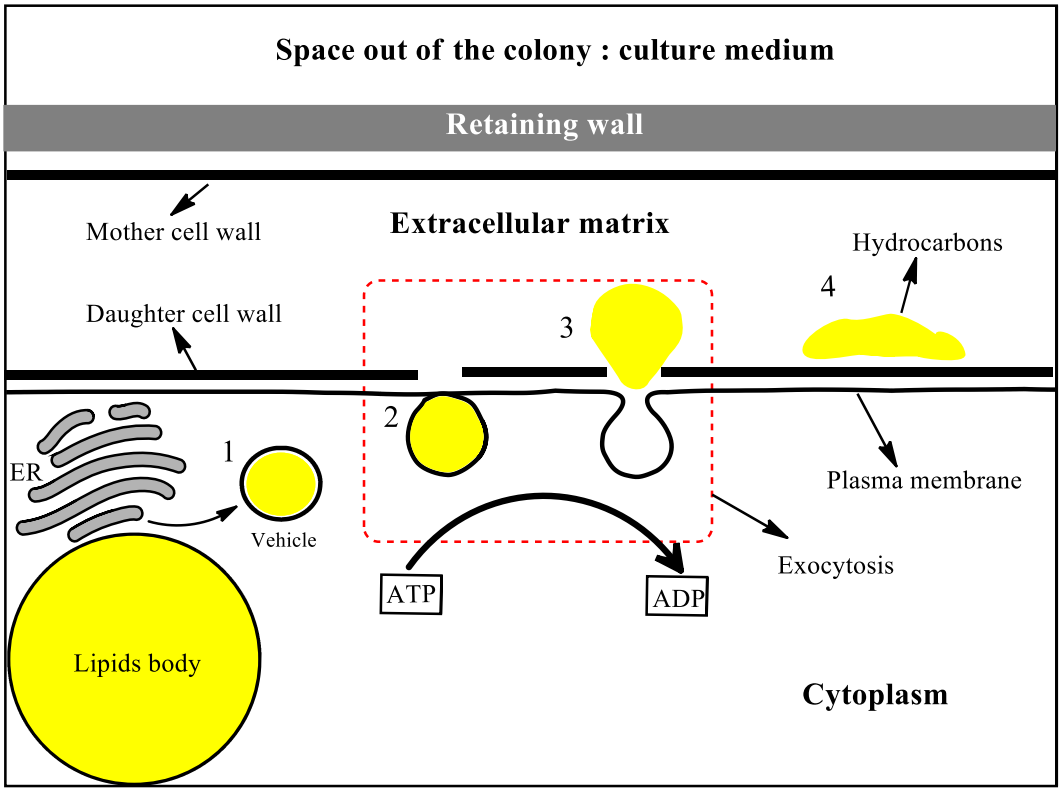


Figure 1-6. Putative process of hydrocarbons oils secretion from intracellular space to extracellular matrix in *B. braunii;* the secretion processes are shown from 1 to 4; vehicle could be derived from ER; figure is drawn according to (Burgoyne and Morgan, 2003; F. Zhang et al., 2011; Molnár et al., 2012; Uno et al., 2015).

In plant, for instance in *Arabidopsis* cell, Rab GTPase family encodes some key proteins for exocytosis (Rutherford and Moore, 2002). In *B. braunii,* ATP-binding cassette transporters were suggested as candidates for the secretory system of hydrocarbons, according to that transcriptome of *B. braunii* contains numerous contigs encoding potential ATP-binding cassette transporters (Molnár et al., 2012). However, ATP-binding cassette transporters are also possible for transportation of other substrates (Davis Jr., 2014). Further analysis to elucidate how vehicles fuse to plasma membrane and secret hydrocarbon oils to the extracellular matrix is expected.

After the hydrocarbons oils are secreted out of the cell, they will be stored and accumulated in the extracellular matrix, which is enclosed by a layer named “retaining wall”

(Weiss et al., 2012). The main structure of retaining wall is unusual polysaccharides comprising of galactose, arabinose, and uronic acid (Atobe et al., 2015). *B. braunii* also has the largest trans-Golgi network among all the organisms and the trans-Golgi vesicles contained several masses of electron-dense substances (Noguchi and Kakami, 1999). These masses could be the materials forming retaining wall and the activated Golgi apparatus seem to be implied in polysaccharides secretion (Weiss et al., 2012). This retaining wall could inhibit hydrocarbon recovery by a nonpolar organic solvent, keeping the colony and protecting the cells (Atobe et al., 2015). Further understating of the retaining wall could give benefits for hydrocarbon extraction from this colonial microalga.

In the retaining wall enclosed the extracellular matrix, except lipid hydrocarbons, there are other cross-linked polymeric substances components. In race A, non-hydrolysable biopolymers are mainly made of branched or unsaturated aliphatic polyaldehydes (Templier et al., 1992; Metzger et al., 1993; Gelin et al., 1994). In race B, these biopolymers are composed of aliphatic polyaldehydes similar to those found in race A. The specificity of the biopolymers in race B is in the acetalization of some aldehyde groups with triterpene diols (Metzger et al., 2007, 2008). The enlargement of the biopolymers network by extending cross-linkages would lead to insolubilization and finally induce its deposition for building up the backbone of extracellular matrix of *B. braunii* colonies (Metzger et al., 2007). Interestingly, proteins were also discovered recently in extracellular polymeric substances components, and seemed to play a more profound role on colony formation (Shen et al., 2014). Further focus is necessary to elucidate the structures and the functions of the extracellular matrix. It is worth to note that other microorganisms, such as bacterial and actinomycetes, could also produce extracellular aliphatic or volatile hydrocarbons (Ladygina et al., 2006). A comprehensive understanding of all these microbial synthesis and secretion of hydrocarbons deserves further investigation.

1.2.4. Cellular growth stages for hydrocarbons production: during cells in division with active growth

In order to obtain high productivity of hydrocarbons from *B. braunii*, it is desirable to investigate production of hydrocarbons at which kind of growth stage. Evidence shows that

hydrocarbons seem to be biosynthesized and secreted when the cells are in division with active growth in both race A and race B.

Firstly, studies in synchronous culture of A-race *B. braunii* revealed the high rate of [^{14}C] acetate incorporation into hydrocarbons occurred during the cells in division, especially just after the septum formation of daughter cells by feeding experiment (Hirose et al., 2013).

Table 1-11
Hydrocarbons production related to transformation of lipid bodies in cell lifecycle in *B. braunii* race A and race B.

Cell life cycle *	*B. braunii* race A *	*B. braunii* race B *
Stage 1: interphase	Lipid bodies are small and not prominent	Lipid bodies occupy space between nucleus and chloroplast
Stage 2: from cell growyh to septum formation	Lipid bodies increase in number reaching maximum total volume; oil droplets appear on cell apex	Lipid bodies increase in number and size reaching maximum total volume
Stage 3: from septum formation to decomposition of mother cell wall	Lipid bodies retain their numbers; electron-transparent regions appear in lipid bodies	Lipid bodies decrease in size and number greatly; oil droplets appear on cell surface
Stage 4: from decomposition of mother cell wall to recovery of chloroplast in daughter cell	Lipid bodies decrease in numbers and begin to appear on cell surface	The lipid bodies are reformed in cytoplasm
Stage 5: from recovery of chloroplast in daughter cell to interphase	Lipid bodies disappear from cytoplasm; oil droplets appear on cell surface	Lipid bodies increase in number and size

* Descriptions in this table are according to (Hirose et al., 2013) and (Suzuki et al., 2013); in order to compare, life cycle of A-race *B. braunii* is also summarized into 5 stages by authors.

In race B, feeding experiments shown that the rate of incorporation of $NaH^{14}CO_3$ into hydrocarbons and the activity of the pentose phosphate pathway producing NADPH were high in early active growth stage (Niitsu et al., 2012). NADPH is involved in hydrocarbons biosynthesis and the process of hydrocarbons secretion needs ATP. If cells growth is limited, there could also

be energy and reducing power limit for hydrocarbons production. Secondly, ESTs associated with energy metabolisms were abundantly expressed in the log-phase fast growing *B. braunii* 779 cells (Fang et al., 2015). Although some published data were controversy, it seems hydrocarbons content and productivity are higher at active growth rate than limited growth rate (Jin et al., 2015).

Moreover, transformation of lipid bodies related to hydrocarbons production in cell lifecycle was observed both in *B. braunii* race A and race B strains, basing on microscopy observation with Nile red staining. Cell lifecycle classified into five or six stages of *B. braunii* strains (Hirose et al., 2013; Suzuki et al., 2013). It was illustrated that there are only a few, small lipid bodies in non-growing cells (Hirose et al., 2013). As shown in **Table 1-11**, secretion processes are occurring when the cells are in division. To our knowledge, not only in *B. braunii*, exocytosis of secretory granules during division is also the case in other cells, for example, in the hormone-storing organelles cells (Bukoreshtliev et al., 2012).

Even though both race A and race B strains shown to produce extracellular hydrocarbons during cells in division, there are some differences between these two races. *B. braunii* race A does not have lipid bodies in the interphase cells, while race B has lipid bodies in this stage. The formation of new lipid bodies in race A occur only during the early growing stage (stage 2), but in race B, new lipid bodies appear to be synthesized during the maturation of the daughter cells (stage 4 and stage 5) as well as stage 2. These differences may due to different hydrocarbons biosynthetic pathways. Understanding the expression and localization of key enzymes in each pathway of these two races could be critical to answer these differences (Suzuki et al., 2013).

As a result, it seems the biosynthesis of hydrocarbons is contrast to other neutral lipids (triglycerides) produced in most of microalgae triggering by unfavorable conditions with limited cell growth, such as nitrogen limitation (Liu et al., 2016). Then, hydrocarbons production in *B. braunii* cultures should be considered to discuss optimal culture conditions enabling high ratio of cells in division with active cell growth for efficient hydrocarbons biosynthesis and secretion.

1.3. Conclusion

Reaching either a positive energy balance or a cost-competitive process for *B. braunii* exploitation could be anticipated on the basis of some specific characteristics. Thanks to its chimiodiversity some strain selections on the basis of the hydrocarbon content and composition, with more than 60 already isolated, have already improved the proportion of some particular hydrocarbons. Thanks to the high calorific value biomass and physical properties of *B.braunii* oil. *B. braunii* oil can be transformed into diesel or jet fuels by hydrocracking with a yield up to 85%. In contrast with other microalgae studied for biofuel production, relatively few works deal with the optimization of culture conditions. Indeed the colonial state of this microalga asks the question of the impact of the nutrient-mass transfer and the photon availability within these cell aggregates in relation to the effect of hydrodynamic stress on the size distribution of the colonies. Then there is a large room for improvement of biomass and hydrocarbon productivity, owing to *B. braunii* strain's capacity for physiological adaptation. A very optimistic estimation of biomass productivity could be extrapolated to 90–100 t $ha^{-1}y^{-1}$. By contrast to triacylglycerol production, hydrocarbon productivity seems to be growth associated; but this positive correlation with the specific growth rate is based on scarce data obtained from batch cultures. Recent advances have highlighted new potential opportunities to reduce the production costs through genetic, photobioreactor or bio-refinery engineering. In line with recent papers and patents, it appears that some of these challenges could be addressed and opportunities could be taken in the near future, for example, the recent development of pilot facilities for effective production and exploitation of *B. braunii* cultures, confirming *B. braunii* is a very promising candidate for biojetfuel production.

Study on hydrocarbons biosynthetic mechanism of microalga *B. braunii* is an undoubted worthy task for increasing its production. The main pathways of hydrocarbons biosynthesis are quite clear both in *B. braunii* race A and race B, except some reactions, such as desaturation in race A and methylation in race B. In race A, with ATP, NADPH and NADH, VLCF acids elongate from oleic acid and then convert to hydrocarbons via VLCF aldehydes. In race B, with ATP, NADPH and CTP, squalene and botryococcene C_{30} synthesize through triterpene synthesis pathway via MEP and then methylate for higher hydrocarbons. While the mechanism of hydrocarbons biosynthesis are relatively unknown in *B. braunii* race L and race S. Study of

hydrocarbons biosynthesis in race L and race S could be interesting for comprehensive understanding the biosynthetic mechanism of *B. braunii.* Genetic engineering could be implied for the improvement of hydrocarbons production, since a simple and effective genetic transformation method for *B. braunii* is available (Berrios et al., 2016) and some key steps of biosynthesis are detected.

As for the biosynthetic localization and secretion, recent finding showed both in *B. braunii* race A and race B strains, hydrocarbons or precursors could be biosynthesized starting from chloroplast, intermediately stored in lipid bodies and then secreted out of cell into the extracellular matrix. The secretion process is probably a vesicle-mediated transport as exocytosis, depending on ATP. ER plays an important role in hydrocarbons biosynthesis and secretion, while Golgi apparatus could imply in polysaccharides secretion. Further study on the expression and localization of key enzymes in the pathways would be meaningful for understanding of the secretion of hydrocarbons from cell to the extracellular matrix. Structure analysis of extracellular matrix could give information for improving hydrocarbons extraction and recovery.

In contrast to other neutral lipids (triglycerides) biosynthesis in most of microalgae increases at stress conditions with limited cell growth, extracellular hydrocarbons produced probably during cells in division with active growth. This could be due to more energy and reducing power, such as ATP and NADPH, could be provide at active cellular growth stage for hydrocarbons biosynthesis. Consequently, it is more considerable to discuss the favorable culture conditions enabling higher cellular growth rate for efficient hydrocarbons production.

B. braunii is a kind of colonial microalga, and the colony is formed through successive cell divisions by asexual binary fission. Even though some high production are reported, the bibliographic analysis shows that there is a need for *kinetics analysis* of growth and hydrocarbons *productions* in well-controlled cultures of this green microalga, since most of previous studies were only in batch cultures.

CHAPTER II: Materials and methods

2.1. Strains and media

2.1.1. Strains

B. braunii race A strain SAG 30.81 is obtained from the Culture Collection of Algae at Göttingen University (Sammlung von Algenkulturen, SAG). This strain is classified as race A strain as identified by their characteristic hydrocarbons by GC-MS (Dayananda et al., 2005, 2006, 2007b; Ranga Rao et al., 2007) and further confirmed by comparison with a typical race A strain LB 572 using random amplified polymorphic DNA analysis (Chandrappa et al., 2010).

B. braunii race B strain BOT 22 is obtained from University of Tsukuba, Japan. This strain has been well identified to produce a high purity $C_{34}H_{58}$ botryococcene by nuclear magnetic resonance (NMR) (Ishimatsu et al., 2012).

Strains were kept on buffered AF-6 agar plates (Shimamura et al., 2012) and sub-cultured in 300 mL column. The subcultures were regularly checked for absence of bacteria and fungi by control on Plate Count Agar (Fluka).

2.1.2. Media

The modified AF-6 medium (Shimamura et al., 2012) with pH buffer 2-(N-morpholino) ethanesulfonic acid (MES, 2 mmol L^{-1}) was further modified by us for batch cultivation. The final concentration of AF-6 medium contains $NaNO_3$ 140 mg L^{-1}, NH_4NO_3 22 mg L^{-1}, $MgSO_4 \cdot 7H_2O$ 30 mg L^{-1}, KH_2PO_4 10 mg L^{-1}, K_2HPO_4 5 mg L^{-1}, $CaCl_2 \cdot 2H_2O$ 10 mg L^{-1}, $FeCl_3 \cdot 6H_2O$ 1 mg L^{-1}, Fe-citrate 2 mg L^{-1}, citric acid 2 mg L^{-1}, $Na_2EDTA \cdot 2H_2O$ 8 mg L^{-1}, microelements ($MnCl_2 \cdot 4H_2O$ 200 µg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 40 µg L^{-1}, $CoCl_2 \cdot 6H_2O$ 8 µg L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 20 µg L^{-1}, NH_4VO_3 1 µg L^{-1}, H_2SeO_3 5µg L^{-1}) and Vitamins (biotin 2 µg L^{-1}, thiamine HCl 10 µg L^{-1}, vitamin B_6 0.1µg L^{-1}, vitamin B_{12} 1µg L^{-1}). Before autoclave, the pH was adjusted to 6.6 using KOH.

For the continuous cultures, a 2 times phosphate enriched medium (2P-AF-6) and a 3 times sodium nitrate and 6 times phosphate concentration enriched medium (3N6P-AF-6) were used in order to avoid phosphate limitation and improve biomass production. The final

concentrations of these media are shown in **Table 2-1**. The feeding medium for continuous cultures was without MES pH buffer, because of pH controlling automatically by PBR system.

Table 2-1
Composition of AF-6, 2P-AF-6 and 3N6P-AF-6 medium (μM) used for *B. braunii* batch or continuous cultures (Shimamura et al., 2012).

	Element	AF-6	2P-AF-6	3N6P-AF-6
Macronutrient	Na	1690	1690	1690
	K	2048	2048	2048
	NO_3	1922	1922	5733
	PO_4	204	408	1224
	Ca	68	68	68
	Cl	149	149	149
	SO_4	122	122	122
	Mg	122	122	122
	Fe	12	12	12
	EDTA	21	21	21
	Citric Acid·H_2O	10	10	10
	Citrate	8	8	8
	NH_4	275	275	275
Micronutrient	Mn	1.01	1.01	1.01
	Zn	0.14	0.14	0.14
	MoO4	0.08	0.08	0.08
	Co	0.03	0.03	0.03
	Se	0.03	0.03	0.03
	V	0.01	0.01	0.01
Vitamin	B1	0.029	0.029	0.029
	B6	0.008	0.008	0.008
	B12	0.0007	0.0007	0.0007
	Biotin	0.0006	0.0006	0.0006

2.2. Culture system and experimental set-up

2.2.1. Batch culture

Batch culture experiments were conducted in 300-mL bubble columns. Aeration was provided by injecting *0.2 µm-filtered air* at the bottom of the columns at a rate of 0.1 vvm (volume per volume per minute). Continuous photon irradiance was provided by cool-white fluorescent lamps (Philipps, Master TLD 18W) and its intensity-adjusted to 150 µmol photon m^{-2} s^{-1} as photosynthetic photon flux density (PFD, µmol photon m^{-2} s^{-1} or µmol m^{-2} s^{-1}) corresponding to photosynthetically active radiation (PAR). Measurements were performed at the surface of the columns using a Li-Cor light meter (LI−250A). Batch cultures were maintained at a temperature of 23 ± 2ºC.

2.2.2. Continuous culture

The torus photobioreactor (PBR) system (**Figure 2-1**), as described by Pruvost et al (Pruvost et al., 2006). The optical surface (front) of the PBR is made of transparent material (polycarbonate), the rest being made of stainless steel (type 316L). The square section of the PBR allows it to be considered as ‘‘flat panel’’. The working volume (V) was 1.4 L with light incident area A of 0.035 m^2 and then a A /V ratio of 25 m^{-1}.

A light-emitting diode (LED) panel composed of 1,500 white light LEDs (NSPW500CS, NICHIA) was used to illuminate the PBR from the front. The PFD was measured using a plane cosine quantum sensor Li-Cor light meter (LI−250A), which measures photosynthetically active radiation (PAR) in the 400–700 nm wavebands. The mean value was the average of measurements made at eight different locations on the reactor front. Then, the specific light supply rate was calculated as the following relation:

$$\text{specific light suppy rate} = \frac{\text{PFD}}{\text{X}}, \text{(mol photon gX}^{-1}\text{ d}^{-1}\text{ or mol gX}^{-1}\text{ d}^{-1}\text{)}$$

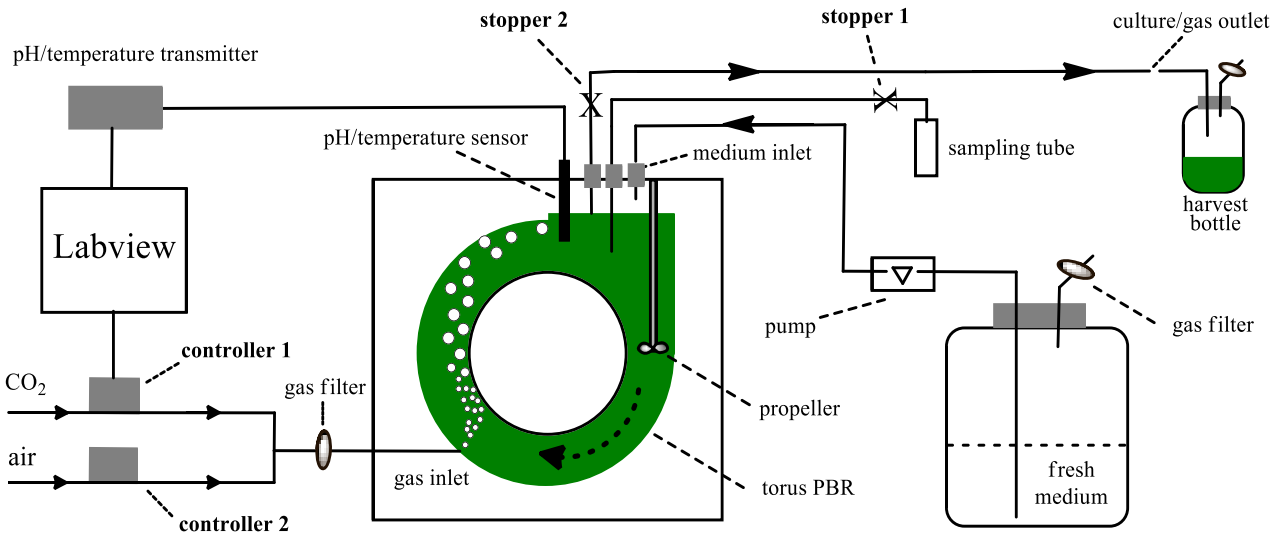


Figure 2-1. Sketch map of the torus PBR system. At sampling, stopper 1 is open and stopper 2 is closed. At other times, stopper 1 is closed and stopper 2 is opened. Sampling tube and harvest bottle were autoclaved (121ºC, 25 min) before being connected. Controller 1 (CO_2 flow controller) will be opened when the pH value detected online is higher than the setpoint pH value, or closed when the pH value is lower than setpoint pH. Controller 2 (air flow controller) is opened all the time at a constant air flow rate (450 mL min^{-1}), with culture pH controlled at the setpoint pH ± 0.1 pH unit.

The speed of the axial flow impellers was 200 rpm by motor. Temperature and pH were monitored with sensor (Mettler Toledo SG 3253). Temperature was controlled at 23 ± 1 ºC. Gas flow rate was 450 mL min^{-1} corresponding to an aeration rate of 0.32 vvm. The pH was controlled by automatic CO_2 injection (pH-controller Mettler Toledo M200) for set values.

Cultures were run in continuous mode. The PBR was sterilized by vapor stream (102 ºC) for 2 hours before inoculation. Sampling tube and harvest bottle were autoclaved (121 ºC, 25 min) before connected. The fresh medium was injected using a peristaltic pump (ISMATEC, ISM832C), and harvesting was by overflowing into a flask by gas pressure, making the reactor volume constant. Dilution rate (D, d^{-1}) was calculated by the following relation:

$$D = \frac{F}{V}$$

Where, F is the flow rate (L d^{-1}). In continuous culture, the biomass concentration in photobioreactor, the specific growth rate (μ) of microalga and the dilution rate are related to the equation as follows:

$$\frac{dX}{dt} = \mu \cdot X - D \cdot X$$

Then, at steady state,

$$\mu = D$$

2.3. Analytical methods and calculations

2.3.1. Determination of biomass concentration and biomass productivity

The cell density was monitored spectrophotometrically at 750 nm in triplicate (Spectrophotometer, Perkin Elmer Lambda 2S) (Sakamoto et al., 2012). Biomass concentration (X, g L^{-1} or g m^{-2}) was determined by dry weight (dw) measurement in triplicate as described before (Ruangsomboon, 2012). Amount of samples (10 mL) were filtered over pre-weighed glass fiber filters (Whatman GF/F) and washed with distilled water. The filters were dried at 105 ºC for 24 h in aluminum trays, cooled down in a desiccator, and the dry weight was measured. The biomass productivity P_x (g L^{-1} d^{-1} or g m^{-2} d^{-1}) was calculated as the equation:

$$P_x = D \cdot X \text{ , (g } L^{-1} d^{-1} \text{ or g } m^{-2} d^{-1})$$

Then, the relation between cell density (OD_{750}) and dry biomass weight could be plotted, as shown in **Figure 2-2.**

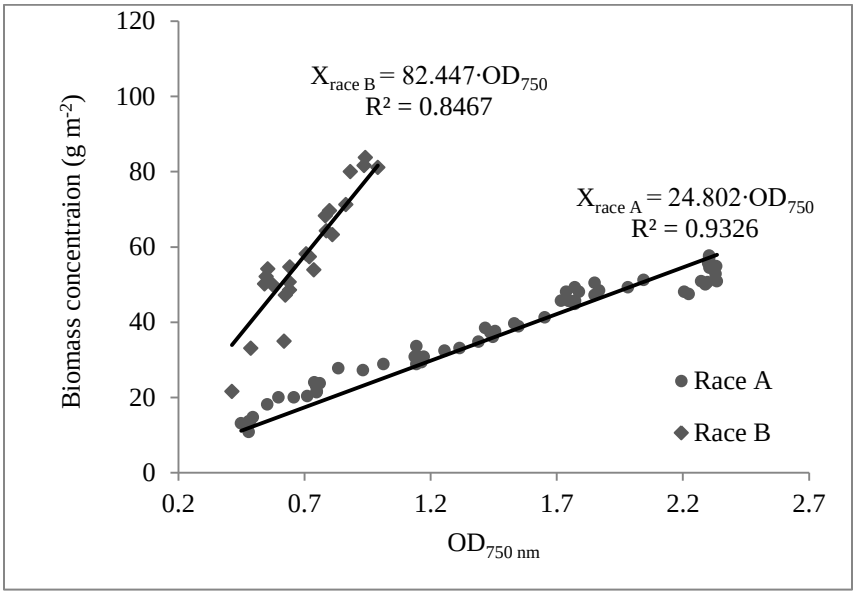


Figure 2-2. Relations between cell density (OD $_{750}$) and dry biomass weight (X) of *B. braunii* race A and race B strains.

The relations were significantly different between race A and race B strains, probably due to different size of the colonies, since the colony size are strain dependent (Eroglu et al., 2011). Although the relation between cell density (OD $_{750}$) and dry biomass weight was in linear relationship, the R^2 was not so high, only 0.9326 for race A and 0.8467 for race B. Then, in order to obtain precise data of the biomass concentration, dry weight was measured by gravimetrically method, but not calculated form the value of OD $_{750}$.

2.3.2. Pigments measurement

Pigments measurement did in in triplicate. For race A, amounts of cells (2 mL) in the culture were harvested by centrifugation (12100 g, 10 min) for race A. For race B, cells (2 mL) were harvested by filtration with glass fiber filters (Whatman GF/F), due to the natural buoyancy and difficult to precipitate in centrifugation, as shown in **Figure 2-3**.

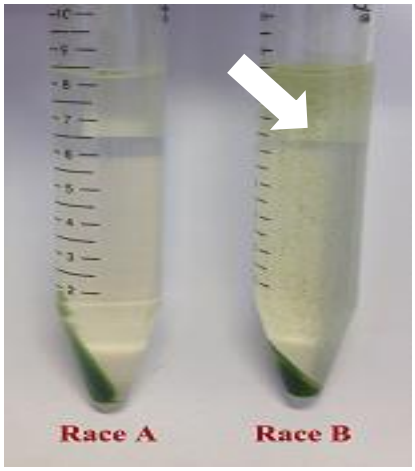


Figure 2-3. Cultures of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 after 10 min centrifugation at 12 100 g; white arrow indicate that some of the colonies still in the supernatant of race B.

About 25% race B biomass still suspended in the supernatant after 10 min centrifugation at 12100 g (**Figure 2-3**) Filtration and the "wet cell cake" approach would be better to remove water and harvest cells than centrifugation and wet pellet measurement (Eroglu et al., 2011).

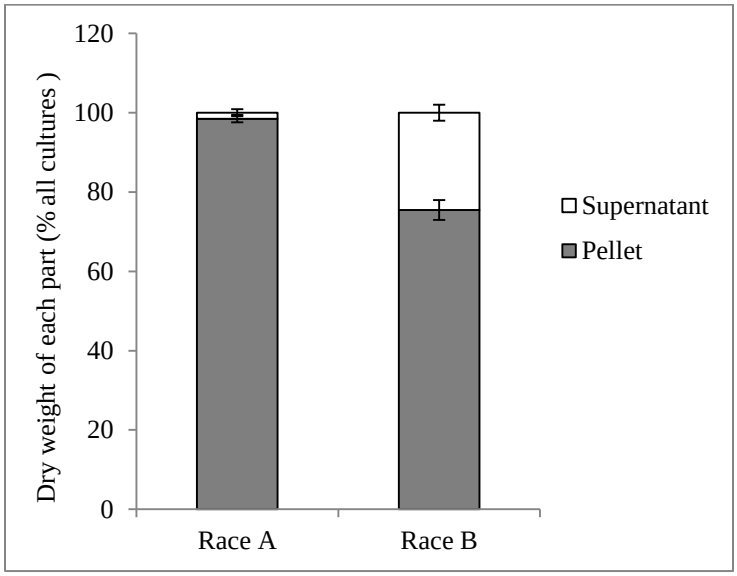


Figure 2-4. Percentage of dry weight in supernatant and pellet of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 all cultures after 10 min centrifugation at 12100 g.

Then, the harvested cells were suspended in 2 mL methanol solution (90 % v/v) by sonication and vortexing (18000 rpm), followed by a dark incubation during 2 h. The supernatant was clarified by centrifugation (12100 g, 10 min) and the optical densities at 470 nm, 652.4 nm,

665.2 nm and 750 nm (Perkin Elmer Lambda 2S spectrophotometer) were used for determining the chlorophylls (Chl, including chlorophyll *a*, Chl *a* and chlorophyll *b*, Chl *b*) and carotenoids (Car) concentrations according the spectrophotometric equations (Lichtenthaler and Buschmann, 2001) and the following equations are used for determination:

$$\text{Chl } a = (OD_{665.2nm} - OD_{750nm}) \cdot 16.82 - (OD_{652.4nm} - OD_{750nm}) \cdot 9.28, \text{ (mg L}^{-1}\text{)}$$

$$\text{Chl } b = (OD_{652.4nm} - OD_{750nm}) \cdot 36.92 - (OD_{665.2nm} - OD_{750nm}) \cdot 16.54, \text{ (mg L}^{-1}\text{)}$$

$$\text{Car} = \frac{1000 \cdot (OD_{470nm} - OD_{750nm}) - 1.91 \cdot \text{Chl } a - 95.15 \cdot \text{Chl } b}{225}, \text{ (mg L}^{-1}\text{)}$$

The total chlorophyll content ([Chl]x, % dw) and Car content ([Car]x, % dw) were calculated as the following relations:

$$[\text{Chl}]_X = \frac{\text{Chl } a + \text{Chl } b}{X} \cdot 100\%, \text{ (\% dw)}$$

$$[\text{Car}]_X = \frac{\text{Car}}{X} \cdot 100\%, \text{ (\% dw)}$$

2.3.3. Estimation of photosystem parameters

In vivo chlorophyll fluorescence of photosystem II (PSII) was measured in triplicate with a Water-PAM (Walz GmbH, Germany). Samples from steady-state cultures were dark adapted for 20 min, since this dark adapted time saturated for Fv/Fm measurement (**Figure 2-5 a**).

Fm determined by exposed to a saturating pulse (800 ms; irradiance of about 2400 μmol photon m^{-2} s^{-1}). The photochemical efficiency (Fv/Fm) was calculated with WinControl-3 Software (Walz GmbH). For photosynthesis-irradiance (P/I) curve determinations, samples of microalgal cells were exposed to eight incremental levels of irradiance (75, 109, 165, 245, 346, 479, 773 and 1127 μmol photon m^{-2} s^{-1}), each with a 3 min light adaptation duration (Cosgrove

and Borowitzka, 2006)(Cosgrove and Borowitzka, 2006) to obtain a relative high ETRmax value (**Figure 2-5 b**).

The electron transport rates (ETR) vs photosynthetic active radiations (PAR) values were fitted as Photosynthesis/Irradiance (P/I) curves according to Platt's equation (Platt et al., 1980) as follows:

$$\mathrm{ETR} = \mathrm{PAR} \cdot \mathrm{Factor}_{ETR} \cdot \frac{P_{\mathrm{PS2}}}{P_{\mathrm{PPS}}} \cdot \mathrm{Y(II)}$$

$$\mathrm{Y(II)} = \frac{F_m\prime - F_0}{F_m\prime}.$$

Where: PAR is photosynthetic active radiation (μmol photon m^{-2} s^{-1}). Default values of Factor_{ETR} and $\frac{P_{\mathrm{PS2}}}{P_{\mathrm{PPS}}}$ are 0.84 and 0.5, respectively. Fm' is Maximum fluorescence level of illuminated sample as induced by saturating pulses which temporarily close all PS II reactions centers. Fo is Minimum fluorescence level excited by very low intensity of measuring light to keep PS II reaction centers open.

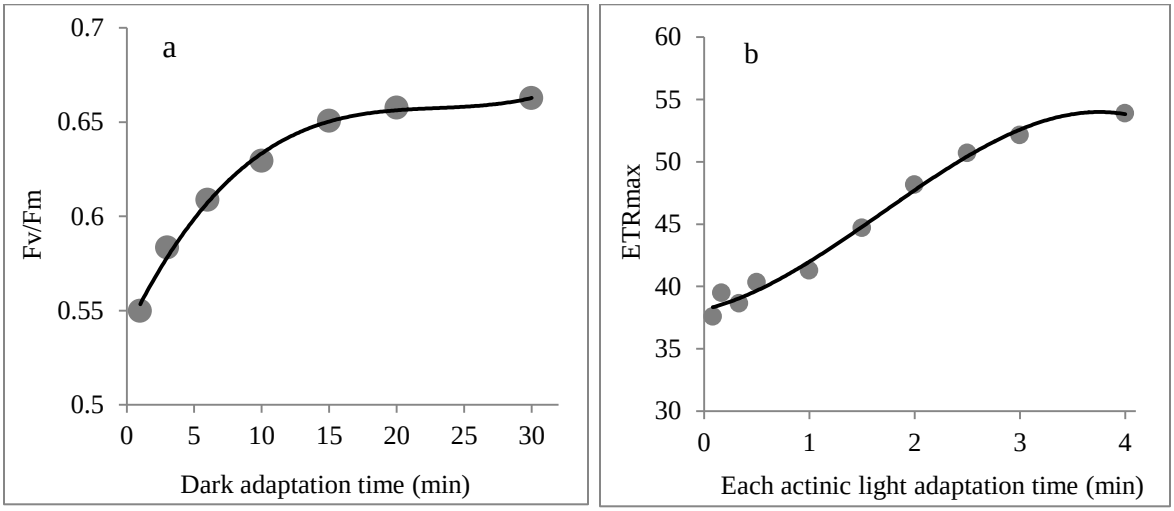


Figure 2-5. The values of Fv/Fm as a function of dark adapted time (a) and ETRmax as a function of duration at each actinic light adaptation (b) of *B. braunii* cultures.

The light curve was fit as Platt's equation, i.e.:

$$\mathrm{ETR} = \mathrm{ETR_{mPot}} \cdot (1 - e^{-\frac{\alpha \cdot \mathrm{PAR}}{\mathrm{ETR_{mPot}}}}) \cdot e^{-\frac{\beta \cdot \mathrm{PAR}}{\mathrm{ETR_{mPot}}}}$$

Where: α is initial slope of LC which is related to quantum efficiency of photosynthesis (electrons/photon), β is photoinhibition parameter. The values of α, β and $\mathrm{ETR_{mPot}}$ are estimated by the fitting procedure.The maximum electron transport rate (ETRm, μmol electrons m^{-2} s^{-1}) is determined by the following equation:

$$\mathrm{ETR_{max}} = \mathrm{ETR_{mPot}} \cdot (\frac{\alpha}{\alpha + \beta}) \cdot (\frac{\beta}{\alpha + \beta})^{\frac{\beta}{\alpha}}$$

2.3.4. Dissolved inorganic carbon determination

Dissolved inorganic carbon (DIC) was determined in triplicate using a TOC Analyzer (Shimadzu TOC-5000A) with $NaHCO_3$ and Na_2CO_3 were used as the standard to do the calibration (**Figure 2-6**). Sampled culture was centrifuged (12100 g, 10 minutes) or filtrated with glass fiber filters (Whatman GF/F) to obtain the supernatant. Then, the supernatant was injected into the IC reaction vessel which contains 25% phosphoric acid solution. The DIC was measured by the reaction of total carbon dioxide present in the form of molecular CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} into gaseous CO_2. Then, CO_2 was detected by an infrared analyzer.

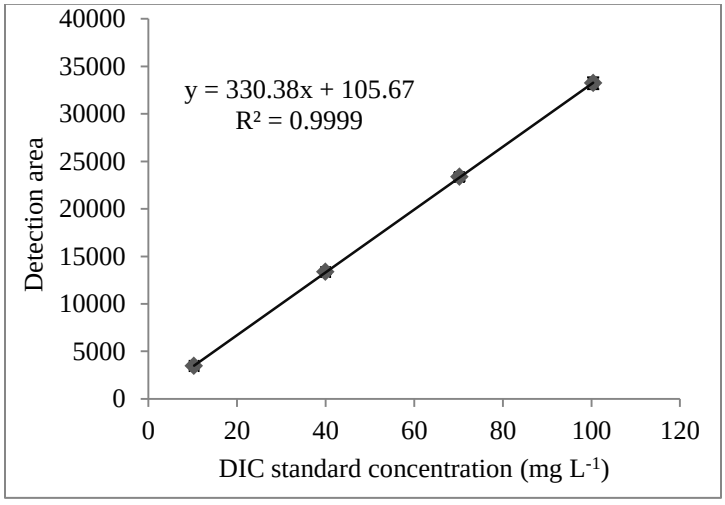


Figure 2-6. Calibration curve of the DIC concentration measurement by TOC Analyzer TOC-5000A; $NaHCO_3$ and Na_2CO_3 were used as the standard.

The relative concentrations of all DIC species, dissolved carbon dioxide ($CO_{2(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) in the culture, were calculated from the DIC and the pH values at each steady state with the equations derived from the following reactions:

$$[\mathrm{DIC}] = [\mathrm{CO_{2(aq)}}] + [\mathrm{H_2CO_3}] + [\mathrm{HCO_3^-}] + [\mathrm{CO_3^{2-}}];$$

At constant pressure and temperature, the following equilibriums between $CO_{2(aq)}$, H_2CO_3, HCO_3^- and CO_3^{2-} species apply:

$$\mathrm{CO_{2(aq)} + H_2O \overset{K_0}{\leftrightarrow} H_2CO_3 \overset{K_1}{\leftrightarrow} H^+ + HCO_3^- \overset{K_2}{\leftrightarrow} 2H^+ + CO_3^{2-}}$$

$$\text{Where } \mathrm{K_o} = \frac{[\mathrm{H_2CO_3}]}{[\mathrm{CO_{2(aq)}}]},$$

$$\mathrm{K_1} = \frac{[\mathrm{HCO_3^-}]\cdot[\mathrm{H^+}]}{[\mathrm{H_2CO_3}]},$$

$$\mathrm{K_2} = \frac{[\mathrm{CO_3^{2-}}]\cdot[\mathrm{H^+}]}{[\mathrm{HCO_3^-}]},$$

The calculations were done with the constants from Millero and Roy (Millero and Roy, 1997; Chen et al., 2016). At equilibrium, $K_0 \approx 1.7 \times 10^{-3}$; then only a small fraction, less than 1 % of the dissolved CO_2 is converted to H_2CO_3, with the main fraction as aqueous molecular $CO_{2(aq)}$. K_1 and K_2 were calculated from the following equations:

$$\mathrm{pK_1} = \frac{6320.813}{\mathrm{T}} + 19.568224 \cdot \mathrm{LnT} - 126.34048$$

$$\mathrm{pK_2} = \frac{5143.692}{\mathrm{T}} + 14.613358 \cdot \mathrm{LnT} - 90.18333$$

$$\text{Then, } [\mathrm{CO_{2(aq)}}] = [\mathrm{DIC}] \cdot \frac{[\mathrm{H^+}]^2}{[\mathrm{H^+}]^2 + \mathrm{K_1}\cdot[\mathrm{H^+}] + \mathrm{K_1}\cdot\mathrm{K_2}}$$

2.3.5. Determination of ions

Nitrogen (N-NO_3^-) and phosphorus (P-PO_4^{3-}) concentrations in cultures were determined in triplicate using ionic chromatography system, including a cationic chromatograph (Dionex-1100-IonPac) equipped with a guard column (CG16) and a separation column (CS16), an anionic chromatograph (Dionex-ICS 900-IonPac) equipped with a guard column (AG9-HC) and a separation column (AS9HC) and an external AMMS (Anion-ICE MicroMembrane Suppressor 300, Dionex) supplied with sulfuric acid (H_2SO_4, 25 mmol L^{-1}, 1.8 mL min^{-1}). Eluents were a solution of 6 mmol L^{-1} Na_2CO_3 and 1 mmol L^{-1} $NaHCO_3$ with a flow of 0.9 mL min^{-1} for the anionic chromatography and a solution of 20 mmol L^{-1} H_2SO_4 with a flow of 0.9 mL min^{-1} for the cationic chromatography. Detection was made by conductivity and data acquisition and processing were accomplished with the software program Chromeleon. Calibrations were done using nitrate and phosphate as standard (**Figure 2-7a and Figure 2-7b**).

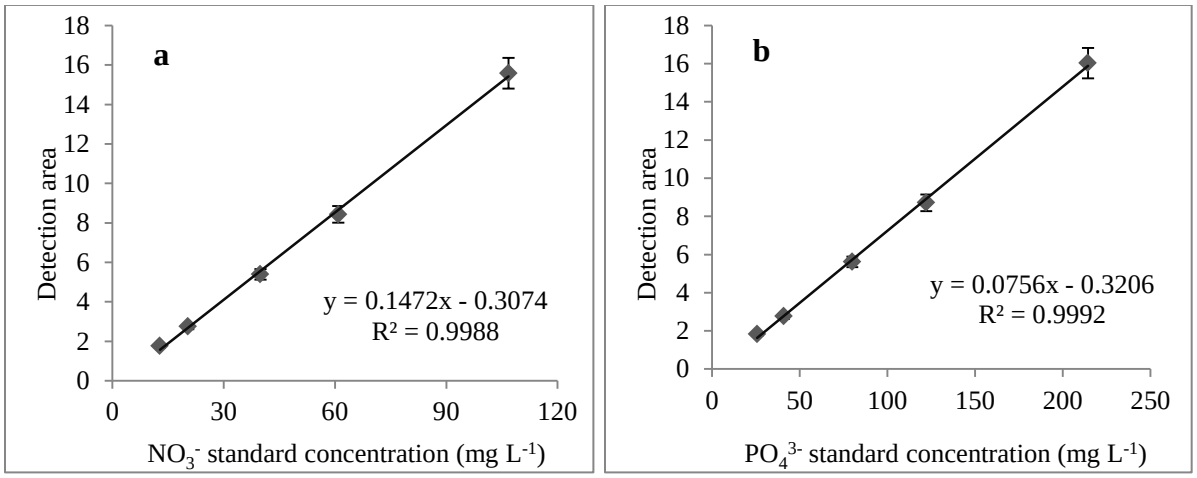


Figure 2-7. Calibration curves of (a) nitrate and (b) phosphate concentration measurement by ionic chromatography.

The nutrients requirement for biomass production (R_{XS}, mg gX^{-1}), the biomass yield from nutrients ($Y_{X/S}$, gX gS^{-1}), nutrients content of biomass ([S] x, % dw) and specific uptake rate ($r_{S/X,}$ mgS gX^{-1} d^{-1}) in terms of biomass production at each steady state was estimated according to the following relations：

$$R_{XS}= \frac{S_{in}-S_{out}}{X} \text{, (mg } gX^{-1}\text{);}$$

$$Y_{X/S} = \frac{X}{S_{in} - S_{out}}, (gX\ gS^{-1})$$

$$[S]_X = \frac{S_{in} - S_{out}}{X} \cdot 100\%, (\%\ dw);$$

$$r_{S/X} = \frac{S_{in} - S_{out}}{X} \cdot D, (mg\ g\ X^{-1}\ d^{-1})$$

Where: S_{in} is the nutrients concentration of the fresh medium flow into the photobioreactor and S_{out} is the concentration of culture sampled from the photobioreactor outlet.

2.3.6. Hexane extraction

Hexane extracts, generally regarded as representative of crude hydrocarbon are measured in triplicate using gravimetric method (Ge et al., 2011). However, it seems controversial as to whether hydrocarbons contents could be overestimated by using raw hexane extracts, when a hydrocarbons purification step by silica chromatography resulted in significantly lower values (Jin et al., 2015). Such method was applied for comparing our data to published ones. Then the lyophilized biomass was soaked in n-hexane at room temperature followed by sonication and vortex (18000 rpm) during 5 minutes. The upper hexane layer containing hydrocarbon was collected by centrifugation (12100 g, 10 min). The supernatants were evaporated under gentle flow of nitrogen gas. Dried hexane extracts were weighted to determine percent of biomass dry weight.

2.3.7. Total lipids extract and hydrocarbon purification

Total lipids were extracted and hydrocarbon was purified as the method described before in triplicate (Ishimatsu et al., 2012). Lyophilized cells of *B. braunii* were dissolved in 6 mL of chloroform: methanol (2:1 [v/v]) and mixed vigorously and the suspension was disrupted by sonic disintegration for 1 min using the Ultrasonic Disruptor. Then the suspension was shook for 6 hours at room temperature. Next, 1.5 mL of 0.9% NaCl were added and vigorously mixed. The

suspension was centrifuged (3700g for 5 min) at 20 ℃ and the bottom layer was obtained and transferred to a pre-weighed glass vial. The solvent was evaporated and dried under gentle flow of nitrogen gas. Total lipids content ($[TL]_{x,}$ % dw) was determined gravimetrically and expressed as percent of biomass dry weight.

After drying and weighing for the calculation of total lipids, the residual oil was dissolved in n-hexane and subjected to silica gel column chromatography with four bed volumes of n-hexane as the mobile phase. Eluates before a color band of pigments were collected, evaporated to remove the solvent, dried under mild nitrogen flow gas, and the hydrocarbon content ($[HC]_{x,}$ % dw) determined gravimetrically. The purity of extracted hydrocarbon was confirmed by thin layer chromatography (TLC). Then, the hydrocarbon concentration (HC, g L^{-1} or g m^{-2}) and hydrocarbon productivity (P_{HC}， g L^{-1} d^{-1} or g m^{-2} d^{-1}) were estimated as:

$$HC = X \cdot [HC]_x \text{ , (g } L^{-1} \text{ or g } m^{-2})$$

$$P_{HC} = P_x \cdot [HC]_x \text{ , (g } L^{-1} \text{ } d^{-1} \text{ or g } m^{-2} \text{ } d^{-1})$$

The content of intracellular lipids ($[IL]_x$, % dw) was estimated according to the following relation:

$$[IL]_X = [TL]_X - [HC]_X, \text{ (\% dw)}$$

2.3.8. Hydrocarbon specific production rate

The empirical Luedeking-Piret model was used for evaluating the relation between specific rate of product formation and cell specific growth rate (Thierie, 2015). The equations were as follows:

$$\frac{dP}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X$$

$$\frac{1}{X} \cdot \frac{dP}{dt} = \alpha \cdot \mu + \beta$$

where P = product concentration

X = biomass concentration

t = time

α and β are coefficients meaning :

α, growth associated product formation coefficient (g g^{-1});

β, non growth-associated product formation coefficient (g g^{-1} d^{-1});

In chemostat cultures at steady states, $\mu = D$, then,

$$\frac{1}{X} \cdot \frac{dP}{dt} = \alpha \cdot D + \beta$$

The specific rate of hydrocarbons production ($\frac{1}{X} \cdot \frac{dHC}{dt}$) was estimated as equivalent to $\frac{P_{HC}}{X}$ (gHC gX^{-1} d^{-1}).

2.3.9. GC-FID for hydrocarbons profiles analysis

Purified hydrocarbon extract was first analyzed in triplicate by gas chromatography coupled to a flame ionization detector (GC-FID) (Moutel et al., 2015). Amount of sample was dissolved using n-hexane. The prepared sample solution and n-alkane standard mixture (C_{10}–C_{40}, all even) standard mark solution were analyzed with a 7820A gas chromatograph (Agilent Technologies, Santa-Clara, CA, USA) equipped with a split/splitless injector (injected quantity: 2 µL, split set at 1/10–10 mL min^{-1}), an HP-5 capillary column (apolar phase 5% phenyl methylpolysiloxane, internal diameter 0.32 mm, film thickness 0.25 µm, length 30 m, Agilent Technologies, Santa-Clara, CA, USA) and a flame ionization detector. The flowrate of carrier gas (hydrogen) during the analysis was constant at 2.0 mL·min^{-1}; the gas was produced using a hydrogen generator (WM-H2, F-DGSi, Evry, France). The chromatograph temperature program was: injector temperature 250 °C, FID 280 °C, oven 75 °C for 3 min, then ramped at 10 °C min^{-1}

to 300 °C for 15 min. Finally at 75 °C min^{-1} to 340 °C for 4 min. Chromatographic data were recorded and identified by comparison of the retention times of the eluting hydrocarbons to the even-number C_{10}-C_{40} standard mark.

2.3.10. GC-MS for hydrocarbons profiles analysis

To determine the hydrocarbon formula, the purified hydrocarbon extract was further analyzed in triplicate by GC-MS (Moutel et al., 2015). The analytical device used was a Trace GC Ultra (Thermo Fisher Scientific, Boston, MA, USA) coupled to a Trace ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific, Boston, MA, USA). The optimized separation conditions for the measurement of the molecules of interest (HCs and FAMEs) were set up as follows: 2 μL of sample was injected onto a TG-5HT column (apolar phase 5% phenyl methylpolysyloxane, internal diameter 30 m × 0.32 mm, film thickness 0.25 μm; Thermo Scientifics, Waltham, MA, USA) using programmed temperature vaporization (PTV), injection set at constant temperature (CT) mode at 280 °C, with a split mode of 1/10 (10 mL min^{-1}). The flow rate of carrier gas (hydrogen produced by a generator; WM-H2, F-DGSi, Evry, France) during the analysis was 1.5 mL·min^{-1}. The oven temperature gradient for the separation of the mixture of hydrocarbons was 75 °C for 3 min, ramped at 10 °C min^{-1} to 300 °C for 1 min, and finally at 100 °C min^{-1} to 340 °C for 4 min for cleaning. The temperature of the transfer line to the MS was 250 °C, and the ion source was set at 300 °C. Electron ionization was conducted at 70 eV. The mass resolution was 1 mass unit throughout the mass range of 50–650 amu. The total GC–MS analysis time was 40 min. Data were post-processed and analyzed using the Xcalibur 2.1 software (Thermo Fisher Scientific, Waltham, MA, USA Thermo-Fisher).

2.3.11. GC-FID for total fatty acid analysis

Fatty acids were analyzed in triplicate. Lyophilized cell biomass was rehydrated by distilled water in glass vial. After adding Butylated hydroxytoluene (BHT, 20g L^{-1}) as antioxidant, cells were re-suspended in 6 mL of a chloroform/methanol mixture (2:1, v/v). To ensure a complete total fatty acid extraction, vials were sonicated in a water bath and maintained 6 h under agitation. The quantity and the quality of total fatty acids were analyzed by gas

chromatography coupled to a flame ionization detector (GCFID) as described before (Moutel et al., 2015).

The total lipids extracts were transesterified before the GC-FID analysis. The transesterification reaction was performed on an aliquot of the total lipid extract to which amonts of glyceryl triheptadecanoate (TAG 17:0) was added as an internal standard. After evaporation to dryness under nitrogen and addition of 1.6 mL of MeOH-boron trifluoride (BF3), the fractions were heated for 10 min at 100 °C. After cooling and adding 1.5 mL of hexane, the organic phase containing the fatty acid methyl esters (FAMEs) was washed three times with 2 mL of water saturated in hexane. FAMEs were then recovered and analyzed with a 7820A gas chromatograph (Agilent Technologies, Santa-Clara, CA, USA) equipped with a split/splitless injector (injected quantity 2 µL, split set at 1/10–10 mL/min), a TR-FAME capillary column (polar phase 70% cyanopropyl polysilphenylene-siloxane, internal diameter 0.25 mm, thickness 0.25 µm film, length 30 m, Thermo Fisher Scientific, Waltham, MA, USA) and a flame ionization detector. The flow rate of carrier gas (hydrogen) during the analysis was constant at 1.0 $mL \cdot min^{-1}$; the gas was produced using a hydrogen generator (WM-H2, F-DGSi, Evry, France). The chromatograph temperature program was: injector temperature 250 °C, FID 280 °C, oven at 80 °C for 1 min, then ramped at 5 °C min^{-1} to 145 °C for 12 min, then at 1 °C min^{-1} to 155 °C for 5 min and finally at 5 °C min^{-1} to 200 °C for 5min. The FAMEs were identified from their retention times compared with known standard mixtures. The FAME quantities were determined from their respective peak areas relative to that of the TAG 17:0 internal standard mark. Integration and calculation were performed using the Agilent software Chemstation v. 0.1.0.4. The absolute quantities of the TL were first expressed in µg of TL-FAs in the analyzed sample and in % dw relative to the biomass concentration of the test sample.

2.3.12. Fluorescent microscopy observations

A bright field microscope and a fluorescence microscope were used for microscopic observations (ZEISS, AXIO scope.A1). Cells were incubated in medium with Nile red stock solution prepared in DMSO for 5 min at room temperature (final concentration of Nile red is 1µg mL^{-1}, DMSO is 10%). Fluorescence was observed under a microscope with the No.20 HE filter

zeiss (excitation, 540-552 nm; emission, 567-647 nm) as the Nile red channel and the No. 5 filter zeiss (excitation, 395-440 nm; emission, 470 nm) as the merge channel of Nile red and chlorophyll auto-fluorescence.

2.4. Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Data were treated by R software (http://www.r-project.org) to test the homogeneity of variance by leveneTest (package Car). Differences between the groups were statistically analyzed by using one-way analysis of variance (ANOVA) and Tukey's 'Honest Significant Difference' method (TukeyHSD) with 95 % family-wise confidence level.

CHAPTER III: Effects of pH and nutrients supply in the cultures of *B. braunii* SAG 30.81

3.1. Introduction

Microalgal biofuels are gaining increasing attention as an alternative energy source. Among microalgae, *Botryococcus braunii* strains have emerged as the most promising potential renewable resource due to the huge amount of extracellular hydrocarbon (HC) they produce, which can reach 75% of dry cell weight (Banerjee et al., 2002). Strains of *B. braunii* are classified into four races, A, B, L and S, according to nature of the hydrocarbons they produce (Kawachi et al., 2012). Hydrocarbon oils of *B. braunii* make a suitable feedstock material for hydrocracking to transport fuels (Banerjee et al., 2002). CO_2 supplementation improves growth and hydrocarbon oil production in *B. brauni* cultures (Ranga Rao et al., 2007; Ge et al., 2011; Yoshimura et al., 2013), which means that *B. braunii*, like other photosynthetic microalgae, can bio-mitigate CO_2 from industrial exhaust gases to help reduce greenhouse gas emissions (Sydney et al., 2010a).

However, successful mitigation of CO_2 and improved production of hydrocarbon oils depend on CO_2 tolerance and the associated decrease in culture pH. Environmental pH is one of the key operational parameters driving algal growth, as it is known to affect photosynthetic activity, availability of inorganic nutrients through carbon and metal speciation, potential enzyme activities within cell walls, and oil production rates (Bartley et al., 2014). Low environmental pH can influence intracellular pH and thereby modify enzyme activities (Satoh et al., 2001), and acidification of the chloroplast is known to cause inhibition of photosynthesis (Tikhonov, 2013). This makes it vital to achieve appropriate pH control to improve the productivity of microalgal cultures, as demonstrated for the triacylglycerol-accumulating microalgae *Nannochloropsis salina* (Bartley et al., 2014). Nevertheless, few studies have investigated the influence of pH on *B. braunii* cultivation (Dayananda et al., 2006, 2007a; Ge et al., 2011; Yoshimura et al., 2013;

Hifney and Abdel-Basset, 2014). It has been reported that maximal production of both biomass and hydrocarbon could be achieved at a pH around 6.3 (Ge et al., 2011) or at pH 7.5 (Dayananda et al., 2007a), whereas another study showed higher biomass could be obtained at pH 6.0 but that hydrocarbon content showed little response to pH in the range 6.0 to 8.5 (Dayananda et al., 2006). Other studies have demonstrated pH effects on pigments (Hifney and Abdel-Basset, 2014) and growth rate (Yoshimura et al., 2013) but did not address pH effect on hydrocarbon production. It appears that *B. braunii* race A are generally cultivated at around pH 7.5 (Talukdar et al., 2013) whereas *B. braunii* race B strains are cultivated at around pH 6.5 (Yoshimura et al., 2013). However, in these studies, culture pH was only adjusted before cultivation (Dayananda et al., 2005, 2006, 2007a) or monitored (Ge et al., 2011; Yoshimura et al., 2013), never held constant by either automatic pH regulation or stoichiometrically-balanced growth media as was done for triacylglycerol-accumulating microalgae (Scherholz and Curtis, 2013). Moreover, these experiments were often carried out in batch cultures for relatively short durations. Batch cultures are non-ideal model systems as they carry a number of inherent drawbacks for quantitative studies on the effects of environmental parameters on microalgal culture performances. Chemostat cultivation makes a far better tool for studies on quantitative aspects under controlled cultivation conditions.

We therefore investigated the effect of pH on biomass and hydrocarbon productivities in continuous controlled cultures of a *B. braunii* race A strain. Chemostat cultures were used at a fixed dilution rate to determine the relation between culture performances at steady-state, and environmental pH was regulated by automatic CO_2 injection into the cell suspensions. This is the first time this approach has been applied to investigate the behavior of *B. braunii* cultures. A change in nitrate and phosphate nutrient composition of the feeding medium was used to compare

the effects of increasing residual nitrate and phosphate concentrations in the cultures on biomass and hydrocarbon productivities according to pH setpoints.

3.2. Experiment design and setup

The experiment design and setup of the batch culture in columns and continuous cultures in torus photobioreactor as shown in **Figure 3-1**.

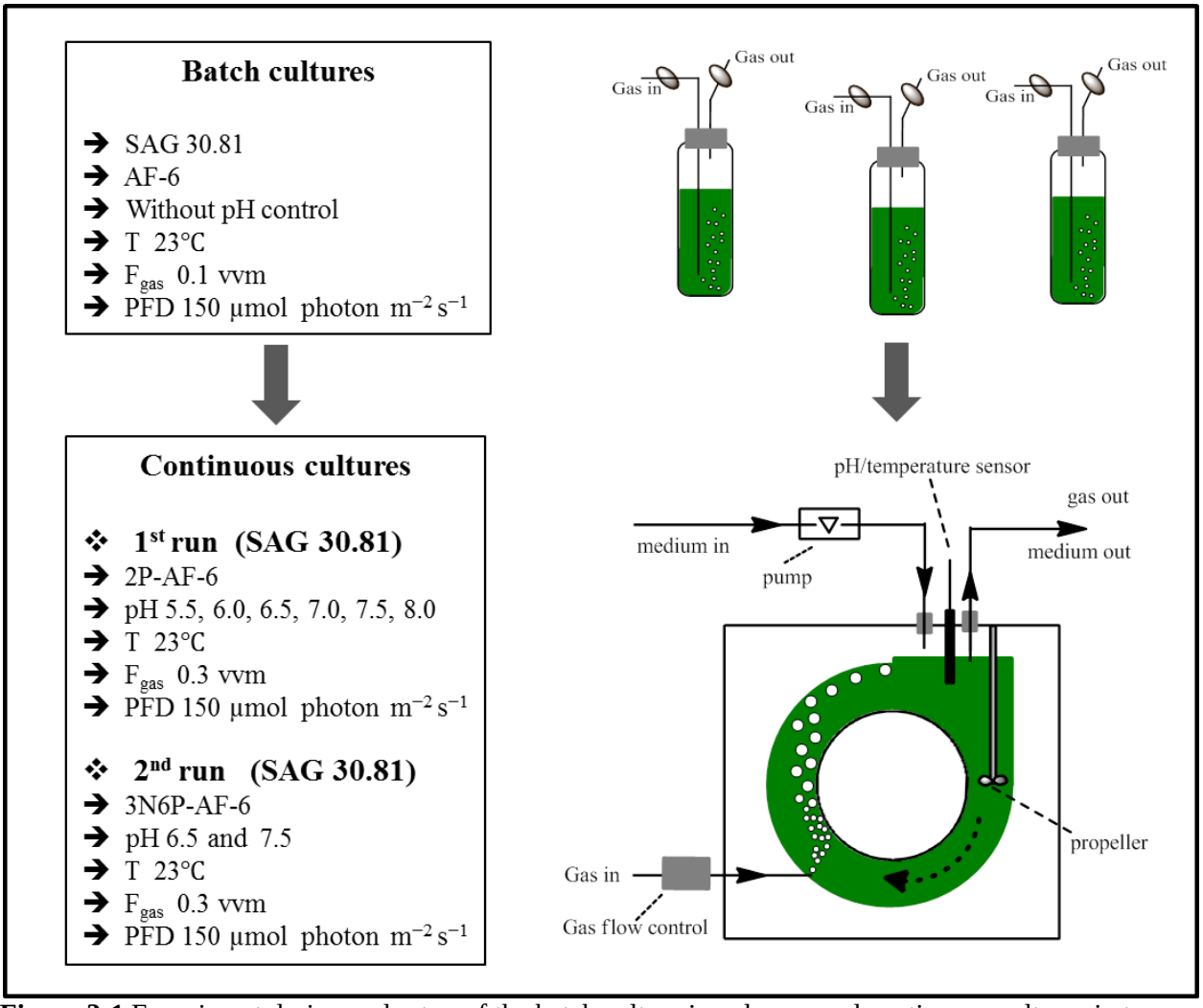


Figure 3-1.Experiment design and setup of the batch culture in columns and continuous cultures in torus photobioreactor.

Experiments were conducted with the strain *B. braunii* Kützing SAG 30.81.The modified AF-6 medium was used for preliminary cultivations. For a first set of continuous experiments, the

feeding medium was 2P-AF-6 in order to avoid phosphate limitation observed during preliminary batch cultures. A second set of experiments was done with 3N6P-AF-6 for improving biomass production.

The batch culture experiments were conducted in 300-mL bubble columns. Aeration was provided by in3jecting *0.2 µm-filtered air* at the bottom of the columns at a rate of 0.1 vvm (volume per volume per minute). Continuous photon irradiance was provided by cool-white fluorescent lamps (Philipps, Master TLD 18W) and its intensity-adjusted to 150 µmol photon m^{-2} s^{-1} as photosynthetic photon flux density (PFD) corresponding to photosynthetically active radiation (PAR). Measurements were performed at the surface of the columns using a Li-Cor light meter (LI−250A). Batch cultures were maintained at a temperature of 23 ± 2ºC.

The chemostat cultures were fed with a medium flow rate (F) 0.28 L d^{-1}, corresponding to a dilution rate D = F/V of 0.2 d^{-1}. Cultures were illuminated continuously at a photon flux density of 150 µmol photon m^{-2} s^{-1} in the 400–700 nm wavebands (photosynthetically active radiation, PAR). The pH was controlled by automatic CO_2 injection (pH-controller Mettler Toledo M200) for values set sequentially at 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, after reaching a steady-state at the operating pH, mean duration of 10 days.

3.3. Results and discussion

The effect of pH on efficiency of *B. braunii* cultures was essentially studied using continuous cultures after preliminary assays in batch cultures to determine the value of the dilution rate and the enrichment level of the basic AF-6 culture medium. The results thus correspond to N and P-enrichment levels of the culture medium at a fixed dilution rate. All data obtained under continuous cultures correspond to steady-state equilibrium, i.e. < 10% variation on at least 10-day periods.

3.3.1. Preliminary characterization of *B. braunii* batch cultures in AF-6 medium

Preliminary cultures were maintained in 300 mL columns with AF-6 medium, as this medium has been demonstrated as suitable for *B. braunii* (Ishimatsu et al., 2012). During 12-day batch cultures, pH increased from 6.6 to 9.5, although the AF-6 medium contained 2 mmol L^{-1} MES, a pH buffer. This corresponds to a specific pH variation from 1.8 UpH to 4.2 UpH per g

biomass produced. This alkalinization of the culture medium, routinely observed in any microalgal cultures with nitrate as N-source, has been reported in other *B. braunii* cultures (Dayananda et al., 2006; Hifney and Abdel-Basset, 2014). Ionic analysis of the culture supernatants after 12-day incubation periods indicated that almost all the initial phosphate concentration (103 µmol L^{-1}), was consumed (**Figure 3-2a**).

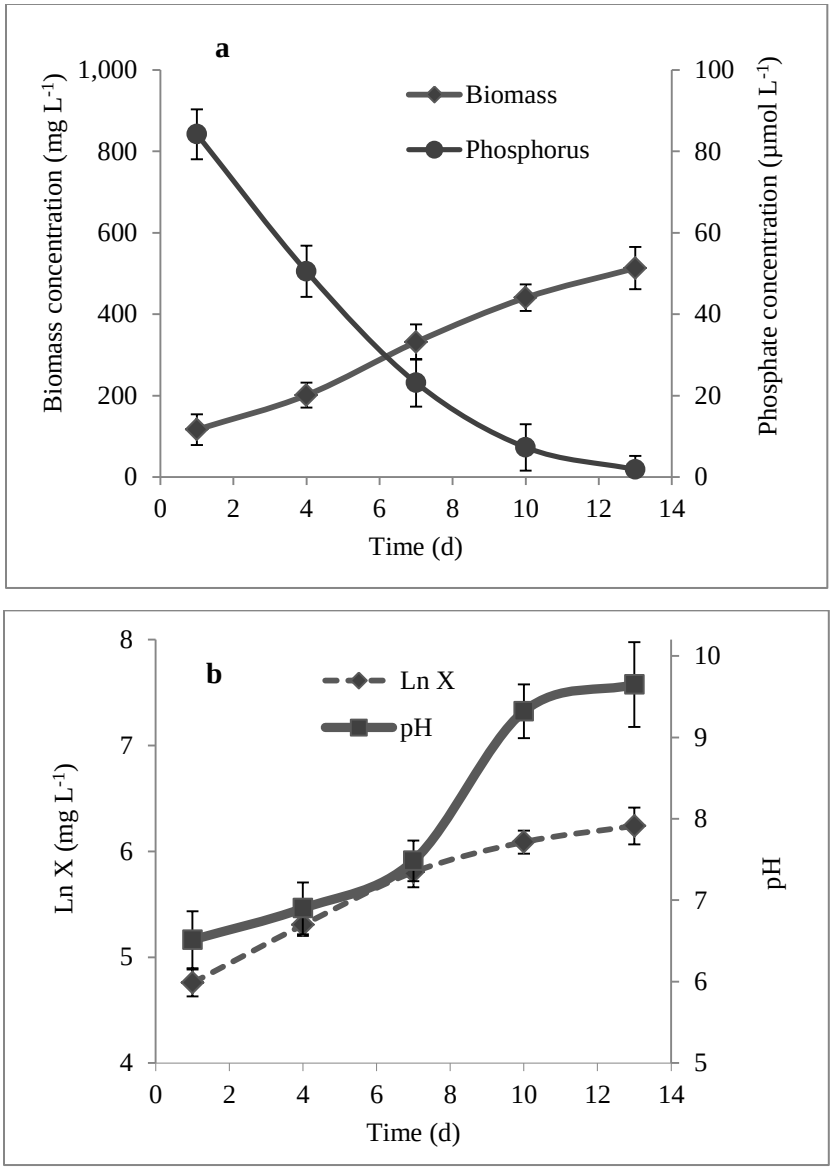


Figure 3-2. Variations of (a) biomass, X, and phosphate concentrations and (b) Ln X and pH as a function of time in a *B. braunii* batch culture. Culture medium was AF-6. The residual concentrations of the other nutrients were non-negligible after 13 days of incubation.

Another study has already shown that phosphate was a potent limiting factor in batch cultures of *B. braunii* 765 with an initial concentration of 175 µmol L^{-1} (Ge et al., 2011). The residual concentrations of the other macronutrients after the 12-day cultivations were still relatively high, such as nitrate at about 300 µmol L^{-1}, except for DIC which in some cultures was under 2 mg L^{-1}. These measured DIC values indicated that inorganic carbon could be limited. Then, during the first set of experiments, the continuous culture was fed with a 2-fold phosphate-enriched AF-6 medium, named 2P-AF-6. Moreover, the end of the log phase seemed to coincide with pH values over 7.5 (see **Figure 3-2b**). Therefore, the pH effects on *B. braunii* biomass and hydrocarbon production were studied with microalgae grown in a photobioreactor where pH was regulated using controlled injection of CO_2 gas flow. Through this approach, the inorganic carbon supply of the cultures was provided, as after injection of a fraction of the CO_2 gas dissolves as $CO_{2\,(aq)}$ according to Henry's law and is then converted to HCO_3^- and CO_3^{2-}. The dilution rate of the continuous cultures was fixed at 0.2 d^{-1}, as the specific growth rates of *B. braunii* cultures are usually in the range 0.1 d^{-1} to 0.3 d^{-1}, with a maximal growth rate of 0.5 d^{-1} (Yoshimura et al., 2013).

3.3.2. Characterization of *B. braunii* continuous cultures in 2P-AF-6 medium

3.3.2.1 Biomass and hydrocarbon productivities in 2P-AF-6 medium as a function of pH

To highlight the effect of environmental pH on production rates, a continuous culture of *B. braunii* was started in a 1.4 L photobioreactor. All the parameters were fixed,, i.e. irradiance (150 µmol photon m^{-2} s^{-1}), temperature (23 °C), stirring (200 rpm), aeration rate (0.32 vvm) and 2P-AF-6 feed medium at a dilution rate of 0.2 d^{-1}, except the pH value which was changed stepwise in 0.5 unit increments from pH 5.5 to pH 8.0. The biomass and lipid concentrations were determined after reaching a steady-state at each pH value. The effects on biomass and total lipid productivities were deduced from these steady-state values.

Environmental pH had a significant effect on total lipid productivity ($P < 0.05$) while having little effect on biomass productivity (**Figure 3-3**). According to the geometric features of the PBR, maximal biomass productivity reached 5.2 g m^{-2} d^{-1} and total lipids areal productivity reached 2.2 g m^{-2} d^{-1} at pH 6.5. Environmental pH not only had an effect on total lipids productivity but also on the total lipids composition and hexane extract contents of dry biomass

(**Figure 3-4**). Total lipid contents were significantly different according to whether microalgal biomass was produced at a pH over or under the threshold of 7.0. The hexane extracts are generally regarded as crude hydrocarbon fractions. As shown in **Figure 3-4**, in contrast with total lipids, the highest values for hexane extract contents were obtained at pH 6.5.

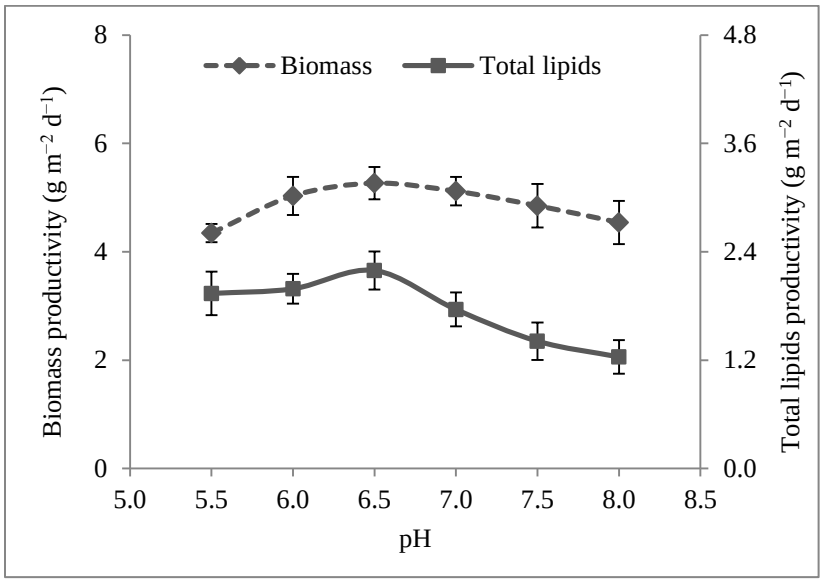


Figure 3-3. Biomass and total lipid productivities as a function of pH in steady-state continuous cultures fed with 2P-AF-6.

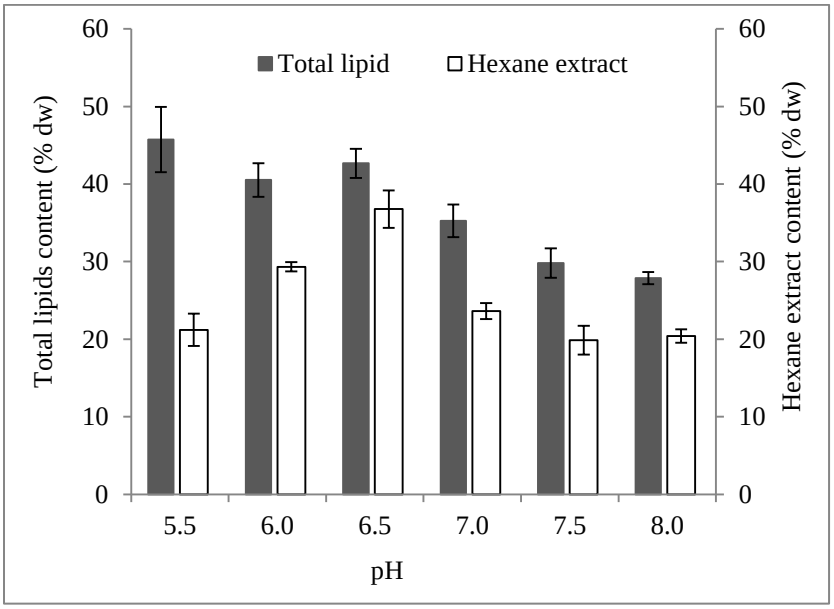


Figure 3-4. Total lipids and hexane extracts contents as a function of pH in steady-state continuous cultures fed with 2P-AF-6.

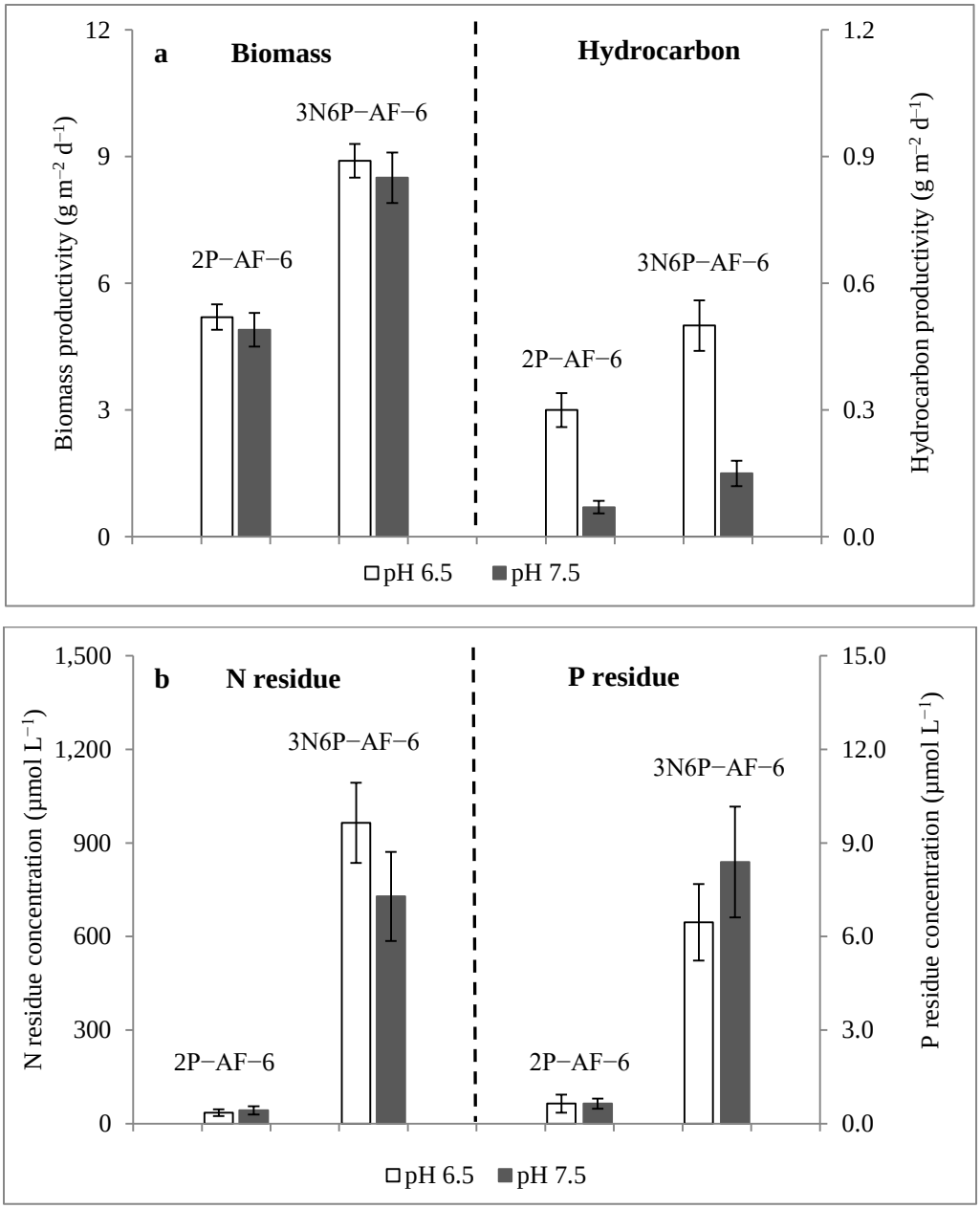


Figure 3-5. Comparison of biomass productivity, purified hydrocarbon productivity, nutrient consumption at pH 6.5 and nutrient consumption at pH 7.5 in steady-state continuous cultures fed with 2P-AF-6 or 3N6P-AF-6. a) biomass and hydrocarbon productivities; b) N and P residue concentrations. N = nitrogen; P = phosphate.

A change of just one pH unit, from pH 7.5 to 6.5, was enough to induce a significant ($P < 0.05$) increase in total lipid and hexane extract contents. Hexane extract content was between 19.9% dw and 36.8% dw, which is consistent with Dayananda et al. (Dayananda et al., 2005)

who found that hexane extract as hydrocarbon ranges from 15% to 50.6% dw of the same SAG 30.81 strain. Note that the hexane extracts could contain other lipids, such as triacylglycerol and aldehydes, and that hydrocarbon accounted for only 20.4% of hexane extracts from an Austin strain of *B. Braunii* (Metzger and Casadevall, 1989). In order to quantify the exact hydrocarbon content of this strain, we purified the hydrocarbon using silica gel chromatography. In addition, pH also had a significant effect on purified-hydrocarbon productivity, which increased fourfold with the change from pH 7.5 to pH 6.5 (**Figure 3-5a**).

At pH 6.5, hydrocarbon productivity reached up to 0.291 g m^{-2} d^{-1} with a hydrocarbon content of 5.7% dwh whereas at pH 7.5, hydrocarbon productivity was only 0.065 g m^{-2} d^{-1} and with a much lower hydrocarbon content of 1.3% dw. Moreover, hydrocarbon-specific production rate was significantly higher at pH 6.5 (11.1 mg HC g X^{-1} d^{-1}) than at pH 7.5 (2.7 mg HC g X^{-1} d^{-1}; $P < 0.05$). The hydrocarbon contents measured in this study were not so high, peaking at 5.7% dw, they are still within the range of values reported in the literature. Hydrocarbon contents in *B. braunii* race A biomass are generally from 1% dw to 10% dw, although reports claim some race-A strains accumulate up to 61% hydrocarbons while others accumulate less than 0.1% dw (Jin et al., 2015). As hydrocarbons are mainly accumulated in the extracellular matrix of *B. braunii*, it was possible to estimate intracellular lipid contents by subtracting purified hydrocarbons content from total lipids content (Jin et al., 2015). The calculation of the ratio of extracellular hydrocarbon to intracellular lipids as a function of pH then confirmed that optimal pH was around pH 6.0–6.5 (**Figure 3-6**).

According to the literature, one of the main precursors for hydrocarbon biosynthesis in *B. braunii* race A is oleic acid, probably via an elongation mechanism involving conversion to very-long-chain fatty acids, as revealed by comparative gene expression analysis in a race A strain of *B. braunii* (Baba et al., 2012a; Ioki et al., 2012a).

There could therefore be a relation between intracellular lipid accumulation and extracellular hydrocarbon accumulation in *B. braunii* cultures. Indeed, when taking in account only the pH range from 6.0 to 8.0, there is a linear relation between extracellular hydrocarbon and the intracellular lipid contents:

$$[HC] = 0.534 \cdot [\text{Intracellular lipid}] - 13.398, \; R^2 = 0.939$$

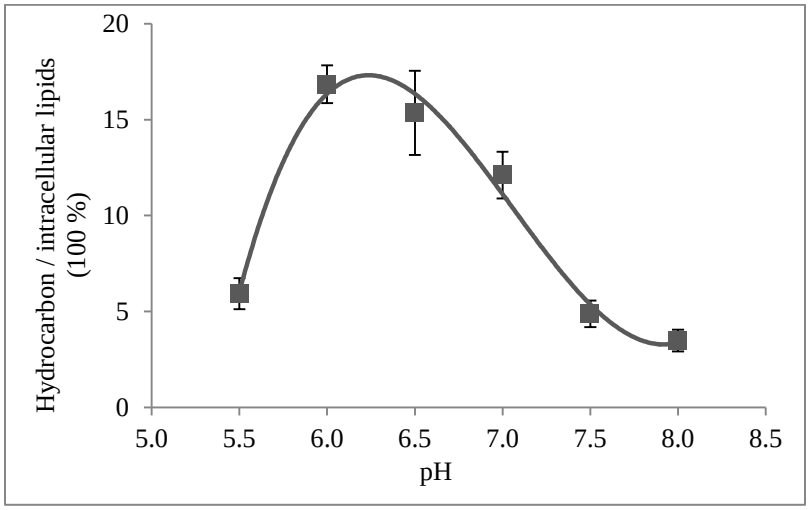


Figure 3-6. Ratio of purified hydrocarbons to intracellular lipids as a function of pH in steady-state continuous cultures fed with 2P-AF-6. Intracellular lipids = total lipids – purified hydrocarbon.

The assumption of a *linear relationship between hydrocarbon and lipid contents is only valid for pH values between 6.0 and 8.0.* This relationship is not valid for the values corresponding to pH 5.5. The hypothesis for this is that the physiological state of *B. braunii* cells could be modified when the algae is grown in such acidic environmental conditions. Then, we also investigated the physiological responses when cultivating at different pH condtions as below.

3.3.2.2. Steady-state photosynthetic activity at pH from 5.5 to 8.0

As *B. braunii* SAG 30.81 was cultivated under photoautotrophic conditions, photosynthetic activity is essential to provide energy and reductive carbon for biomass and hydrocarbon production (Sakamoto et al., 2012). The photosynthetic characteristics of the cell populations were determined at each steady-state. The photochemical efficiency of photosystem II (PSII), expressed as Fv/Fm and ETR_{max}, was relatively unaffected by pH of the culture from pH 6.0 to 8.0, even if a slight increase was observed at pH 6.5 for the continuous culture fed with 2P-AF-6 (**Figure 3-7**).

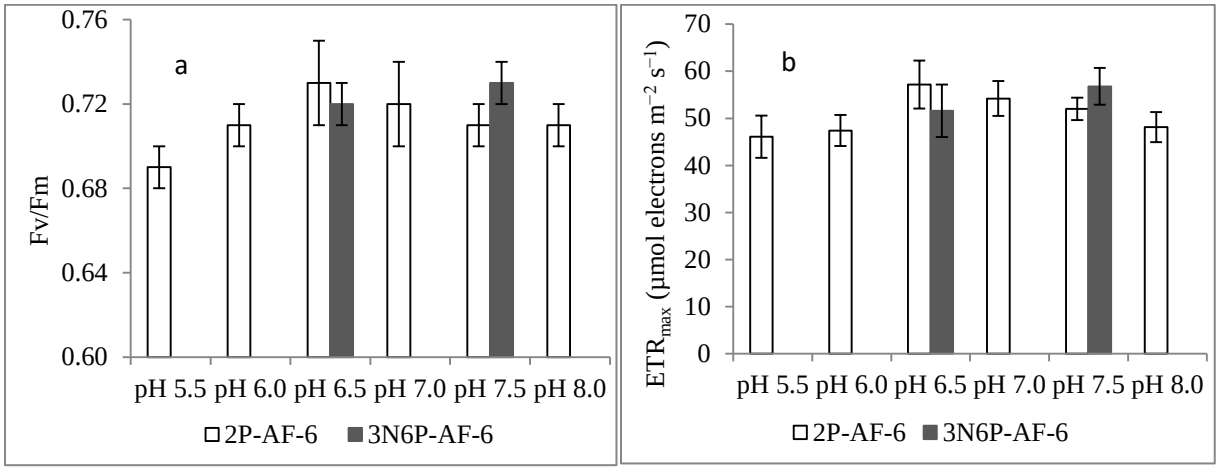


Figure 3-7. Steady-state Fv/Fm and ETR_{max} of the P/I curve as a function of pH in continuous cultures of *B. braunii* at a fixed dilution rate of 0.2 day^{-1}. Means ± SD. P/I curve = photosynthesis-irradiance curve.

The parameters Fv/Fm and ETRmax are commonly used to evaluate the impact of stresses such as nutrient or heat stress on microalgae (Claquin et al., 2008; Moheimani et al., 2014). In our experimental conditions, the absence of any sharp decline in Fv/Fm and ETR_{max} values suggests *B. braunii* was not stressed at any pH in the range 6.0 to 8.0 (**Figure 3-7**). However, the slight decrease of Fv/Fm at pH 5.5 indicates an inhibition of microalgal photosynthesis by this more acidic environment—an effect that could be related to the decrease in biomass concentration at pH 5.5. The decrease of extracellular pH induced by a high CO_2 supply could also affect intracellular pH, although green microalgae are known to maintain their intracellular pH at around 7.2 even when grown in relatively acidic media of pH down to 3.0 (Balkos and Colman, 2007). For example, when cells of *Chlorococcum littorale* were transferred from low CO_2, i.e. 0.04% in air, to high CO_2, i.e. up to 40%, intracellular pH value decreased from 7.0 to 6.4 with an environmental pH value of 5.5 (Satoh et al., 2001). This acidification induced an inhibition of photosynthetic carbon fixation in *C. littorale* cells exposed to excess concentrations of CO_2 (Satoh et al., 2001).

3.3.2.3. Steady-state pigment composition at pH from 5.5 to 8.0

Microalgae like *B. braunii* have two major classes of *pigments,* chlorophylls (Chl) and carotenoids (Car), that are generally associated to photosynthetic activities, except in some race B strains that accumulate Car within the extracellular matrix (Eroglu and Melis, 2010).

The race A strain SAG 30.81, like other green algae, contains Chl *a*, Chl *b* and Car. Chl *a*, Chl *b* and Car contents are known to depend on culture conditions, particularly nutrients and light. Eroglu et al. (Eroglu et al., 2011) observed values from 0.38% to 0.86% (w/w) for chlorophylls and from 0.10% to 0.54% (w/w) for carotenoids according to species of *B. braunii* grown under air. Sydney et al. (Sydney et al., 2010a) found 13.05% (w/w) pigments in *B. braunii* SAG 30.81 cultivated under CO_2-enriched (5%) air.

Table 3-1
Steady-state pigment composition of biomass as a function of pH in continuous cultures of *B. braunii* at a fixed dilution rate of 0.2 d^{-1}.

pH	Feeding medium	Total Chl (% dw)	Chl *a* / Chl *b* (mol / mol)	Total Car (% dw)	Car / Chl (w / w)
5.5	2P-AF-6	1.3±0.1*	2.1±0.2	0.24±0.02*	0.19±0.02
6.0	2P-AF-6	2.0±0.2	2.3±0.2	0.37±0.03	0.19±0.02
6.5	2P-AF-6	2.1±0.1	2.4±0.3	0.37±0.03	0.18±0.01
7.0	2P-AF-6	1.8±0.1	2.2±0.1	0.33±0.02	0.18±0.01
7.5	2P-AF-6	1.8±0.2	2.3±0.3	0.33±0.02	0.18±0.01
8.0	2P-AF-6	1.7±0.1	2.2±0.2	0.32±0.02	0.19±0.02
6.5	3N6P-AF-6	2.2±0.2	2.1±0.3	0.38±0.03	0.18±0.01
7.5	3N6P-AF-6	1.8±0.2	2.1±0.2	0.34±0.02	0.19±0.02

Means ± SD. Chl = chlorophylls; Car = carotenoids; * = significantly different from other conditions ($P < 0.05$).

As shown in **Table 3-1**, the pigment contents obtained here at each steady-state were within the range of values obtained by Ranga Rao et al. (Ranga Rao et al., 2007), at 1.16% w/w total Chl and 0.27% w/w Car. Chl *a*/Chl *b* ratio and Car/Chl ratios are widely used indicators of photosynthetic cell response to changes in irradiance, nutrients, temperature or salinity (Solovchenko et al., 2009). Plant cells present Chl *a*/Chl *b* ratios of 3.2–4.0 when exposed to high irradiance and 2.5–2.9 when grown under low irradiance (Lichtenthaler and Babani, 2004). As reported for other Chl *b*-containing plant cells, the Chl *a*/Chl *b* values of microalgae are generally between 2 and 3, with little if any effect of pH (Wood, 1979).

The Car/Chl ratio (0.24) of *B. braunii* obtained by Ranga Rao et al. (Ranga Rao et al., 2007) in cultures fed at a 2% CO_2 concentration is relatively close to our data. Note that a lower Car/Chl ratio (0.15) was also observed in other *B. braunii* species in absence of CO_2 supplementation (Eroglu et al., 2011). The evidence thus suggests there was apparently no stress from pH 5.5 to 8.0, as environmental pH had no significant influence on Chl *a*/Chl *b* and Car/Chl ratios. However, under steady-state, total chlorophyll and carotenoid contents were significantly decreased at pH 5.5 ($P < 0.05$), thus confirming that the cells were stressed by the more acidic conditions.

3.3.2.4. Nutrient requirement for biomass production at steady-state from pH 5.5 to 8.0

As residual concentrations of macronutrients—mainly phosphate and nitrate—in the outflow from the continuous culture were relatively low (**Figure 3-5b**), we cannot exclude that the pH effect could be related to some nutrient limitations. Nutrient limitations are known to induce physiological responses, particularly in certain photosynthetic characteristics related to PSII (White et al., 2011). Indeed, nitrogen ($N\text{-}NO_3^-$) and phosphorus ($P\text{-}PO_4^{3-}$) count among the main components of many microalgal culture media as well as the most effective factors for regulating secondary metabolism in microalgal cells (Peccia et al., 2013).

Table 3-2
Nutrient requirement for biomass production (Rxs, mg gX^{-1}) during growth in steady-state continuous cultures at a fixed dilution rate of 0.2 d^{-1} but at different pH levels.

pH	Feed medium	$N\text{-}NO_3^-$	$P\text{-}PO_4^{3-}$
5.5	2P-AF-6	57.5±3.2	13.5±1.2
6.0	2P-AF-6	59.0±2.2	13.0±1.0
6.5	2P-AF-6	56.5±4.0	12.0±0.9
7.0	2P-AF-6	58.5±2.8	12.5±1.0
7.5	2P-AF-6	61.5±2.6	12.5±1.1
8.0	2P-AF-6	63.5±4.4	13.5±1.0
6.5	3N6P-AF-6	42.2±3.8	15.9±1.2
7.5	3N6P-AF-6	48.1±5.1	16.6±1.5

Means ± SD.

N and P requirement for biomass production were calculated for all the successive steady-states of the continuous culture fed with 2P-AF-6 medium (**Table 3-2**). pH changes had no significant effect on nutrient consumption yields determined from data on each steady-state continuous culture.

3.3.3. Biomass and hydrocarbon productivities at pH 6.5 and 7.5 with 3N6P-AF-6 medium

We led a second set of experiments characterized by threefold-higher N and P concentrations in the feed medium (3N6P-AF-6) and focusing on two successive steady-states at pH 6.5 then 7.5. In these conditions, N and P concentrations increased in the PBR outlet regardless of pH setpoint (**Figure 3-5b**). As shown in **Figure 3-5a**, both biomass and hydrocarbon productivities were increased by feeding the culture with the 3N6P-AF-6 nutrient-enriched medium compared to 2P-AF-6 medium. Biomass and hydrocarbon productivities increased up to 8.9 g m^{-2} d^{-1} and 0.50 g m^{-2} d^{-1}, respectively, at pH 6.5, and up to 8.5 g m^{-2} d^{-1} and 0.15 g m^{-2} d^{-1}, respectively, at pH 7.5. Note that the increase in purified hydrocarbon productivity was related to the increase in biomass productivity by increasing the N and P supply. Indeed, purified hydrocarbon content showed no significant change, at 5.6% dw at pH 6.5 and 1.7% dw at pH 7.5. These data confirm the results of Xu et al. (Xu et al., 2012b) showing that higher biomass and hydrocarbon productivities in *B. braunii* cultures were obtained by increasing the N and P supplies. Therefore, according to these data, the effect of pH on biomass and hydrocarbon contents was similar whatever the N and P concentrations.

However, nitrogen requirement for biomass production significantly decreased when the culture medium contained threefold more N (**Table 3-2**). It seems that enriching N and P in the feed medium could decrease the N needs for biomass. Sydney et al. (Sydney et al., 2010a) worked on the same *B. braunii* SAG 30.81 strain as here and reported a relatively similar nitrogen requirement for biomass production of 40.72 mg g X^{-1}. However, phosphorus requirement for biomass increased slightly but significantly in the cultures grown on 3N6P-AF-6 medium (**Table 3-2**). This observation could be interpreted by a relatively classical luxury phosphate uptake process, with intracellular accumulation of polyphosphate as an intracellular nutrient pool in different microalgae (Procházková et al., 2014), particularly in *B. braunii* (Casadevall et al., 1985). Further studies are needed to improve the formulation of a culture

medium for an optimal production of biomass for the different *B. braunii* strains.

There was no significant difference in Fv/Fm and ETR_{max} between feeding with 2P-AF-6 and 3N6P-AF-6 media in steady-state continuous cultures at pH ≥ 6.0 (**Figure 3-7**). Total lipid productivity was significantly higher at pH 6.5 (3.4 g m^{-2} d^{-1}) than pH 7.5 (2.8 g m^{-2} d^{-1}). Hydrocarbon productivities were also significantly higher at pH 6.5 ($P < 0.05$) regardless of media used (2P-AF-6 or 3N6P-AF-6) (**Figure 3-5a**).

In addition, extracellular hydrocarbons-to-intracellular lipids ratio was also higher at pH 6.5 than at pH 7.5, in line with the results on feeding with 2P-AF-6 medium. Extracellular hydrocarbons and intracellular lipids were fitted together with data previously obtained. Even if they appeared to follow a linear relationship:

$$[HC] = 0.557 \cdot [\text{Intracellular lipids}] - 141$$

The low R^2 value (= 0.769) indicates the relation between these cellular components would need more detailed studies, involving for instance some lipid classes rather than total intracellular lipids.

Table 3-3
Comparison of *B. braunii* biomass productivity, total lipid productivity and hydrocarbon productivity during studies within pH-controlled photobioreactors.

Strain	Race	pH control	Biomass productivity (mg L^{-1} d^{-1})	Hydrocarbon productivity (mg L^{-1} d^{-1})	Total lipid productivity (mg L^{-1} d^{-1})	Reference
GUBIOTJTBB1	--	7.5	13	6.8[a]	7.4	(Talukdar et al., 2013)
SAG 30.81	A	7.2	207[b]	--	68	(Sydney et al., 2010a)
SAG 30.81	A	6.5	223	12.5[c]	84	This study
SAG 30.81	A	7.5	212	3.8[c]	70	This study

[a], hexane extract as crude hydrocarbon; [b], average productivity calculated from figure; [c], purified hydrocarbon; --, no data available.

There are relatively few published studies on *B. braunii* cultures within photobioreactors, and only two in which pH was controlled by automated CO_2 injection, at setpoint values of pH 7.2 and 7.5, respectively ((Sydney et al., 2010a; Talukdar et al., 2013); see **Table 3-3**). Our data at pH 7.5 were similar to results from another study at pH 7.2 on the same strain but much higher on biomass productivity and lipids productivity and different on hydrocarbon productivity compared to strain GUBIOTJTBB1 at pH 7.5.

This difference could be due to other cultivation conditions or could be strain-dependent. Further, our study found that the highest hydrocarbon and total lipid productivities were reached at pH 6.5, but pH is also known to influence the speciation and bioavailability of different nutrients in the culture media, particularly DIC forms.

3.3.4 Relation between dissolved carbon dioxide and hydrocarbon production

Hydrocarbon is considered as a growth-associated product in *B. braunii* cultures (Yoshimura et al., 2013). However, our preliminary results suggest that environmental pH can stimulate hydrocarbon production with little effect on biomass productivity. The large increase (>70%) in biomass concentration found here when continuous cultures were fed with 3N6P-AF-6 medium compared to 2P-AF-6 medium indicates that certain N and P limitations control biomass accumulations without change of hydrocarbon content within the PBR. The controlling factor here was not light availability, as PFD at the PBR was constant. Indeed higher volumic productivities could be reached by increasing PFD and decreasing the light path within the PBR, as already shown in a trickle-film configuration (Jin et al., 2015).

B. braunii hydrocarbon content is mainly related to its exocellular hydrocarbons (Banerjee et al., 2002; Saga et al., 2015). Indeed, the biomass of this colonial species is essentially composed of cells surrounded by a complex extracellular matrix of polymerized hydrocarbons (Weiss et al., 2012) that accumulates liquid hydrocarbons, mainly alkadienes and alkatrienes in race A strain SAG 30.81. The extracellular fraction has been evaluated to about 95% of total hydrocarbon (Eroglu et al., 2011). We can use these characteristics to assume that the liquid hydrocarbons as exocellular products could be dissociated from the biomass and the matrix. Then, as biomass concentration at steady-state seems relatively unaffected by pH compared to the

change in hydrocarbon biosynthesis, the stimulation of hydrocarbon productivity at pH 6.5 could be triggered by either increased hydrocarbon biosynthesis activities, the excretion process, or the adsorption capacity of the matrix. In the first case, pH 6.5 would be optimal for hydrocarbon biosynthesis activities that resulting in overproduction. Another hypothesis is that this stimulation could be caused by an effect of external pH on internal pH, as some intracellular enzymes, such as the decarbonylase, associated with hydrocarbon biosynthesis, are highly pH-dependent (Dennis and Kolattukudy, 1991). In the second case, pH 6.5 could impact the rate of globule content exocytosis through the plasma membrane and cell wall of *B. braunii*. In the third case, the polymer's capacity to trap extracellular hydrocarbon would presumably be pH-dependent, like the effect of NaCl on efficiency of hydrocarbons extraction from *B. braunii* biomass observed by Furuhashi et al. (Furuhashi et al., 2013). Moreover, we cannot rule out an effect of pH on chemical composition of the liquid hydrocarbon fraction.

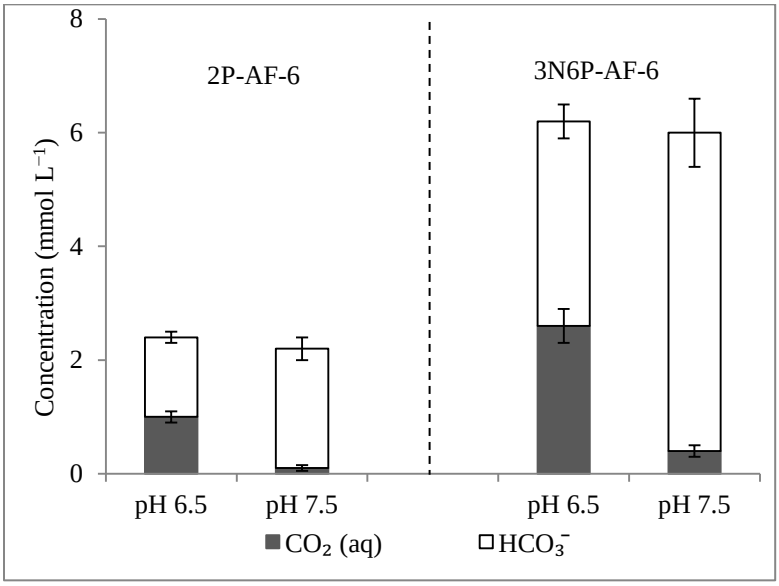


Figure 3-8. Inorganic carbon dioxide composition of the supernatant of *B. braunii* cultures in 2P-AF-6 and 3N6P-AF-6 media at pH 6.5 and 7.5 at the constant dilution rate of 0.2 d^{-1}; $CO_{2(aq)}$ values were calculated from DIC data.

However, we observed that with pH controlled by CO_2 injection, DIC concentrations in the supernatants of steady-state cultures seemed to be relatively constant (i) at pH 6.5 and 7.5 regardless of feed medium (2P-AF-6 or 3N6P-AF-6) (**Figure 3-8**), and (ii) between pH 6.0 and 8.0, with a net RSD of 13% and very little fluctuations of DIC concentration during each steady

state (RSD ≤ 6.8%). Thus, at first glance, there was visibly no DIC limitation of growth and hydrocarbon production occurred in our experimental conditions. On the basis of biomass elemental composition, stoichiometry equations on *B. braunii* have shown that hydrocarbon-enriched biomass production needs about 30% more CO_2 for strain BOT 22 than for *Chlorella* sp. or *Spirulina* sp. (Jin et al., 2015). Not only the necessary amounts but also species of DIC have to be provided to drive increased growth of hydrocarbon-enriched biomass. The significant difference in hydrocarbon production at pH 6.5 *vs* pH 7.5 could thus be related to the effect of pH on inorganic carbon speciation, i.e. $CO_{2(aq)}$ or H_2CO_3, HCO_3^-, and CO_3^{2-} (Danckwerts and Kennedy, 1958; Grima et al., 1993; Nedbal et al., 2010; Raven, 2010).

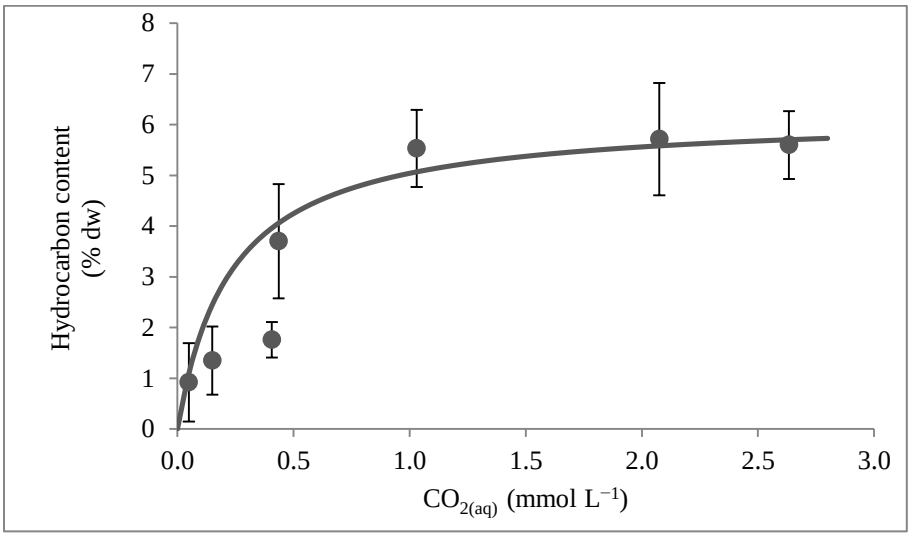


Figure 3-9. Hydrocarbon content of *B. braunii* biomass as a function of residual $CO_{2(aq)}$ in the culture under steady-states conditions from pH 6.0 to 8.0 in continuous cultures fed with 2P-AF-6 or 3N6P-AF-6; $CO_{2(aq)}$ values calculated from DIC data.

Figure 3-9 shows that hydrocarbon accumulation in *B. braunii* colony biomass is seems to be limited at dissolved CO_2 concentrations < 1 mmol L^{-1}. A maximal hydrocarbon content of 5.7% dw was reached at and above this concentration. This DIC range is comparable with other DIC values related to biomass productivities in cultured microalgae such as *Isochrysis galbana* and *Phaeodactylum tricornutum*, where DIC limitations but not CO_2 limitations were observed for biomass productivity but not metabolite productivity at DIC values < 0.5 mmol L^{-1} (Clark et al., 1999).

After factoring in all the data obtained here but excluding data with physiological inhibition on cells at pH 5.5, hydrocarbon productivity appears linearly related to $CO_{2(aq)}$ up to 2.5 mmol L^{-1}, whatever the N and P content of the culture medium (**Figure 3-10**), according to the relation:

$$P_{HC} = 0.146 \cdot CO_{2(aq)} + 0.077,\ R^2=0.869$$

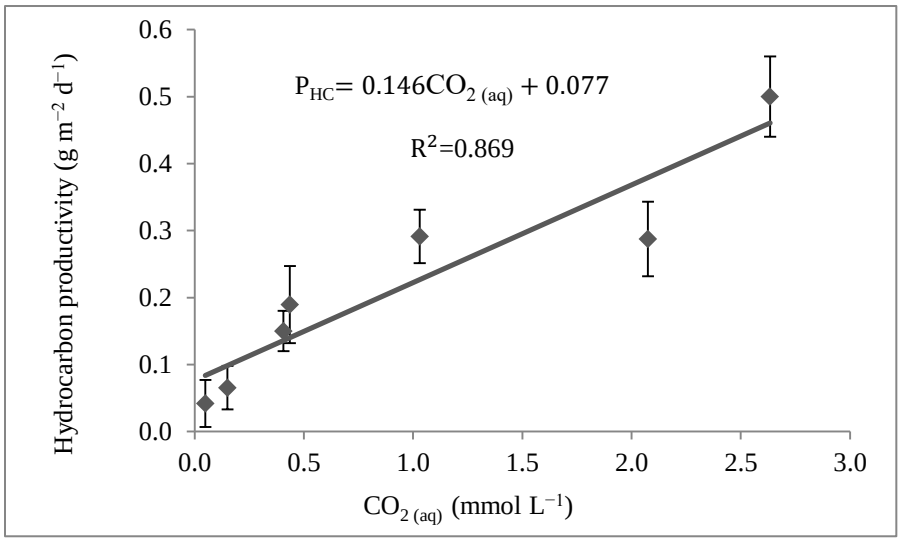


Figure 3-10. Relation between hydrocarbon productivity and residual dissolved carbon dioxide in the culture medium at each steady state; $CO_{2(aq)}$ values calculated from DIC data.

As a result, pH effects on hydrocarbon productivity are probably related to how pH influences distribution of DIC speciation, and $CO_{2(aq)}$ could be the key driver of hydrocarbon production.

3.4. Conclusion

Successive steady-state continuous cultures of *B. braunii* SAG 30.81 at a constant dilution rate have shown that hydrocarbon productivity but not biomass productivity is pH-dependent in the range 5.5 to 8.0. At pH 5.5, the inhibition of PSII activities could be related to a reduction in hydrocarbon production. Maximal hydrocarbon productivities were obtained at pH 6.5 in two modified AF-6 media with nitrate and phosphate enrichments. The pH effect on hydrocarbon

production was shown to be mainly related to dissolved $CO_{2\ (aq)}$ concentration. This study also indicated that in unstressed, i.e. stable PSII activity, extracellular hydrocarbon accumulation appeared to be linked to intracellular lipid accumulation. Further comparative studies between race-A and race-B *B. braunii* strains cultivated at different dilution rates and PFD could valuably help determine how dissolved CO_2 availability, hydrocarbon productivity and intracellular lipid productivity are co-related.

CHAPTER IV: Characteristics of *B. braunii* SAG 30.81 cultures at different dilution rates

4.1. Introduction

A number of microalgae have been reported for their high biomass productivities under photoautotrophic conditions, thanks to their efficient photosynthetic activities, from extrapolated data obtained with labscale photobioreactors. However, for inducing metabolites accumulation such as pigments or lipids, they generally need to be cultivated under unfavorable conditions, such as nitrogen limitation, resulting in partial growth inhibition (Liu et al., 2016). The development of microalgal biodiesel is still hampered by some drawbacks; one of them is the constraint of overproducing triacyglycerol only under restricted culture growth. By contrast, even if *some uncertainties remain* about the relationships between their nutrient status, culture growth rate and product accumulation, the biosynthesis of long chain hydrocarbons by the microalgal species *Botryococcus braunii* was reported to be growth associated (Kojima and Zhang, 1999). Indeed according to some studies hydrocarbon contents and productivities seemed growth associated as shown by a positive correlation with the specific growth rate (Yoshimura et al., 2013). Moreover, repeated feeding of nitrate and other nutrient in a fed batch culture stimulated cells growth of *B. braunii* IPE 001 resulting in an increase of biomass production from 1.82 g L^{-1} to 2.87 g L^{-1}, as well as an increase of hydrocarbon content from 58.7 % to 64.3% (Xu et al., 2012a). Conversely, other studies on *B. braunii* have shown that hydrocarbon contents could increase when the growth of *B. braunii* cultures was inhibited by nitrogen limitation (Singh and Kumar, 1992; Cheng et al., 2013, 2014). For example, transferring *B. braunii* from nitrogen sufficient medium to nitrogen deficient medium induced a decrease of biomass dry weight from 4.2 g $L^{-1}$to 2.3 g L^{-1}, while hydrocarbon content increased from 21.8 %to 39.7 % dw (Singh and Kumar, 1992).

It is worth mentioning that most of previous studies on *B. braunii* were carried out in batch cultures, mainly without pH control. In this mode of culture, the environmental conditions are continuously changing. Such an unsteady-state system could mask subtle physiological differences and trends (Hoskisson, 2005). Indeed continuous variations of environmental parameters, such as pH or specific light supply, could confuse the relations between specific growth rates and hydrocarbon production. To overcome such drawbacks, more reliable data could

be obtained from continuous cultures at successive steady−states (Hoskisson, 2005; Tang et al., 2012). Only one study reported data obtained from continuous cultures of the *B. braunii* race A strain CCC 807/1; those experiments were done without pH control and at only two dilution rates (0.25 d^{-1} and 0.30 d^{-1}) (Casadevall et al., 1985). Another work on the *B. braunii* race B strain Showa tried to achieve pseudo-steady−states by semi−continuous culture and also without pH control (Khatri et al., 2014).

In the present study, the axenic strain, *B. braunii* race A SAG 30.81, was cultivated in a torus photobioreactor with pH control. This strain is known to produce polyalkadiene and polyalkatriene hydrocarbons of potential interest for sustainable chemistry, e.g; plastic or rubber synthesis, pharmaceutical intermediates or as biofuel (Pollard and Woodley, 2007; Jin et al., 2015). The relationships between the specific growth rate, hydrocarbon and total lipids production were analyzed, as well as nitrogen supply and consumption according to different values of the dilution rate. To our knowledge, this is the first time to use continuous cultures system with full environmental conditions control for obtaining data in steady−states at different dilution rates in *B. braunii*. In contrast to triacylglycerol accumulation in most of oleaginous microalgae at limited growth rate, hydrocarbon productivity was correlated linearly to biomass productivity and hydrocarbon specific production rate was demonstrated in linear correlation to cell specific growth rate of *B. braunii* irrespective of nitrogen conditions.

In addition to hydrocarbon, lipids of microalgae are also the crucial factors in making algal biofuels economically viable (Griffiths and Harrison, 2009). Especially in *B. braunii* race A strain, active carbon exchange could take place between the biosynthesis of lipids and alkadiene and alkatriene hydrocarbons via oleic acid, which is the precursor for hydrocarbons biosynthesis, probably via an elongation mechanism involving conversion to very long chain fatty acids followed by decarbonation reaction (Baba et al., 2012a; Ioki et al., 2012a). Then, this study describes the relation between the kinetics of biomass, intracellular lipids and extracellular hydrocarbon productions in *B. braunii* cell suspensions cultivated at different dilution rates.

From another perspective, a deeper knowledge of the energy balance of microalgae growth in a photobioreactor (PBR) plays a crucial role both to better understand the energetic

constraints of photosynthesis and to optimize the efficiency of the production process. To minimize the operation cost, to optimize biomass yield on light energy in photobioreactors, an optimum between low and high light supply rates should be found. However, it seems no studies on biomass and hydrocarbon yield on light in *B. braunii*, like triglyceride yield on light were carried out in *Nannochloropsis* sp. (Benvenuti et al., 2016).

To optimize these biological systems for efficient bioproduction, it is imperative to gain better knowledge of their physiological and bioenergetic processes. In fact, light availability is the factor with a seminal influence on productivity because autotrophic algal growth is fully supported by light energy (Carvalho et al., 2011). Light reveals to be the major factor controlling, and often limiting, the productivity of bioprocess involving photosynthetic microorganisms. They are already some of the studies focused on light optimization of *B. braunii, such as the* growth of *B. braunii* BOT-22(race B) were determined at light intensity of 20, 40, 100 and 200 μmol m^{-2} s^{-1} (Sakamoto et al., 2012) and *B. braunii* Strain Showa(race B) were tested in the light intensity levels from 0 to 2000 μmol m^{-2} s^{-1} (Yoshimura et al., 2013)(Yoshimura et al., 2013). However, in most studies, the light intensity is the absolute data that irradiate to the culture, without consideration of biomass concentration. Then, it is difficult to compare results published in different papers. Thus, to our knowledge, it is better to use the term "specific light supply rate" to describe the light intensity condition which means amounts of photon supplied to per gram dry biomass per day, as used for energy requirements for photoautotrophic growth and maintenance of *Chlamydomonas reinhardtii* (Kliphuis et al., 2012). Therefore, in this study, we also aimed to quantify the impact of specific light sully rate on biomass and hydrocarbon yields in *B. braunii*, as well as physiological responses.

4.2. Experiment design and setup

Experiment was designed as maintaining continuous cultures in torus photobioreactor as shown in **Figure 4-1.** Experiments were conducted with the strain *B. braunii* Kützing SAG 30.81. The continuous culture was conducted in the torus photobioreactor (PBR) system as described before (Pruvost et al., 2006) . Cultures illuminated continuously at a photon flux density of 150 μmol photon m^{-2} s^{-1} in the 400–700 nm wavebands (photosynthetically active radiation, PAR). The speed of the axial flow impellers was 200 rpm. Temperature and pH were monitored with

sensor (Mettler Toledo SG 3253) with temperature at 23 ± 1 ºC and pH at 6.5 by automatic CO_2 injection. Gas flow rate was 450 mL min^{-1} corresponding to an aeration rate of 0.32 vvm.

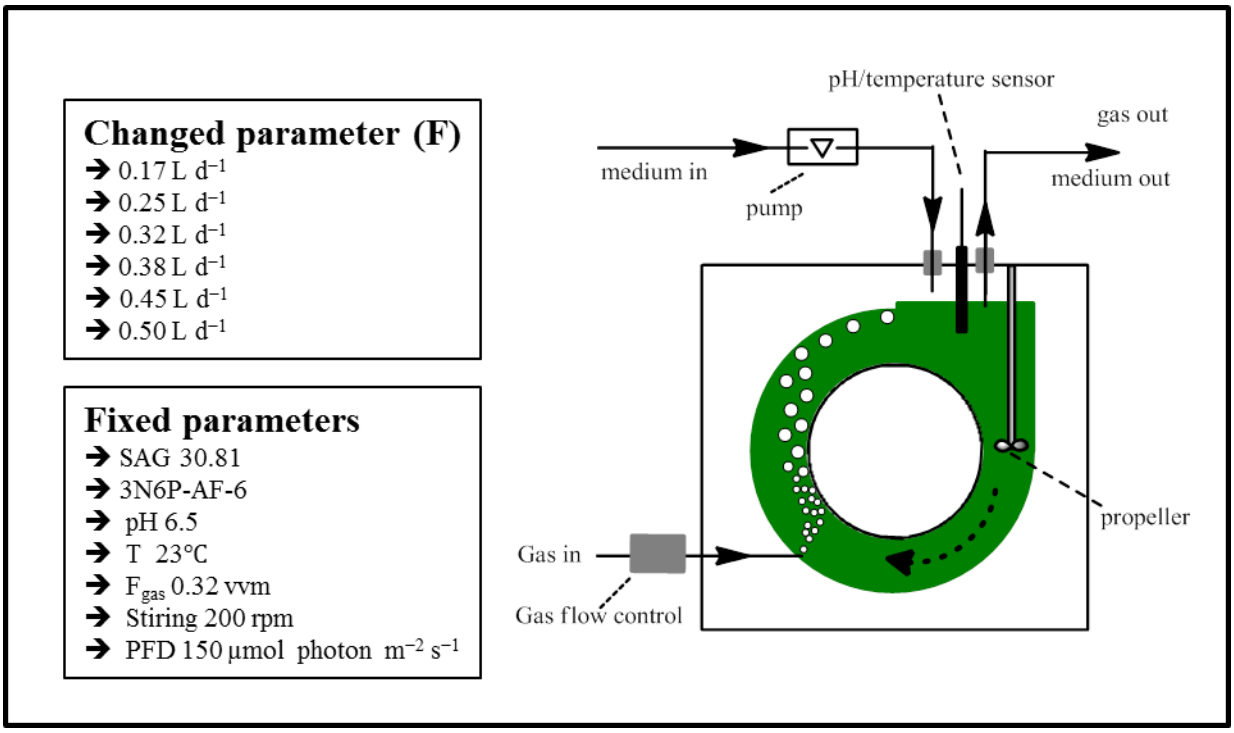


Figure 4-1. Experiment design and setup of the continuous cultures at different dilution rates in torus photobioreactor.

The continuous cultures were fed with 3N6P−AF−6 medium with different flow rates (F, L d^{-1}), from 0.17 L d^{-1} to 0.50 L d^{-1}, after reaching a steady−state at operating flow rate. Dilution rate (D, d^{-1}) was calculated by the following relation:

$$D = \frac{F}{V}$$

Then, the experiments conducted at six dilution rates, 0.12 d^{-1}, 0.18 d^{-1}, 0.23 d^{-1}, 0.27 d^{-1}, 0.32 d^{-1} and 0.36 d^{-1}. In continuous culture, the biomass concentration in photobioreactor, the specific growth rate (µ) of microalga and the dilution rate are related to the equation as follows:

$$\frac{dX}{dt} = \mu \cdot X - D \cdot X$$

At steady state,

$$\mu = D$$

Then, at steady state, the *B.braunii* cells were maintained at the different specific growth rate. In order to characterize the actual light supplied to the cells, the term "specific light supply rate" to describe the light intensity condition which means amount of photon supplied to per gram dry biomass per day. The specific light supply rate was calculated as the following relation:

$$\text{specific light supply rate} = \frac{PFD}{X}, \text{ (mol photon } gX^{-1}\ d^{-1} \text{ or mol } gX^{-1}\ d^{-1})$$

This study is based on the Luedeking–Piret model for growth associated and non-growth associated lactic acid production (Luedeking and Piret, 1959). These authors considered the relationship of cell growth to product formation for lactic acid production in continuous cultures of *Lactobacillus delbrueckij*; they detailed a model which included both a 'growth associated' and a 'non-growth associated' term for lactic acid production. Indeed, when some constituents of biomass, which are proportional to cell mass, are the product(s), the rate of product formation could be directly related to the rate of growth. Some products are known to start formation only when growth stops, i.e. when the limiting nutrient falls under a threshold concentration. The Luedeking-Piret equation with empirical constants α and β correlates product formation, d*P*/d*t* with cell growth rate, d*X*/d*t* and cell concentration, *X*, respectively. Applied to the hydrocarbon production in *B. braunii* cultures, the equation is:

$$\frac{dHC}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X$$

$$\frac{1}{X} \cdot \frac{dHC}{dt} = \alpha \cdot \mu + \beta$$

In chemostat cultures at steady states, μ = D, then,

$$\frac{1}{X} \cdot \frac{dHC}{dt} = \alpha \cdot D + \beta$$

The specific rate of hydrocarbons production ($\frac{1}{X} \cdot \frac{dHC}{dt}$) was estimated as equivalent to $\frac{P_{HC}}{X}$ (gHC gX^{-1} d^{-1}).

with α and β are coefficients meaning :

α , growth associated product formation coefficient (g g^{-1});

β , nongrowth-associated product formation coefficient (g g^{-1} d^{-1});

4.3. Results and discussion

In order to highlight the effect of growth rate in steady−state, a continuous culture of *B. braunii* was started in a 1.4 L photobioreactor by changing dilution rate. Environmental conditions were kept in constant during all the continuous cultivation, including temperature (23 °C), pH (6.5), stirring (200 rpm), aeration rate (0.32 vvm) and a continuous150 µmol m^{-2} s^{-1} light intensity at the surface of photobioreactor.

4.3.1. Growth and biomass production

Six steady−states were obtained at dilution rate 0.12 d^{-1}, 0.18 d^{-1}, 0.23 d^{-1}, 0.27 d^{-1}, 0.32 d^{-1} and 0.36 d^{-1}, during the growth of *B. braunii* in present 103 days continuous cultures. Continuous cultures could offer advantages over biologically heterogeneous batch cultures (Hoskisson, 2005), and the specific growth rate is equated to dilution rate at steady state, that means six specific growth rates in steady−state were obtained in present study and the following analyses were mainly focused on these steady−states. The green alga *B. braunii* is generally regarded as a slow-growing microalga, except that a few investigations have shown this species could grow at specific rates up to 0.5 d^{-1} in batch cultures (Jin et al., 2015) . This work illustrates that *B. braunii* could maintain steady−state at dilution rate of 0.36 d^{-1} without washing out, showing a relatively high growth rate potential.

As **Figure 4-2** shows, with the dilution rate increasing from 0.12 d^{-1} to 0.36 d^{-1}, the steady−state biomass concentration decreased linearly in *B. braunii* continuous culture. At low dilution rate, the rate of removal of cells from photobioreactor was lower, resulting to a high biomass concentration, reaching 56.0 g m^{-2}. As increasing of the dilution rate, the rate of removal of cells was also higher and so the biomass concentration fell successively, eventually to 13.8 g m^{-2}. This kind of biomass concentration decreasing with increasing of dilution rate is general, for example, biomass concentration of microalga *Isochrysis galbana* also decreased linearly at steady state from dilution rate 0.12 d^{-1} to 0.96 d^{-1} in continuous cultures (Grima et al., 1994).

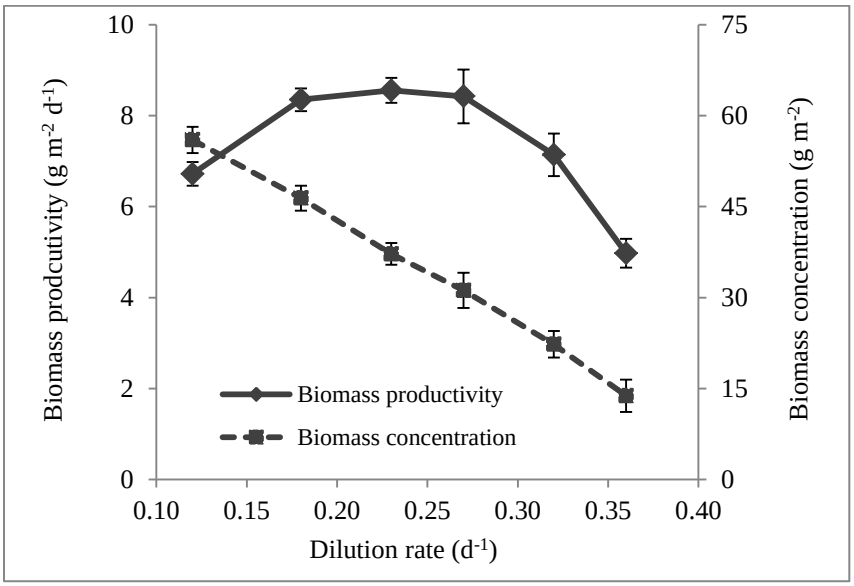


Figure 4-2. The productivity and concentration of biomass as a function of dilution rate at steady-states in *B. braunii* continuous cultures.

Biomass productivity was increased, with the increasing of dilution rate from 0.12 d^{-1} to 0.18 d^{-1}. Dilution rate in the range of 0.18 d^{-1} to 0.27 d^{-1} could be regarded as the maximum productivity dilution rate range at present works for the *B. braunii* race A strain SAG 30.81, which is much higher than a race B strain in semi−continuous culture with the dilution rate at 0.1 d^{-1} for maximum productivity (Khatri et al., 2014). Maximal biomass productivity was 8.5 g m^{-2} d^{-1} (214 mg L^{-1} d^{-1}), similar to previous cultivation of the same strain in BioFlo Fermentor with average biomass productivity of 207 mg L^{-1} d^{-1} (Sydney et al., 2010a). A further increasing dilution rate above 0.27 d^{-1} would decrease the productivity **(Figure 4-2**), due to rapid removal

of the culture from photobioreactor. This curve of productivity as a function of dilution rate is classic in chemostat cultures, in consistent with a *B. braunii* race B strain Showa (Khatri et al., 2014) and other microalgae such as *Chlorella minutissima* and *Dunaliella tertiolecta* (Tang et al., 2012). Nonetheless, the maximum productivity dilution rate of *B. braunii* is still lower than some other microalgae, such as for *C. minutissima* and *D. tertiolecta* which could obtain the maximum productivity dilution rate at 0.32 d^{-1} and 0.42 d^{-1}, respectively (Tang et al., 2012). Further optimization of culture conditions for obtaining higher productivity could be meaningful for industry application of this hydrocarbon-rich microalga.

4.3.2. Nitrogen supply and consumption

Growth of cell and biomass production of microalgae depends on the availability of multiple nutrients. Nitrogen is the main component of different cultures media used for growing microalgae, as well as the most effective factor to regulate the secondary metabolism in microalgal cells (Cheng et al., 2013, 2014). The residual concentration of nitrogen in the photobioreacotr outlet was monitored when cells in steady−states. As **Table 4-1** shows, nitrogen residue was presented at very low concentrations in the chemostat cultures operating at a low dilution rate, especially at dilution rate 0.12 day^{-1}, which means most of nitrogen converted into cells. As the dilution rate was stepwise increased from 0.18 day^{-1} to 0.36 day^{-1}, the availability of nitrogen increased, which should be sufficient for growth at high dilution rate.

Table 4-1
The residual concentration of nitrogen and biomass yield from nitrogen at steady-states of different dilution rates in *B. braunii* continuous cultures.

Dilution rate (d^{-1})	0.12	0.18	0.23	0.27	0.32	0.36
N_{out} (mmol L^{-1})	0.01± 0.01	1.27± 0.11	1.87± 0.19	2.68±0.22	3.44± 0.23	4.19± 0.30
$Y_{X/N}$ (gX gN^{-1})	20.9±1.8	21.7±1.9	22.7±1.5	26.3±2.7	29.4±2.8	37.0±3.2

N_{out} = residual concentration of nitrogen in photobioreactor; $Y_{X/N}$ = biomass yield from nitrogen.

Nitrogen content of biomass was calculated according to mass balance during all the successive steady−states of the continuous culture, as well as the nitrogen specific consumption rate (**Figure 4-3**).

Generally, increase of nitrogen concentration in culture medium can stimulate the growth of *B. braunii* and improve biomass production (Singh and Kumar, 1992; Xu et al., 2012a; Cheng et al., 2013). For example, feeding nitrate and other nutrient into batch culture stimulated cells growth of *B. braunii* IPE 001 and increased biomass production from 1.82 g L^{-1} to 2.87 g L^{-1} (Xu et al., 2012a). While there are some exceptions reported in *B. braunii* cultures, for instance, biomass production of a new isolated strain *B. braunii* KMITL 2 from a freshwater reservoir in central Thailand is not significantly affected by the nitrogen concentration after 30 days of cultivation (Ruangsomboon, 2012). Present study also found that biomass yield from nitrogen is not constant, but increased from 20.9 gX gN^{-1} to 37.0 gX gN^{-1} by increasing dilution rates. The influences of nitrate on growth of *B. braunii* and the biomass yield from nitrogen still need to be elucidated.

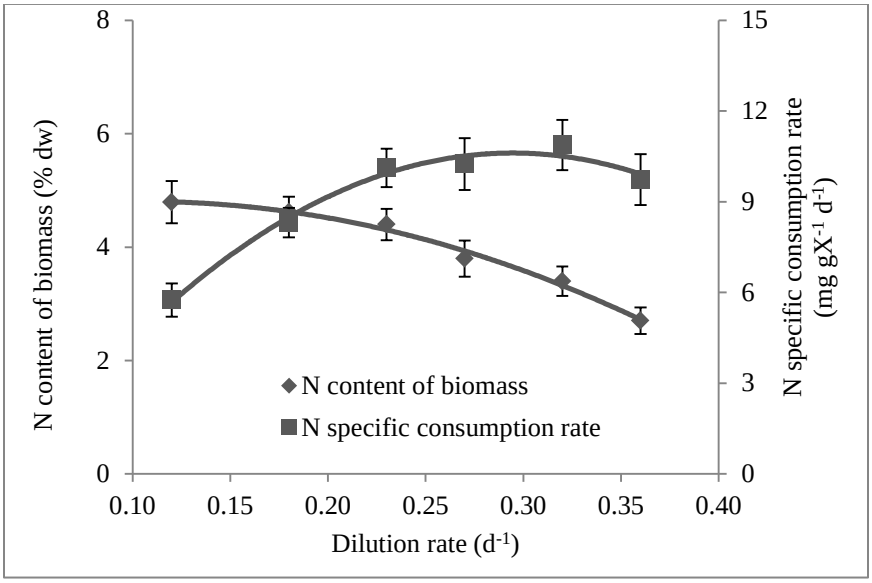


Figure 4-3. The content and specific consumption rates of nitrogen at steady-states as function of dilution rate in *B. braunii* continuous cultures; N, nitrogen.

Nitrogen content of biomass in the range of 2.7 % to 4.8 % dw according to dilution rate, in consistent with previous investigation showing a 4.1 % dw of *B. braunii* SAG 30.81 (Sydney

et al., 2010a). It seems that increasing dilution rate could be related to a decrease in the needs of nitrogen for producing hydrocarbon enriched biomass, since the nitrogen content of biomass decreased at high dilution rate. Moreover, **Figure 4-3** shows the specific consumption rates of nitrogen increased with dilution rate and the maximum consumption rate occurred at high dilution rates (specific growth rates). These characteristics could be used for the waste water treatments and *B. braunii* have already shown the potential for nitrogen removal in domestic sewage (Sydney et al., 2011), soybean curd wastewater (Yonezawa et al., 2012) and swine lagoon wastewater (Liu et al., 2013).

4.3.3. Hydrocarbon content, intracellular lipids content and their relationship

B. braunii race A could produce high content of hydrocarbon, up to 61% of dry biomass. Exceptionally, there were also some strains of *B. braunii* which produce hydrocarbon as low as 1% of dry weight (Jin et al., 2015). In the present study, **Figure 4-4 a** reveals that hydrocarbon content is relative constant with a 5% of dry biomass in *B. braunii* ASG 30.81 cultures at different dilution rates (specific growth rates), whatever high or low nitrogen conditions. However, previous published data on the relationship of growth rate, nitrogen condition and hydrocarbon content of *B. braunii* are not consistent. Positive correlation of hydrocarbon content with specific growth rate is found in a *B. braunii* race B strain Showa (Yoshimura et al., 2013) and strategic feeding nitrate to batch culture could slight increases the hydrocarbon content of *B. braunii* strain IPE 001 from 58.7 % to 63.5 % dw (Xu et al., 2012a), while by transferring *B. braunii* from nitrogen sufficient to nitrogen deficient condition with limiting the cell growth rate, hydrocarbon content increased from 21.8% to 39.7% dw in a Varanasi isolated *B. braunii* strain (Singh and Kumar, 1992), increased from 1.6% to 2.9% dw in *B. braunii* IPPAS H−252 strain (Zhila et al., 2005)(Zhila et al., 2005) and increased from 19.4% to 34.3% dw of *B. braunii* FACHB 357 (Cheng et al., 2013). This kind of diversity could be due to strain dependent. From another perspective, the culture systems should be also taken into consideration for steering clear of this kind of controversy. Most of previous works on *B. braunii* were conducted in Erlenmeyer flasks or in photobioreactors without pH control, which exerts a critical influence on hydrocarbon production by our preliminary results. To our knowledge, present work was the first time to use

continuous cultures system with pH and other environmental factors control for acquisition of data in steady−states at different dilution rates, providing advantages for better understanding the relationship of hydrocarbon content and nitrogen conditions. Our results indicate that the nitrogen concentration does not influence the hydrocarbon content in *B. braunii* SAG 30.81 cultures, in consistent with the study of *B. braunii* GCC 807/1 showing no much effect on hydrocarbon content in two dilution rates continuous cultures (Casadevall et al., 1985).

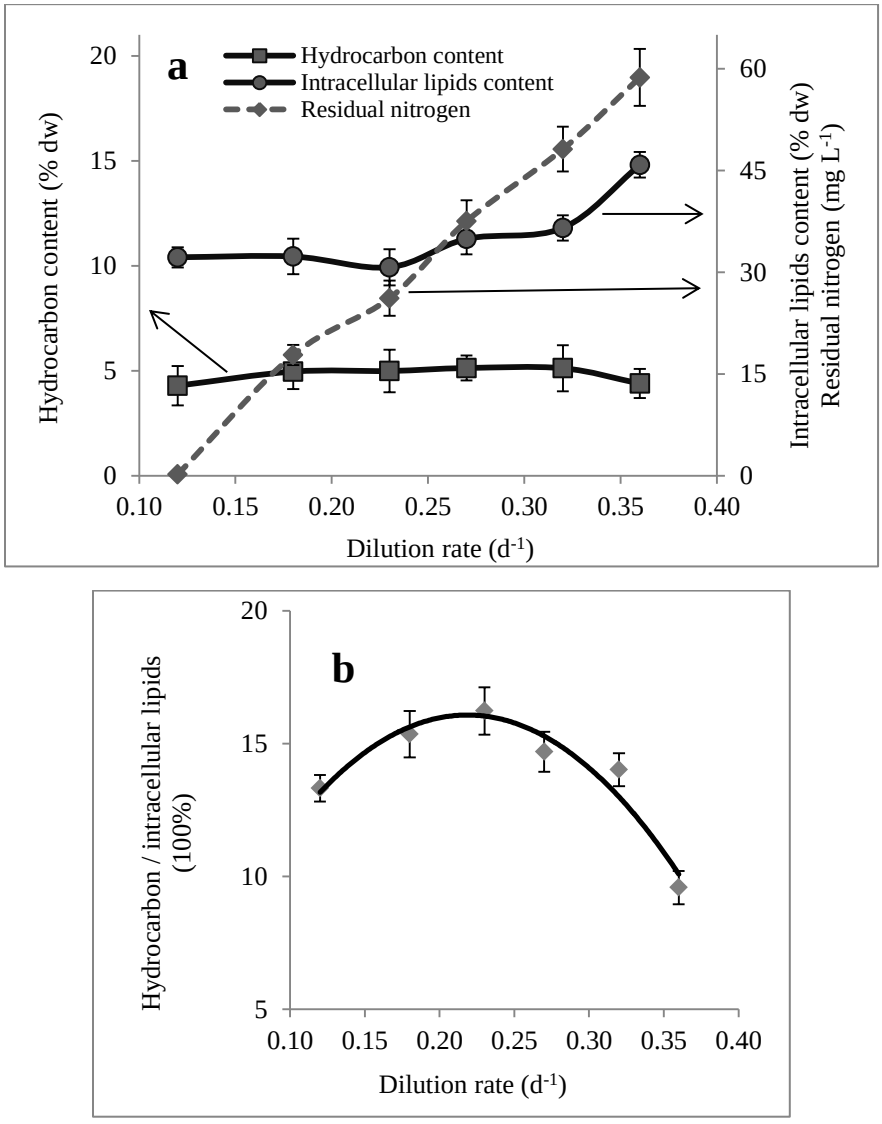


Figure 4-4. Hydrocarbon content, intracellular lipids content and residual nitrogen concentration (a), the ratio of hydrocarbon content to intracellular lipids content (b) as a function of dilution rate at steady−states in *B. braunii* continuous cultures.

In addition to hydrocarbon, lipids of microalgae are also the crucial factors in making algal biofuels economically viable (Griffiths and Harrison, 2009). For the majority of oleaginous microalgae, lipid biosynthesis and accumulation stimulated by nitrogen limitation condition with limiting cell growth (Liu et al., 2016). However, the ideal microalgal strain should be able to produce lipids, without a decrease in biomass. Seldom microalgae could produce high content of lipids at high growth rate, except a newly isolated species, the marine microalga *Isochrysis zhangjiangensis,* which can achieve maximum lipid content of 53% in the presence of high nitrate concentrations in the cuture medium, then in cultures at relative high growth rates (Feng et al., 2011). Other investigations have been done on targeted metabolic engineering of lipid catabolism to induce an increase in microalgal lipid accumulation without inhibition of culture growth (Trentacoste et al., 2013).

Surprisingly, *B. braunii* race A SAG 30.81 strain could biosynthesize intracellular lipids up to 45 % dw, with about 5 % extracellular hydrocarbons, at high dilution rate (specific growth rate) under nitrogen sufficient conditions (**Figure 4-4a**). Here the overproduction of lipids at high dilution rate seems related with a decrease of nitrogen content in biomass (**Figure 4-3**), consistent with the fact that nitrogen is mainly involved in protein but not lipid biosynthesis.

The cell of this colonial microalga *B. braunii* is essentially surrounded by a complex extracellular matrix, in which liquid hydrocarbon, mainly alkadienes and alkatrienes for the race A strain SAG 30.81, are accumulated. Hydrocarbon is mainly related to the extracellular fraction, which is evaluated to about 95 % of total hydrocarbon (Jin et al., 2015). We can use these characteristics to assume that the intracellular lipids content could be estimated by subtracting the purified hydrocarbon content from that of the total lipids. In *B. braunii* race A strain, oleic acid is reported as the precursor for hydrocarbon biosynthesis, probably via an elongation mechanism involving conversion to very long chain fatty acids and active carbon exchange could take place between hydrocarbon and intracellular lipids via oleic acid (Baba et al., 2012a; Ioki et al., 2012a). Therefore, an interesting aspect of hydrocarbon biosynthesis is the question whether the ratio of hydrocarbon content to intracellular lipids content is constant or not. In present study, the ratio of hydrocarbon content to intracellular lipids content changed according to dilution rate and no linear relation was found (**Figure 4-4b**). It seems that more intracellular lipids convert to hydrocarbon also occurs at the maximal dilution rate range for biomass productivity, from 0.18 d^{-1} to 0.27 d^{-1}. It is also possible that there are some other relationships currently unknown between

hydrocarbon and intracellular lipids production. A further exploration to identify the lipids class of this hydrocarbon-rich microalga could be considerable.

4.3.4. Hydrocarbon production associated to biomass production and cell specific growth rate

Figure 4-5a demonstrates that hydrocarbon productivity increased from dilution rate 0.12 d^{-1} to 0.18 d^{-1} and maximal hydrocarbon productivity was 0.43 g m^{-2} d^{-1}, obtaining in the dilution rate range from 0.18 d^{-1} to 0.27 d^{-1}.

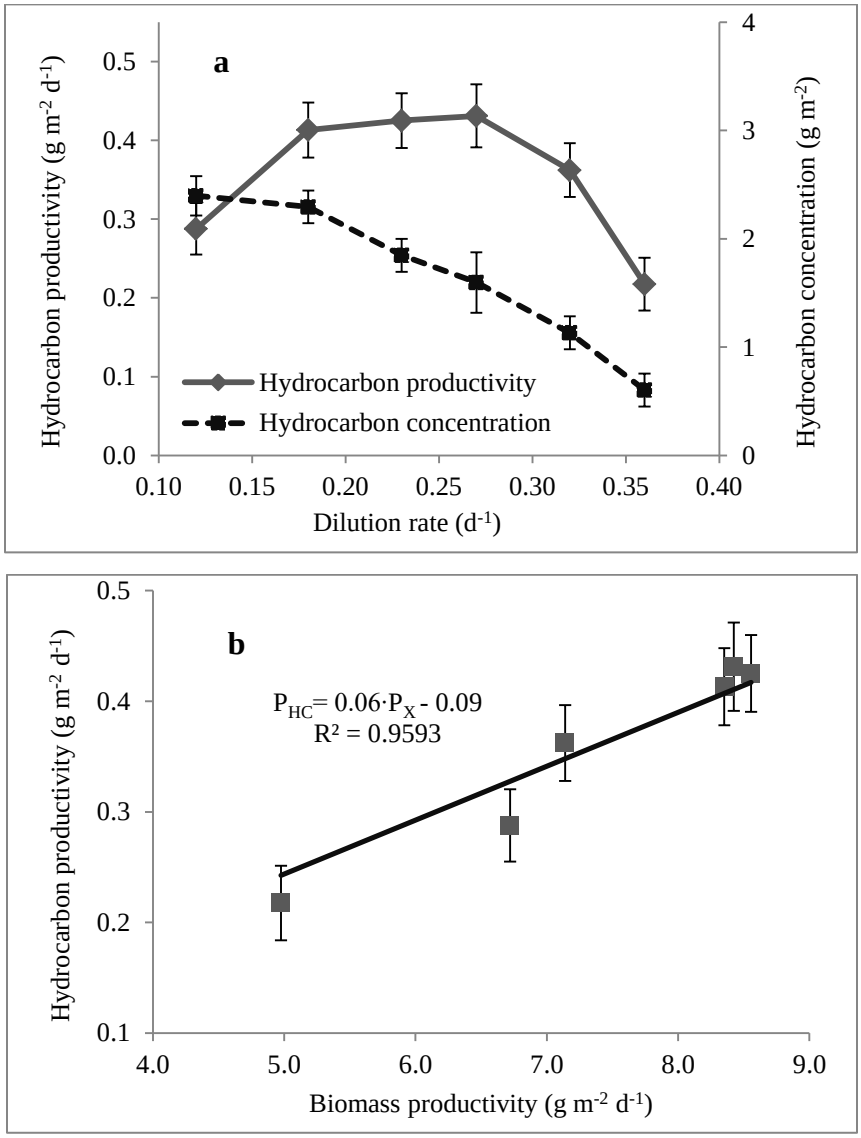


Figure 4-5. The productivity and concentration of hydrocarbon as a function of dilution rate (a), hydrocarbon productivity as a function of biomass productivity (b) at steady-states in *B. braunii* continuous cultures.

Further increasing dilution rate leaded to the decreasing of hydrocarbon productivity. Hydrocarbon concentration decreased from 2.4 g m^{-2} to 0.6 g m^{-2} with the increase of dilution rate. Comparing **Figure 4-2** with **Figure 4-5a**, hydrocarbon productivity and concentration were shown the same tendency of biomass productivity and concentration in present continuous culture. Hydrocarbon productivity is linear correlated to biomass productivity in *B. braunii* culture, with R^2 up to 0.9593, shown in **Figure 4-5b**. A comparing with other studies in batch or continuous cultures of race B (Xu et al., 2012a; Yoshimura et al., 2013; Khatri et al., 2014), it seems the same characteristic was found on hydrocarbon productivity in present investigation in race A. It means that hydrocarbon productivity is associated to biomass productivity for both races.

Table 4-2
Comparison with past studies and this work on *B. braunii* SAG 30.81.

Cultivation mode	Medium	N_{in} [a] (mmol L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Hexane extract (% dw)	Hydrocarbon (% dw)	Lipids (% dw)	Reference
Batch culture	M-Chu 13	1.87	21 [b]	48	--	--	Dayanand a et al., 2005
Batch culture	M-Chu 13	1.87	10 [b]	35	--	--	Dayanand a et al., 2006
Batch culture	M-Chu 13	1.99	53.6 [b]	17.9	--	--	Ranga Rao et al., 2007
Batch culture	BG11	17.65	48 [b]	46	--	--	Dayanand a et al., 2007b
Batch culture	3N-MBM	7.42	610 [c]	--	--	39.6	Sydney et al., 2010a
Continuous culture	3N6P-AF-6	5.73	211	31.7	5.1	40.1	This study

[a], nitrogen concentration in initial medium or in feeding medium; [b], average productivity calculated form figure or table; [c], maximal productivity; --, no data available.

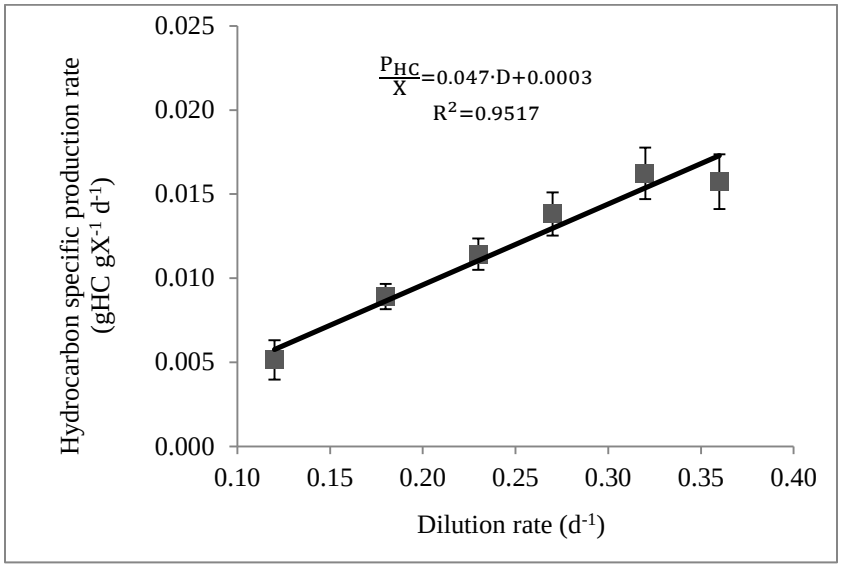


Figure 4-6. The relations between hydrocarbon specific production rate and specific growth rate of *B. braunii* at steady-states in continuous cultures by using Luedeking-Piret model.

The strain *B. braunii* SAG 30.81 was investigated in some other studies before, as summarized in **Table 4-2.** This strain could be cultivated in several culture media with different nitrogen concentrations, such as in modified Chu 13, BG11 and 3N-MBM. Most of the previous studies used hexane extract as the crude hydrocarbon (Dayananda et al., 2005, 2006, 2007b) or only total lipids content (Sydney et al., 2010a). The hexane content and total lipids at dilution rate 0.27 d^{-1} in our study could reach 31.7 % dw and 40.1 % dw, respectively, in the same range of previous studies. It is worth to note that hexane extract could contain other lipids, such as triacylglycerol and aldehydes, and hydrocarbon content could be overestimated by using hexane extract (Jin et al., 2015). Then, in order to quantify the exact hydrocarbon content of this strain, present work not only measured the hexane extract content, but also the purified hydrocarbon by using silica chromatography. Purified hydrocarbon content is 5.1% dw, only 16 % of hexane extract, that means there are some other components in hexane extract. A further investigation to identify what are the components and the ratio in hexane extract of the strain SAG 30.81 could be considerable. Also, the index purified hydrocarbon could more meaningful to evaluate this hydrocarbon-rich *B. braunii* for biofuels application. In addition, it seems increasing nitrogen concentration could increase biomass productivity by comparison with the studies on the same strain SAG 30.81, although there is an exception by using BG11 in **Table 4-2** and other factors could also exert influences on biomass production.

The Luedeking–Piret model is widely used to evaluate specific production rates of the metabolites or other products in cultured cell, as much to prokaryotes as to eukaryotes (Thierie, 2015). To our knowledge, the present work was the first time to introduce this concept in *B. braunii* cultures to identify the relationship between extracellular hydrocarbon production and specific growth rate. The hydrocarbon specific production rate was up to 0.016 gHC gX^{-1} d^{-1} at high dilution rate, which was significantly higher than that at low dilution rate with only 0.005 gHC gX^{-1} d^{-1} **(Figure 4-6)**. The relation between hydrocarbon production rate and dilution rate is linear associated, showing as the simulated equation:

$$\frac{P_{HC}}{X} = 0.0471 \cdot D + 0.0003,\ R^2 = 9517$$

Then, at steady states in continuous culture, dilution rate is equal to cell specific growth rate. Therefore, the equation could be developed to be:

$$\frac{P_{HC}}{X} = 0.0471 \cdot \mu + 0.0003,\ R^2 = 9517$$

As a result, we observed that hydrocarbon production is mainly growth associated; the very low value of β, 0.0003, corresponds to a non-growth associated part of hydrocarbon formation. This hydrocarbon production could be interpreted as leaks of very low concentrations of hydrocarbon from the cell colonies. Indeed we have estimated that less than 1 % of the total hydrocarbon can be found in the supernatant of the cultures. The concentrations of these exo-colonial hydrocarbons are above the level of detection but under the level of quantitation (LOQ). These small leaks could be inferred either to some cell death in the microalgal cultures or to some shear stress dependent production of exo-colonial hydrocarbons. Finding a technological mean to induce continuous leaks of hydrocarbon from colonies would be related to an increase of the β value and could be used for producing hydrocarbons under nongrowth conditions, for instance within culture systems with immobilized cells.

That hydrocarbon production is mainly growth associated is supported by some pieces of evidence as shown by previous studies:

- the rate of incorporation of $NaH^{14}CO_3$ into hydrocarbons was highest in early active growth stage of *B. braunii* B70 (Niitsu et al., 2012);
- the high rate of [^{14}C] acetate incorporation into hydrocarbons occurred in active growth cell of *B. braunii* UTEX 2441 (Hirose et al., 2013);
- ESTs associated to energy metabolism were abundantly expressed in the fast growing log‑phase of *B. braunii* 779 (Fang et al., 2015)(Fang et al., 2015) .

As a consequence, more efficient hydrocarbon production could be achieved at optimal environmental conditions enabling higher specific growth rate in *B. braunii* cultures. Then, producing hydrocarbons by *B. braunii* could be more convenient than producing triacylglycerol by other oleaginous microalgae, these one requiring a two stages or a two steps process: first for biomass production and second for lipids accumulation. This special characteristic of hydrocarbon biosynthesis and accumulation make the microalgae *B. braunii* an interesting candidate for industrial applications.

4.3.5. Biomass concentration and specific light supply rate

Six steady−states were obtained at dilution rate 0.12 d^{-1} to 0.36 d^{-1}, during the growth of *B. braunii* in present continuous cultures. As **Figure 4-7** shows, the steady−state biomass concentration decreased linearly in *B. braunii* continuous culture and each dilution rate corresponded to a different biomass concentration. At low dilution rate, the rate of removal of cells from photobioreactor was lower, resulting to a high biomass concentration, reaching 56.0 g m^{-2}. As increasing of the dilution rate, the rate of removal of cells was also higher and so the biomass concentration fell successively, eventually to 13.8 g m^{-2}. This kind of biomass concentration decreasing with increasing of dilution rate is general, and could be observed in other microalgal continuous cultures, such as *Chlorella minutissima* and *Dunaliella tertiolecta* (Tang et al., 2012).

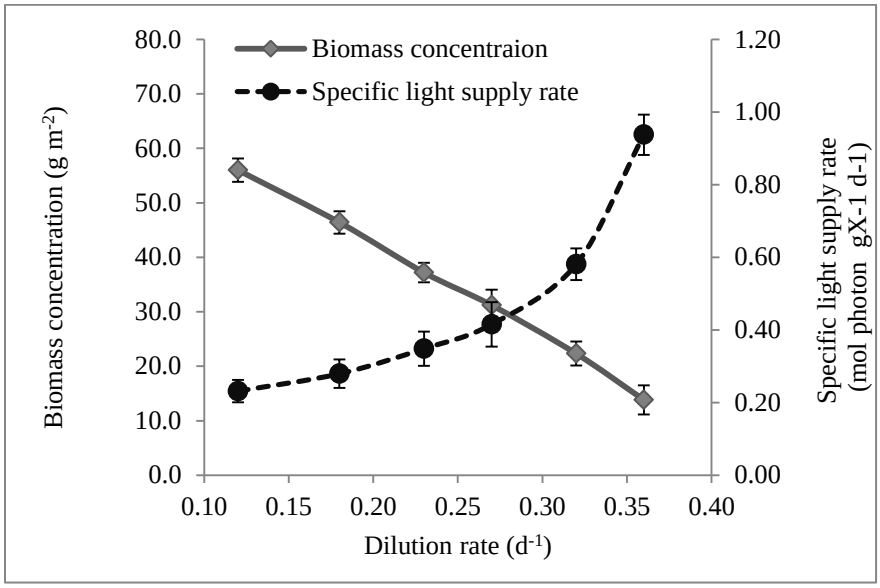


Figure 4-7. The biomass concentration and specific light supply rate as a function of dilution rate at steady-states in *B. braunii* continuous cultures.

By increasing the dilution rate to decrease the biomass concentration in PBR, the cells gradually received increased local light intensity, with fixed light intensity at the photobioreactor surface. Then, in order to quantify the light supply stat for *B. braunii* cells, we used the term "specific light supply rate (mol photon gX^{-1} d^{-1})" to describe the light intensity condition which defined as the amount of photon supplied to per gram dry biomass per day. As a result, cells were cultivated in six specific light supply rate conditions, successively increased from 0.23 mol photon gX^{-1} d^{-1}, 0.28 mol photon gX^{-1} d^{-1}, 0.35 mol photon gX^{-1} d^{-1}, 0.42 mol photon gX^{-1} d^{-1}, 0.58 mol photon gX^{-1} d^{-1} to 0.94 mol photon gX^{-1} d^{-1} (**Figure 4-7**). The following analysis of the first run continuous cultures are focused on the physiological responses, biomass and hydrocarbon yields at steady state of these six specific light supply rates.

4.3.6. Biomass and hydrocarbon yields on specific light supply rate

The ability to efficiently convert available nutrient and energy sources into cell biomass or economically valuable end products is a desirable characteristic for microbes to be used in bioproduction. Thus, an evaluation of biomass and product yields is key to determine the suitability of candidate systems. Studies of biomass yield on light have been conducted for other

microalgae, for example, observed biomass yield on light energy during *Dunaliella tertiolecta* cultivations (Zijffers et al., 2010). Since light intensity can influence metabolic processes in photosynthetic organisms, we studied the specific light supply rate effects on biomass and hydrocarbon yield to better understand the impact of light on *B. braunii* physiology. The biomass and hydrocarbon yields on specific light supply rate were calculated and plotted as a function of the specific light supply rate, as shown in **Figure 4-8.**

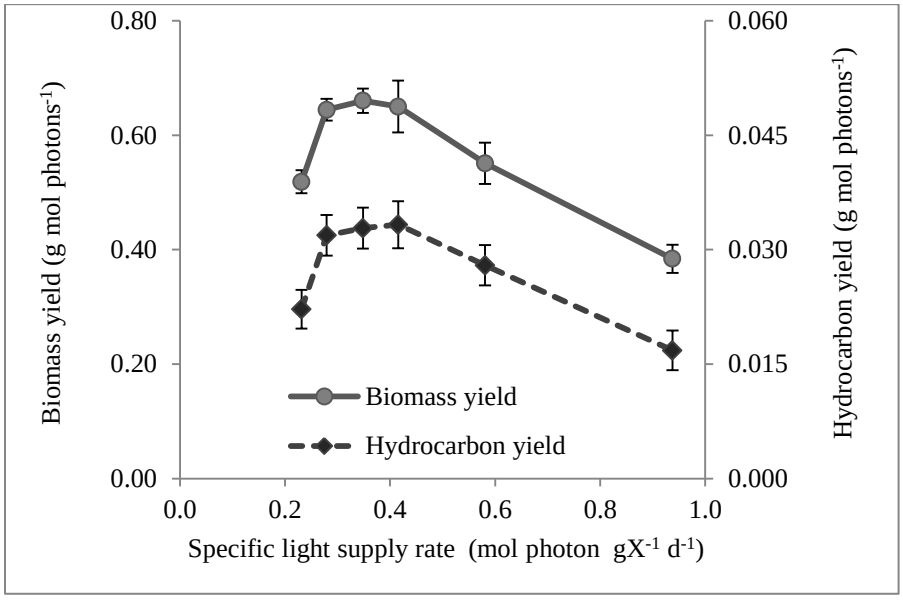


Figure 4-8. The biomass and hydrocarbon yields as a function of specific light supply rate at steady-states in *B. braunii* continuous cultures.

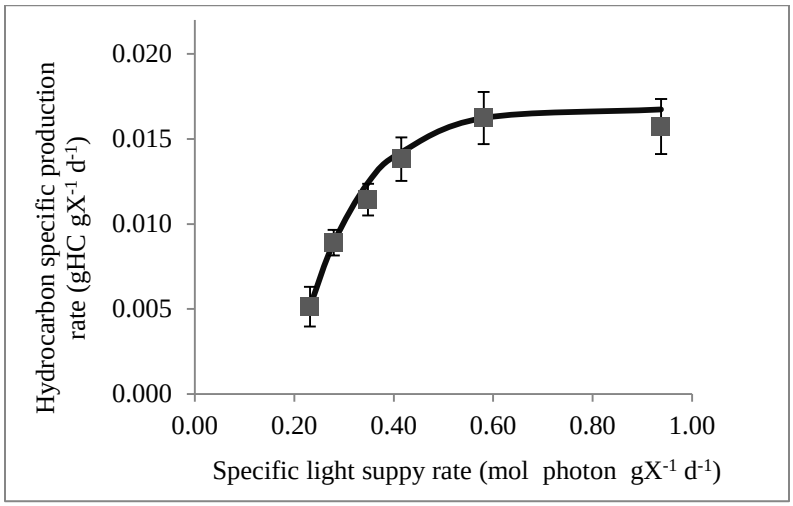


Figure 4-9. Hydrocarbon specific production rate as a function of specific light supply rate at steady-states in *B. braunii* continuous cultures.

With the specific light supply rate increased from 0.23 mol photon gX^{-1} d^{-1} to 0.28 mol photon gX^{-1} d^{-1}, both biomass and hydrocarbon yields significantly increased ($P < 0.05$). However, a further increase of light available per amount of biomass from 0.28 mol photon gX^{-1} d^{-1} to 0.42 mol photon gX^{-1} d^{-1} seems exerting no much effects on biomass and hydrocarbon yields. The maximal value of biomass and hydrocarbon yields were 0.66 gX mol photon $^{-1}$ and 0.033 gHC mol photon $^{-1}$, obtaining in the specific light supply rate 0.35 mol photon gX^{-1} d^{-1}. The yields of biomass and hydrocarbon decreased when the specific light supply rate over 0.42 mol photon gX^{-1} d^{-1}, indicating the saturation of light supply for biomass and hydrocarbon production. Moreover, the hydrocarbon specific production rate also seemed to be saturated at specific light supply rate over 0.42 mol photon gX^{-1} d^{-1} (**Figure 4-9**). Therefore, controlling a specific light supply rate in the range of 0.28 mol photon gX^{-1} d^{-1} to 0.42 mol photon gX^{-1} d^{-1} could be effective for biomass and hydrocarbon production.

4.3.7. Hydrocarbon and intracellular lipids production on specific light supply rate

The efficiency of hydrocarbon accumulation seems to be strain and environmental conditions dependent as hydrocarbon contents of *B. braunii* race A have been reported in a large range from 1% to 61% of dry biomass (Jin et al., 2015).

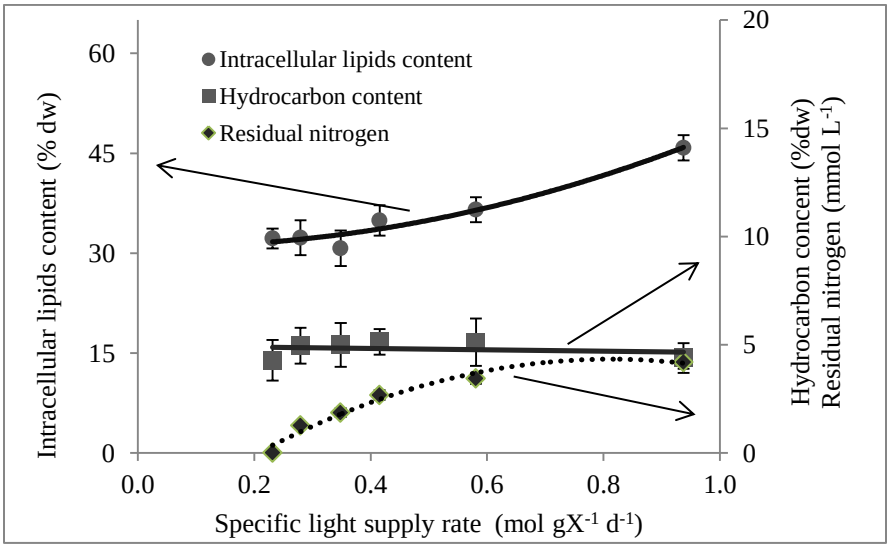


Figure 4-10. The intracellular lipids, hydrocarbon content of biomass and the residual nitrogen concentration as a function of specific light supply rate at steady-states in *B. braunii* continuous cultures.

For photoautotrophic conditions, light is often a factor affecting the biosynthesis and storage of products in microalgae.Then another impact of the specific light supply rate could be on the hydrocarbon content and the intracellular lipids content of *B. braunii* biomass. However, according to the **Figure 4-10** hydrocarbon content seems to be relative constant, while the content of intracellular lipids significantly increases at high specific light supply rate conditions. Different factors can trigger lipid accumulation in other oleaginous microalgae, the most commonly studied are nutrients, mainly nitrogen, limitation or starvation (Liu et al., 2016). Only one report has observed that lipids content of four *Botryococcus* spp. strains increased from 5.7-25.8 % dw in nitrogen-rich condition to 14.7-35.9 % dw in nitrogen deficiency condition (Yeesang and Cheirsilp, 2011). However, it was not the case in our continuous conditions, because the increase of lipids content at high specific light supply rate was with more available nitrogen (**Figure 4-10**). Then, *B. braunii* may store in the form of lipid when excessive light is available. However, what is the real factor in the present, the increased nitrogen availability or the specific light supply rate, still needs to be investigated.

4.4. Conclusion

Working at six steady states of different dilution rates at fixed incident light supply gave access both to a range of nitrate availability and to a range of specific light supply, thanks to the dilution effect. Here the chemostat cultures was used to obtain preliminary results in order to characterize relations between nitrogen conditions, cell growth state and hydrocarbon production in *B. braunii*. In contrast to triacylglycerol accumulation in most of oleaginous microalgae at limited growth rate with nitrogen limitation, hydrocarbon content was relative constant irrespective of growth rates and nitrogen conditions. Hydrocarbon productivity was correlated linearly to biomass productivity as well as hydrocarbon specific production rate to specific growth rate.

Maintaining optimal environmental conditions enabling high specific growth rate could be meaningful for efficient hydrocarbon production in *B. braunii* culture. Indeed the optimal range of specific light supply rate was from 0.28 mol photon gX^{-1} d^{-1} to 0.42 mol photon gX^{-1} d^{-1} .The hydrocarbon specific production rate also seemed to be saturated at specific light supply rate over 0.42 mol photon gX^{-1} d^{-1}. Therefore, controlling a specific light supply rate in the range of

0.28 mol photon gX^{-1} d^{-1} to 0.42 mol gX^{-1} d^{-1} could be effective for biomass and hydrocarbon production. It seems that intracellular lipids overproduction in *B. braunii* occurred by excessive assimilation photon and then stored in the form of lipid at high specific light supply rate conditions. The present sudy shows that further studies will have to characterize the separate and mutual impact on hydrocarbon productivity of nitrogen availability and of specific light supply, for instance by using photobioreactors with a higher surface over volume ratio.

Using the photobioreactor configuration, the next step was to study the effect of increasing the intensity of the incident photon flux density.

CHAPTER V: Kinetics of *B. braunii* SAG 30.81 in chemostat exposure to high light density

5.1. Introduction

The colonial microalga *Botryococccus braunii*, which could biosynthesize high contents of hydrocarbon is attracting increasing attention for biofuels production. *B. braunii* could secrete hydrocarbons into the extracellular matrix and then accumulate hydrocarbon in the extracellular matrix (Jin et al., 2015). Strains of *B. braunii* are classified into four races (A, B, L and S) according to hydrocarbons structures they produced. Race A and race B strains are more abundant in nature and generally exhibit higher hydrocarbons contents than others (Li et al., 2013). Race A strains produce alkadienes and alkatrienes; race B strains produce triterpenoid hydrocarbons (Jin et al., 2015). There are advantages of using bio−hydrocarbon fuels, which could provide more energy with high caloric values and it is unnecessary to modify engines, pumps or distribution networks to accommodate the new renewable liquids in the transportation sector (Serrano-Ruiz and Dumesic, 2011).

However，*B. braunii* is generally regarded as a slow-growing microalga and the biomass production is relatively inefficient by comparison with kinetics obtained in cultures of species such as *Chlorella* sp. There is only a report of biomass concentration reaching 20 g L^{-1} in semi-continuous cultures (Khatri et al., 2014). How to improve biomass and hydrocarbon production of *B. braunii* is meaningful. Among all the environmental factors, irradiance reveals to be one of the main factors controlling the productivity of bioprocess involving photosynthetic microorganisms. Generally, low light intensity causes a reduction in dry weight, whereas too high light intensities ccould induce transient photo-inhibition or, according to the physiological state of the culture the death of the cell culture (Scott et al., 2010). Already some studies have investigated some effects of irradiance on *B. brauni.* Nevertheless, there was no consistent conclusion on the effect of irradiance on this colonial alga. Some studies have shown that *B. braunii* can be photo-inhibited at high irradiance and seemed to grow faster only at relatively low irradiance conditions. For example, growth of *B. braunii* BOT-22 was shown to be saturated at low irradiance condition 100 μmol photon m^{-2} s^{-1} (Sakamoto et al., 2012). The growth rate of cultures of the CHN *B. braunii* strain was higher at 60 W m^{-2} than at 300 W m^{-2} (Qin and Li, 2006). Higher biomass concentrations were obtained in cultures of *B. braunii* KMITL 2 exposed to 87.5 μmol photon

m^{-2} s^{-1} than to 200 and 538 μmol photon m^{-2} s^{-1} (Ruangsomboon, 2012). In contrast, other studies have shown that *B. braunii* could keep high growth rate at very high light conditions without much photo-inhibition. For instance, the growth of two *B. braunii* strains UK and JAP are not significantly affected by irradiance in the range of 60-300 W m^{-2} (Qin and Li, 2006).The reported maximum specific growth rate 0.5 day^{-1} of *B. braunii* is obtained at relative high irradiance condition with 850 μmol photon m^{-2} s^{-1} and only slightly decreased maintaining at 95% of the maximum even at 1770 μmol photon m^{-2} s^{-1} (Yoshimura et al., 2013).

It is worth to note that most of previous studies of irradiance on *B. braunii* mainly focused on biomass or lipids, but not on hydrocarbon production. Especially, to our knowledge, it seems no target works on race A strains showing kinetic process of photo-inhibition or photo-adaptation, increasing or decreasing biomass and hydrocarbon production in *B. braunii* culture at high light conditions. In addition, most of reported studies were carried out in batch cultures, where secondary growth and stress effects can often mask subtle physiological differences and trends (Hoskisson, 2005).

Therefore, in the present study, a race A strain of *B. braunii* SAG 30.81 was cultivated in a torus photobioreacotr in continuous mode at fixed dilution rate. Two conditions were chosen to test the irradiance influences on *B. braunii* cells, including a low irradiance condition 150 μmol photon m^{-2} s^{-1} and a high irradiance condition 900 μmol photon m^{-2} s^{-1}. The kinetic data at steady states and transient state well demonstrated the process of photo-adaptation, hydrocarbon and lipids production in *B. braunii* chemostat exposure to high light density.

5.2. Experiment design and setup

Experiment was designed as maintaining continuous cultures in torus photobioreactor as shown in **Figure 5-1.**

Experiments were conducted with the strain *B. braunii* Kützing SAG 30.81. The continuous culture was conducted in the torus photobioreactor (PBR) system as described in (Pruvost et al., 2006). The speed of the axial flow impellers was 200 rpm. Temperature and pH were monitored with sensor (Mettler Toledo SG 3253) with temperature at 23 ± 1 ºC and pH at

6.5 by automatic CO_2 injection. Gas flow rate was 450 mL min^{-1} corresponding to an aeration rate of 0.32 vvm. Cultures were run in continuous mode feeding with 3N6P-AF-6 at fixed dilution rate 0.23 d^{-1}.

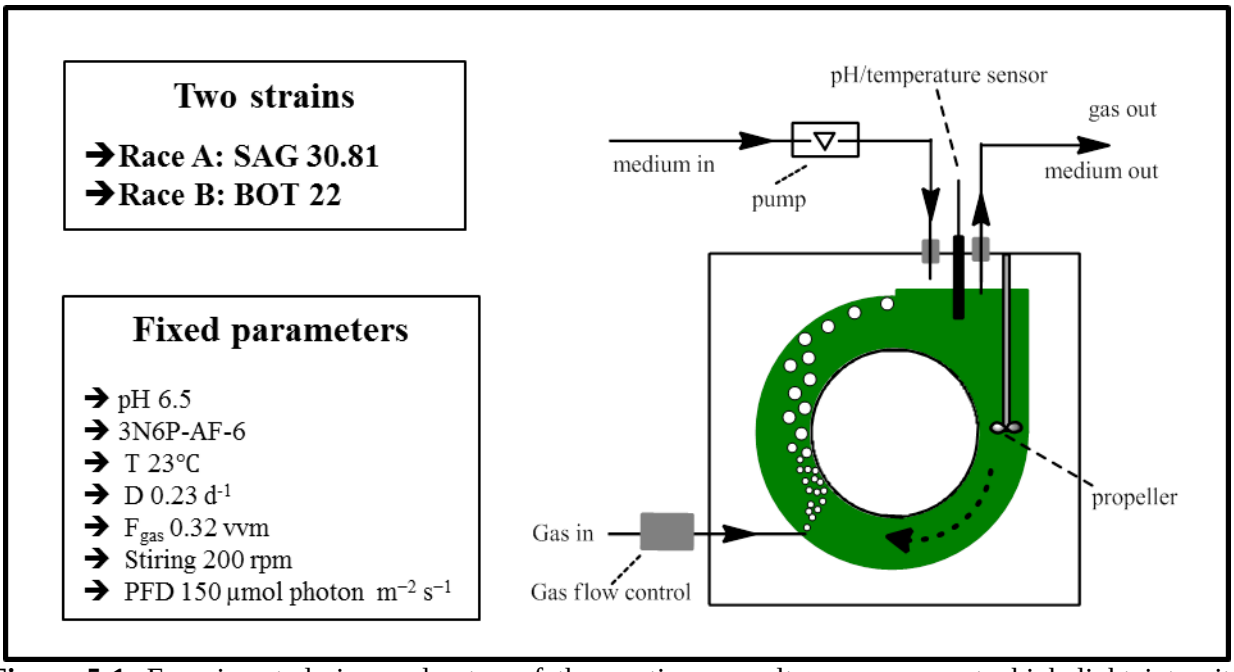


Figure 5-1. Experiment design and setup of the continuous cultures exposure to high light intensity condition in torus photobioreactor.

A light-emitting diode (LED) panel composed of 1,500 white light LEDs (NSPW500CS, NICHIA) was used to illuminate the PBR from the front. The PFD was measured using a plane quantum sensor Li-Cor light meter (LI−250A), which measures photosynthetically active radiation (PAR) in the 400–700 nm wavebands. The mean value was the average of measurements made at eight different locations on the reactor front. The irradiance firstly set at 150 µmol photon m^{-2} s^{-1}, keeping at steady state about 10 days, and then changed to be 900 µmol photon m^{-2} s^{-1}.

5.3. Results and discussion

In order to highlight the effect of irradiance on *B. braunii*, a continuous culture was started in a 1.4 L photobioreactor by only changing light condition at the surface of photobioreactor from 150 µmol photon m^{-2} s^{-1} to 900 µmol photon m^{-2} s^{-1}. Environmental

conditions were kept in constant during all the continuous cultivation, including temperature (23 °C), pH (6.5), stirring (200 rpm), aeration rate (0.32 vvm) and a fixed dilution rate 0.23 d^{-1}.

5.3.1 Biomass, hydrocarbon and intracellular lipids production

The intensity of photosynthetic active radiations (PAR) is considered as one of the major factors controlling the biomass production of photosynthetic microalgae. As shown in **Figure 5-2**, increasing light supply induce an increase of the biomass concentration in photobioreactor. The biomass concentration was only 31.8 g m^{-2} at steady state at 150 µmol photon m^{-2} s^{-1} condition, while the cell growth was stimulated during a transient period after changing the light to 900 µmol photon m^{-2} s^{-1} to reach a biomass concentration of 63.3 g m^{-2} at steady state.

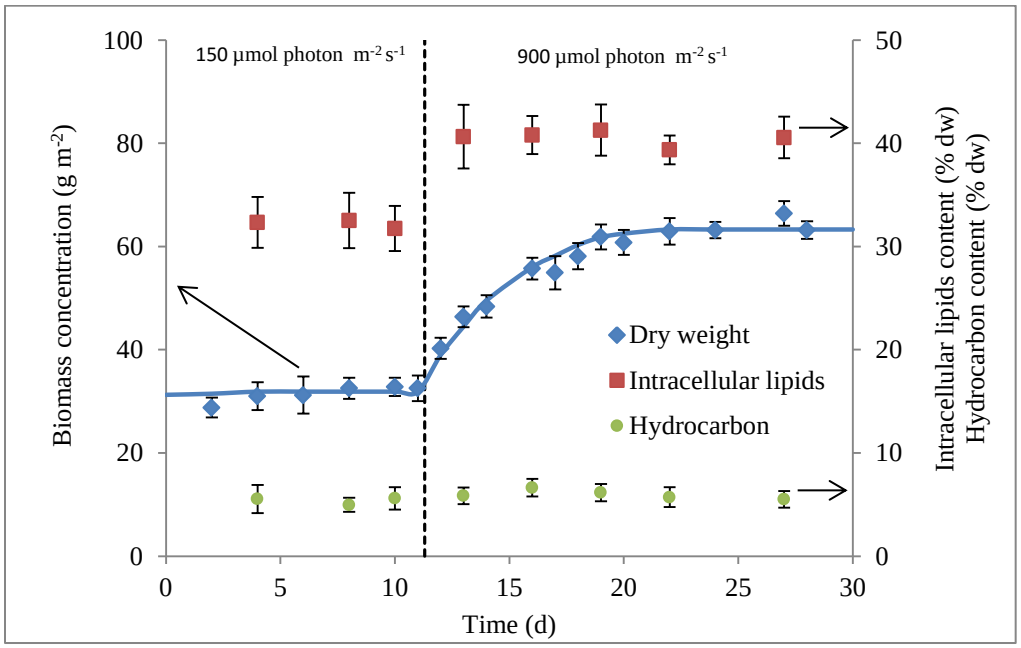


Figure 5-2. Biomass concentration, hydrocarbon content and intracellular lipid content of biomass as a function of time in *B. braunii* continuous cultures from 150 µmol photon m^{-2} s^{-1} to 900 µmol photon m^{-2} s^{-1}; irradiance increased at the time of dash line.

However, from previous studies, it seems there is ambiguity on the effect on cell growth and biomass production in the culture of this colonial alga by exposure to high irradiance. The *B. braunii* race B strain Showa shows a maximum specific growth rate 0.5 d^{-1} at relative high irradiance condition with 850 µmol photon m^{-2} s^{-1} (Yoshimura et al., 2013), while another race B

strain BOT-22 is saturated at low irradiance condition with only 100 µmol m^{-2} s^{-1} (Sakamoto et al., 2012). The UK and Japanese strains were not significantly affected by irradiance in the range of 60-300 W m^{-2} (equal to 275- 1377 µmol m^{-2} s^{-1}), while the CHN strain grew fastest at 60 W m^{-2} (275µmol photon m^{-2} s^{-1}) and slowest at 300 W m^{-2} (1377 µmol photon m^{-2} s^{-1}) (Qin and Li, 2006) and cultures of *B. braunii* KMITL 2 exposed to low light intensity (87.5 µmol photon m^{-2} s^{-1}) showed a higher biomass compared to others irradiance conditions at 200 or 538 µmol photon m^{-2} s^{-1} (Ruangsomboon, 2012). The reasons for *these apparent contradictory results* are not clear; they could be attributed to a strain effect. At steady states in the present continuous culture of *B. braunii* SAG 30.81, the biomass productivity significantly increased from 7.3 g m^{-2} d^{-1} at 150 µmol photon m^{-2} s^{-1} to 14.6 g m^{-2} d^{-1} at 900 µmol photon m^{-2} s^{-1} ($P < 0.05$, **Figure 5-3**) .

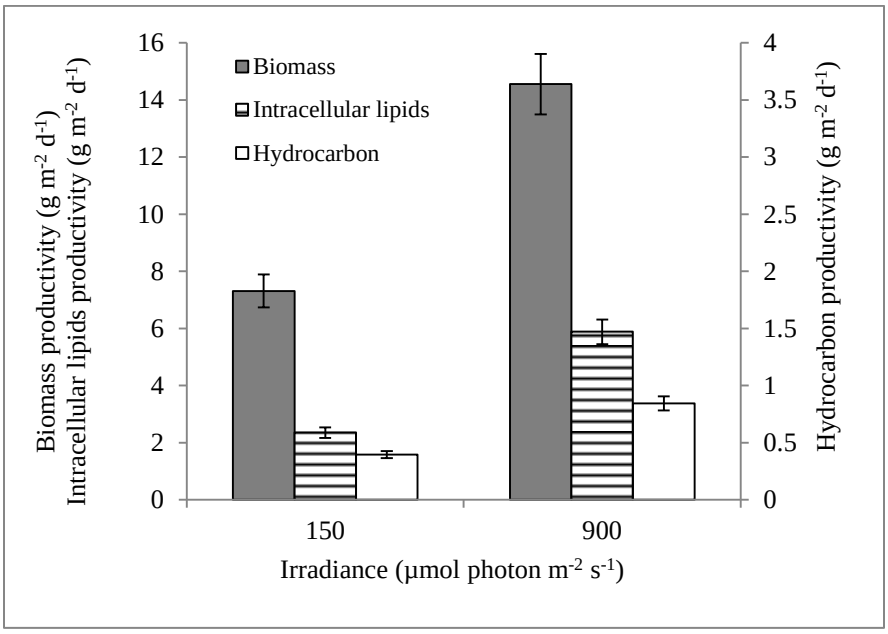


Figure 5-3. The productivity of biomass, intracellular lipids and hydrocarbon at steady states in *B. braunii* continuous cultures supplying with 150 µmol photon m^{-2} s^{-1} or 900 µmol photon m^{-2} s^{-1}.

The microalga *B. braunii* race A strain is well known as producer of alkadiene and alkatriene hydrocarbons (Jin et al., 2015). However, it seems there are few studies on how irradiance affects the hydrocarbon biosynthesis and production in race A. Kojima and Zhang (Kojima and Zhang, 1999) tested a race B strain in a bubble column cultivated under different irradiance conditions ; whatever the value of irradiance, the hydrocarbon content in this race B

strain was constant. Another quantitative analysis of *B. braunii* BOT-22 (race B) showed that the hydrocarbons contents were approximately at 27 % and nearly constant in a large range of light intensity of 20, 40, 100 and 200 µmol photon m^{-2} s^{-1} (Sakamoto et al., 2012). Our results in *B. braunii* race A strain also demonstrated that the hydrocarbon content was relatively constant irrespective to light conditions, from the steady state of 150 µmol photon m^{-2} s^{-1} to the transient state and steady state of 900 µmol photon m^{-2} s^{-1} (**Figure 5-2**), showing that hydrocarbon contents in race A and race B strains have the same behaviour. The large range of hydrocarbon contents among *B. braunii* race A strains, from 1% to 61% of dry biomass, seems to be attributed to the reliability of some analytical methods, the efficiency of cultivation conditions or the characteristics of the working strains (Jin et al., 2015). Hexane extracts are generally regarded as hydrocarbon, but actually contains other lipids, such as triacylglycerol and aldehydes, and hydrocarbon is only 20.4 % of hexane extract as reported in a Austin strain of *B. Braunii* (Metzger and Casadevall, 1989). Hexane extracts of the present strain SAG 30.81 were in the range from 15 % to 50.6 % dw ever measured by Dayananda et al.(Dayananda et al., 2005). In order to further quantify the exact hydrocarbon content of this strain, hydrocarbon was purified by silica chromatography in present study. Even though steady-state hydrocarbon productivity increased from 0.39 g m^{-2} d^{-1} at 150 µmol photon m^{-2} s^{-1} to 0.84 g m^{-2} d^{-1} at 900 µmol photon m^{-2} s^{-1} (**Figure 5-3**), hydrocarbon content kept a constant value of ca 5% on the basis of the dry biomass. Then, it seems unlikely to improve hydrocarbon content by only regulating light intensity. Other environmental factors control or metabolic manipulation, therefore, would be essential for improvement of hydrocarbon biosynthesis in this race A strain.

In addition to hydrocarbon, lipids of microalgae are also the crucial factors in making algal biofuels economically viable (Griffiths and Harrison, 2009). Light is an usual factor affecting the lipid accumulation in microalgae cell. As shown in **Figure 5-2**, the content of intracellular lipid increased from 32.2 % dw to 40.4 % dw in two days after changing the irradiance from 150 µmol photon m^{-2} s^{-1} to at 900 µmol photon m^{-2} s^{-1}, and kept this relative high level from transient state to steady state at high irradiance condition. The steady-state intracellular lipids productivity at 900 µmol photon m^{-2} s^{-1} was also significantly higher than that at 150 µmol photon m^{-2} s^{-1} ($P < 0.05$, **Figure 5-3**). Except a few strains, such as CHN and UK strains, which produced a higher level of lipids at low light condition than at high light condition

(Qin and Li, 2006), it appears that most of *B. Braunii* strains could produce more lipids at high irradiance condition. For example, the highest lipid yield of *B. braunii* KMITL 2 was also obtained by cultivation at 538 µmol photon m^{-2} s^{-1} comparing with 87.5 µmol photon m^{-2} s^{-1} and 200 µmol photon m^{-2} s^{-1} (Ruangsomboon, 2012). In addition, fluorescence microscopic observations of Nile Red-stained *B. braunii* BOT-22 showed that the number of intracellular lipid bodies increased at high light intensities (Sakamoto et al., 2012). Therefore, increasing irradiance supply could stimulate lipids biosynthesis and enhance the lipids production in *B. braunii.*

5.3.2. Supply and consumption of nitrogen and phosphorus

Nitrogen and phosphorus are among the main components of the cultures media used for growing microalgae, as well as the most effective factors to regulate the secondary metabolism in microalgal cells (Cheng et al., 2014). Their residual concentrations were monitored through the continuous culture.

As shown in **Figure 5-4**, nitrogen and phosphorus concentrations were at relative high level at steady state of 150 µmol photon m^{-2} s^{-1}. After changing the light to 900 µmol photon m^{-2} s^{-1}, their residual concentrations decreased gradually, and eventually presented at very low concentrations, to the limit of quantification of ion chromatography. Then, at the steady state of 900 µmol photon m^{-2} s^{-1}, nitrogen and phosphorus could be the limit factor for biomass production.

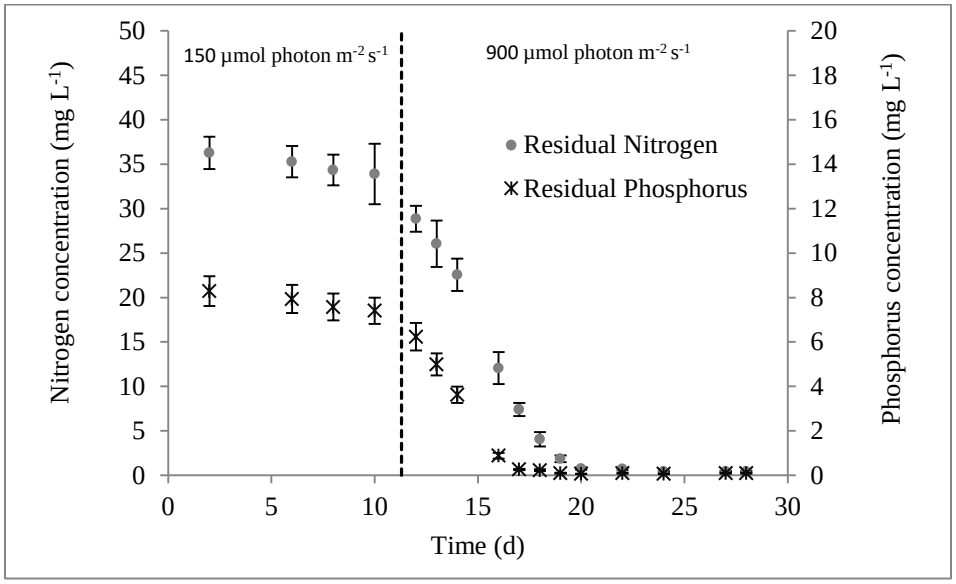


Figure 5-4. The residual nitrogen and phosphorus concentration as a function of time in *B. braunii* continuous cultures from 150 µmol photon m^{-2} s^{-1} to 900 µmol photon m^{-2} s^{-1}; irradiance increased at the time where dash line is.

Average nitrogen and phosphorus contents in biomass were calculated during steady−states of the continuous culture, as well as their yields for biomass production (**Table 5-1**). There was no significant difference of nitrogen content of biomass at low or high irradiance conditions, in consistent with a previous investigation showing a 4.1 % dw of *B. braunii* SAG 30.81 (Sydney et al., 2010a).

Table 5-1
Steady state N and P content of biomass and biomass yield from N and P in continuous cultures of *B. braunii* at different light conditions.

PFD (µmol photon m^{2-} s^{-l})	N content (% dw)	P content (% dw)	$Y_{X/N}$ ($gX\ gN^{-1}$)	$Y_{X/P}$ ($gX\ gP^{-1}$)
150	4.43±0.40	1.41±0.13	22.59±1.92	70.73±6.25
900	4.41±0.35	1.21±0.09	22.68±2.09	82.39±7.67

Means ± SD. N = nitrogen; P = phosphorus.

The nitrogen yield for biomass production also was not so much affected by irradiance. However, the phosphorus content in biomass was higher at 150 µmol photon m^{-2} s^{-1} than that at

900 µmol photon m^{-2} s^{-1}, corresponding to a higher yield for biomass at high irradiance condition. This could be interpreted by a luxury phosphate uptake process, with intracellular accumulation of polyphosphate as an intracellular nutrient pool in *B. braunii* (Casadevall et al., 1985). The luxury phosphorus uptake process resulted in an increase in the ratio of P/N and P/X. This change in N- and P-biomass ratios was observed by other authors, also in the case of the microalga *Scenedesmus* (Martínez Sancho et al., 1999), where an increased P/X ratio at high-growth rate was found, suggesting a higher internal P content as a result of increasing cellular nucleic acids and high energy compounds under high growth- rate conditions (Sforza et al., 2014).

5.3.3. Is there photo-acclimation during the transient period?

As *B. braunii* was cultivated in photoautotrophic conditions, photosynthesis is essential for providing energy and reduced carbon for biomass and hydrocarbon production. The value of Fv/Fm, maximum photochemical quantum yield of PS II, is commonly used for evaluating the impact of stress on microalgae, such as nutrient or photo-inhibition stresses. For example, the value of Fv/Fm decreased from 0.84 to 0.67 in microalgae *Chlorella* sp. culture with nutrient stress (White et al., 2011) and decreased from 0.67 to 0.40 in microalgae *Isochrysis zhangjiangensis* culture at nitrate depletion conditions (Feng et al., 2011).

As shown in **Figure 5-5**, steady-state Fv/Fm at 150 µmol photon m^{-2} s^{-1} is 0.745, and decreased to be 0.707 only one day after increasing the light to 900 µmol photon m^{-2} s^{-1}. This decreasing in Fv/Fm could not be due to nutrient starvation, because the culture was in continuous mode and residual nutrients were still remained. Then, the probably reason could be slight photo-inhibition in a short time exposure to high light density. Surprisingly, during the transient state at 900 µmol photon m^{-2} s^{-1}, the Fv/Fm did not continue to decrease by photo-inhibition, but seems to be recovered. The steady-state value of Fv/Fm at 900 µmol photon m^{-2} s^{-1} increased gradually from 0.707 to be 0.725. Then，it seems the cells finally photoadapted to the high irradiance and were not seriously stressed by photo-inhibition. In addition, the values of ETR_{max} in the range of 40 to 70 without significant difference in present studies through all the cultivation.

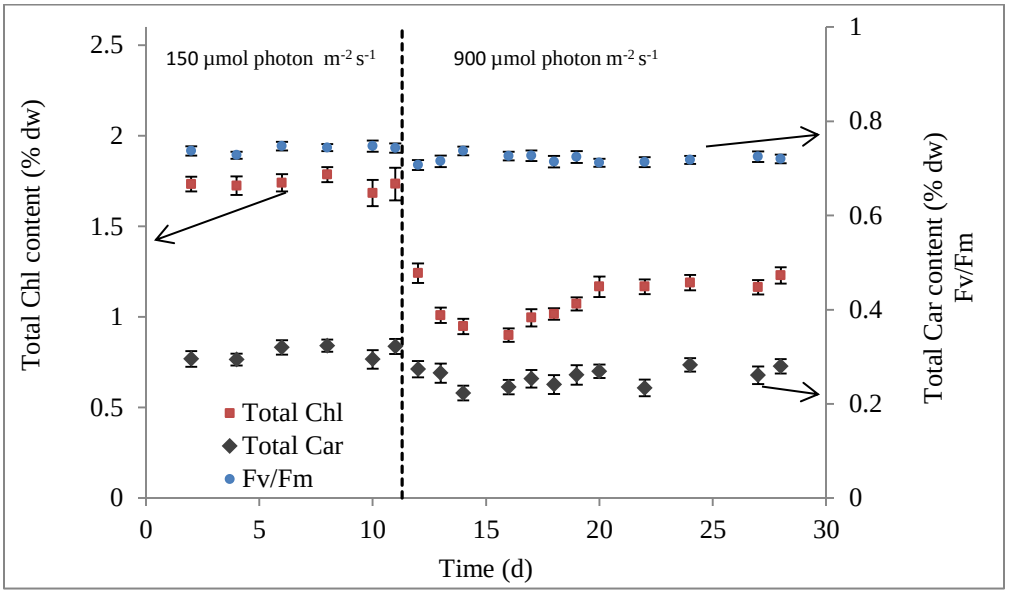


Figure 5-5. The total Chl, Car content of biomass and Fv/Fm as a function of time in *B. braunii* continuous cultures from 150 µmol photon m^{-2} s^{-1} to 900 µmol photon m^{-2} s^{-1}; Chl, chlorophyll; Car, carotenoids; Fv/Fm, maximum photochemical quantum yield of PSII; irradiance increased at the time of dash line.

Another indicator of the physiological state of microalgal cell is the content in photosynthetic pigments. **Figure 5-5** shows that both chlorophyll and carotenoids contents decreased significantly after increasing the irradiance in a short time, and thereafter showed the tendency to recover as Fv/Fm. A further analysis of steady-state average pigments composition showed that pigments content is higher at low irradiance than that at high irradiance condition (**Table 5-2**).

Table 5-2
Steady state pigments composition of biomass in continuous cultures of *B. braunii* at different light conditions.

PFD (µmol photon m^{2-} s^{-1})	Total Chl (% dw)	Chl *a* / Chl *b* (mol / mol)	Total Car (% dw)	Car / Chl (w / w)
150	1.73 ± 0.08	2.1 ± 0.18	0.31 ± 0.02	0.18 ± 0.02
900	1.18 ± 0.06	2.0 ± 0.12	0.26 ± 0.02	0.23 ± 0.02

Means ± SD. Chl = chlorophylls; Car = carotenoids.

The average chlorophyll and carotenoids contents at 150 µmol photon m^{-2} s^{-1} were 1.73 % dw and 0.31 % dw, respectively, while that at 900 µmol photon m^{-2} s^{-1} were decreased only to be 1.18 % dw and 0.26 % dw, respectively. As shown in **Table 5-2**, the ratio of Chl *a* / Chl *b* was not significantly affected by irradiance, while the ratio of Car / Chl slightly increased from 0.18 to 0.23 from low irradiance to high irradiance. Comparing with pigments data in previous studies in *B. braunii*, it seems the pigments contents were in the same range of values. Accordingly it seems that the microalgae were not stressed. For instance, colonies of *B. braunii* Bot 22 changed color from green to yellowish without obvious photo-inhibition when the light intensity was increased from low to high (Sakamoto et al., 2012). For chlorophyll, relative low values of content from 0.38 % dw to 0.86% dw (Eroglu et al., 2011) and 1.16% dw (Ranga Rao et al., 2007) were obtained. For carotenoids, relative large diversity of content from 0.10 % dw to 0.54 % dw were measured (Eroglu et al., 2011). Also, high pigments content of 13.05 % dw were observed (Sydney et al., 2010a). The Chl *a*/Chl *b* values of microalgae are generally between 2 and 3 and the Car/Chl ratio of *B. branunii* could differ from 0.15 to 0.24, according to cultivation conditions (Ranga Rao et al., 2007; Eroglu et al., 2011). Then, it appears that the cells of *B. braunii* in present continuous culture were photo-acclimated when exposed to the high light conditions.

It worth to note that high photon flux densities could inhibit the growth of microalgae cultures (Scott et al., 2010). Such process depends of the incident light supply but also of the level and of the physiological state of the cell population. When working in chemostat cultures, the transient step before reaching a new steady state after a 6-fold increase of incident PFD, from 150 to 900 µmol photon m^{-2} s^{-1}, could be interpretated as a mechanism of photo-adaptation. Indeed different strategies could be involved: increasing biomass concentration or colony size to promote self-shading or converting excess light to chemical energy to avoid photo-oxidative damage. In the first case, light attenuation increased in the microalgae culture, taking into consideration the increase of biomass concentration after changing irradiance condition (**Figure 5-2).** Then, the number of photons available for each microalgal cell actually decreased with the increase in biomass concentration. In the second case, cells of *B. braunii* aggregate together forming colonies enclosed by “retaining wall” composed of polysaccharides and polymerized hydrocarbons (Weiss et al., 2012). Colonies could be enlarged at high irradiance conditions due

to neoformation of extracellular matrix material at high light intensity (Zhang and Kojima, 1998), resulting in another way of selfshading from excess photon fluxes within large cell colonies. In the third case, *B. braunii* may excessively assimilate photons and then store the excess energy in the form of lipids (Solovchenko et al., 2008). This opinion is supported by fast increase in intracellular lipids contents after switching from 150 to 900 µmol photon m^{-2} s^{-1}.

5.3.4. Fatty acids profile and productivity

The fatty acid profiles indicated mainly the presence of C13:0, C16:0, C16:1, C18:1, C18:2(n-6), C 18:3(n-3), C 22:0 and C 24:1 at both steady states of 150 µmol photon m^{-2} s^{-1} and 900 µmol photon m^{-2} s^{-1} conditions (**Table 5 -3**).

Table 5-3
Steady state fatty acids profile in continuous cultures of *B. braunii* at different light conditions.

Fatty acids	Common name	150 µmol photon m^{-2} s^{-1} (% total fatty acids)	900 µmol photon m^{-2} s^{-1} (% total fatty acids)
Total		12.3 ± 0.58 *	15.9 ± 0.66 *
C 13:0	Tridecanoic acid	0.88 ± 0.06	0.46 ± 0.05
C 16:0	Palmitic acid	13.00 ± 0.32	9.39 ± 0.14
C 16:1	Palmitoleic acid	1.79 ± 0.15	1.42 ± 0.09
C 18:1	Oleic acid	61.34 ± 4.40	68.91 ± 1.59
C 18:2(n-6)	Linoleic acid	1.42 ± 0.09	0.90 ± 0.10
C 18:3(n-3)	α-Linolenic acid	13.31 ± 0.60	10.03 ± 0.59
C 22:0	Behenic acid	3.59 ± 0.12	2.95 ± 0.21
C 24:1	Nervonic acid	4.55 ± 4.13	5.51 ± 0.29

*, total fatty acids content of dry biomass (% dw); Means ± SD.

Among the fatty acids, the oleic acid was estimated as the main component. Palmitic acid and α-Linolenic acid took up about 10% of total fatty acids, whereas tridecanoic acid, palmitoleic acid, linoleic acid, behenic acid and nervonic acid were existed as minor fatty acids. The carbon number usually is even number, but also the odd number carbon fatty acids C13:0 could be found in present study, in consistent with another *B. braunii* KMITL strain with minor odd number carbon fatty acids , such as C11:0, C13:0, C15:0, C17:0 (Ruangsomboon, 2015). Such odd number carbon fatty acids could indicate the prence of bacteria, such as the recently described endosymbiont bacteria found in the matrix of *B. braunii*. Most of microalgae showed a peak for oleic acid (18:1) in the range 15 to 24 % dw (Talebi et al., 2013). Oleic acid is also the precursor of the non-isoprenoid hydrocarbons produced by race A strain of *B. braunii* and involved in the formation of very long fatty acid derivatives through chain elongation (Baba et al., 2012a; Ioki et al., 2012a). The microalga *B. braunii* is known to produce high content of oleic acid as the main component of fatty acids, reaching 84 % of total fatty acids (Choi et al., 2011), except a strain *B. braunii* KMITL which mainly produce palmitic acid (Ruangsomboon, 2015).

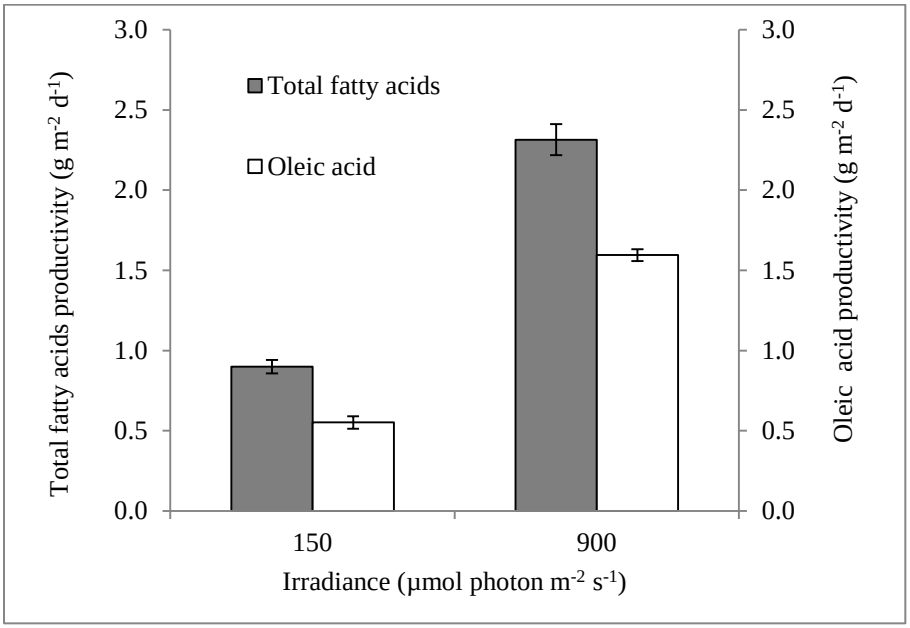


Figure 5-6. The productivity of total fatty acids and oleic acid at steady states in *B. braunii* continuous cultures supplying with 150 µmol photon m^{-2} s^{-1} or 900 µmol photon m^{-2} s^{-1}.

The biosynthesis of oleic acid is dependent of the cultivation conditions. It seemed to be stimulated by increasing irradiance. The proportion of oleic acid in the present strain SAG 30.81 with values of 61.34 or 68.91 % is similar to those in the strain LB 572 with 59.55 % to 81.79 % (Cabanelas et al., 2015), but higher than the strain LB 807/1 with 21.8 % to 46.0 % (Kalacheva et al., 2002) and strains from Thailand with 28.51 % to 37.68 % (Yeesang and Cheirsilp, 2011).

In addition to hydrocarbon, fatty acids in *B. braunii* could be used to produce biodiesel by transesterification. The quality of biodiesel is considerably affected by the composition of the fatty acids in the biodiesel (Knothe, 2008). Generally, cetane number, heat of combustion and viscosity of biodiesel increase with increasing fatty acid chain length, meaning that the long chain fatty acid, oleic acid, is more preferable as a biodiesel fuel (Francisco et al., 2010). For the content in biomass, both total fatty acids and oleic acid were increased supplying with high irradiance instead of low irradiance (**Table 5-3**). The increasing of the content of fatty acids is in consistent with the increasing of intracellular lipids content at higher irradiance. Moreover, **Figure 5-6** shows that the productivity of total fatty acids and oleic acid significantly increase from 0.90 g m^{-2} d^{-1} and 0.55 g m^{-2} d^{-1} at 150 µmol photon m^{-2} s^{-1}, respectively, to 2.31 g m^{-2} d^{-1} and 1.60 g m^{-2} d^{-1} at 900 µmol photon m^{-2} s^{-1}, respectively ($P < 0.05$). Therefore, supplying high irradiance to *B. braunii* could be an appropriate method for the production of excellent quality biodiesel, due to the high fatty acids content and oleic acid proportion.

5.3.5. Biomass and hydrocarbon yields on specific light supply rate

In order to confirm the characteristics of biomass, hydrocarbon and intracellular lipids production of the strain *B. braunii*, the analysis of biomass and hydrocarbon yields on light energy was carried out at this set of chemostat fixed at a dilution rate of 0.23 d^{-1} with changing light intensity at PBR surface from 150 µmol photon m^{-2} s^{-1} to 900 µmol photon m^{-2} s^{-1}. As shown in **Table 5-4**, at steady state, the biomass concentration reached 63.3 g m^{-2} at 900 µmol photon m^{-2} s^{-1}, which was significantly higher than that at 150 µmol photon m^{-2} s^{-1} with 31.8 g m^{-2}. Then, according to the calculation equation of specific light supply rate, two conditions with different specific light supply rates were obtained. With 150 µmol photon m^{-2} s^{-1} at the photobioreactor surface, the specific light supply rate was 0.4 mol photon gX^{-1} d^{-1}, while

increasing the light intensity with 900 µmol photon m^{-2} s^{-1} at the photobioreactor surface, the specific light supply rate increased to be 1.2 mol photon gX^{-1} d^{-1} (**Table 5-4**).

Table 5-4
The biomass concentration and specific light supply rate at 150 and 900 µmol photon m^{-2} s^{-1} conditions at steady-states in *B. braunii* continuous cultures.

PFD (µmol photon m^{-2} s^{-1})	Biomass concentration ($gX\ m^{-2}$)	Specific light supply rate (mol photon gX^{-1} d^{-1})
150	31.8 ±2.5	0.4±0.03
900	63.3 ±4.6	1.2±0.09

Means ± SD.

Then, as shown in **Figure 5-7**, both biomass and hydrocarbon yields decreased at very high specific light supply rate 1.2 mol photon gX^{-1} d^{-1}. Therefore, the characteristic of biomass and hydrocarbon yields has been confirmed at fixed dilution rate with increasing the light supply at photobioreactor surface.

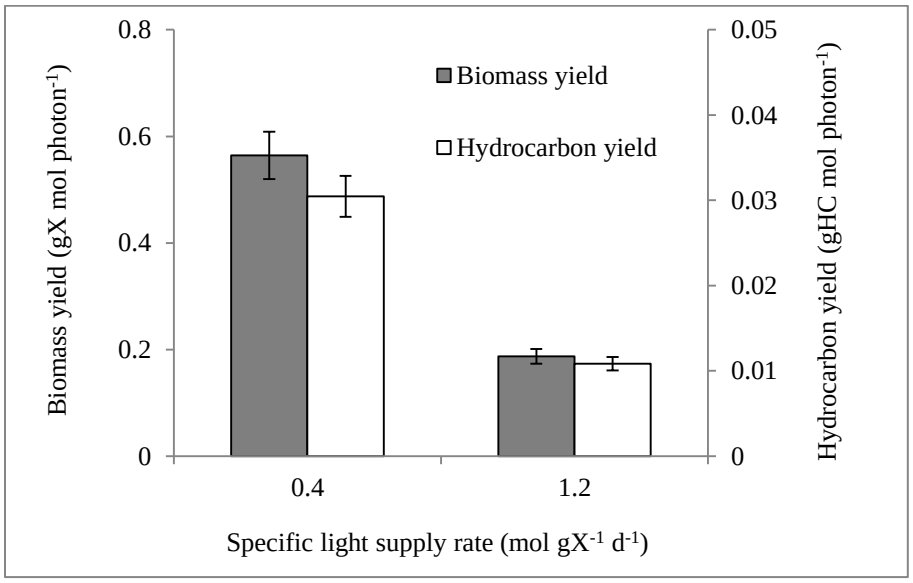


Figure 5-7. The biomass and hydrocarbon yields on light at two specific light supply conditions at steady-states in *B. braunii* continuous cultures; dilution rate fixed at 0.23 d^{-1} and light intensity at the photobioreactor surface changed from 150 to 900 µmol photon m^{-2} s^{-1}.

As a result, cultivating microalgae at very high light intensity is inefficient for light utilization resulting in low biomass and hydrocarbon yields. Triglyceride yield on light were carried out in *Nannochloropsis* sp. and the triglyceride yield also could be decreased when light supply increased to a high value (Benvenuti et al., 2016).

5.3.6. Hydrocarbon and intracellular lipids production on specific light supply rate

During the kinetic processes of *B. braunii* cultures exposed to high light intensity, the pigments contents also decreased at high specific light supply rate conditions, just as the previous investigation by increasing dilution rate. At the same time, Fv/Fm still above 7.2 during all the present run continuous culture indicated no significant photo-inhibition occurred to the cells.

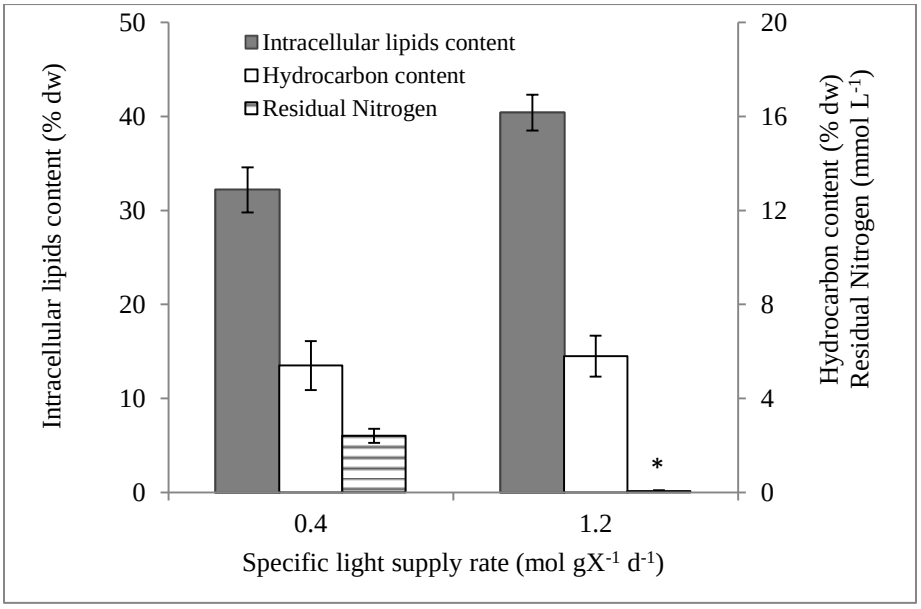


Figure 5-8. The intracellular lipids and hydrocarbon content on light at two specific light supply conditions at steady-states in *B. braunii* continuous cultures; dilution rate fixed at 0.23 d^{-1} and light intensity at the photobioreactor surface changed from 150 to 900 µmol photon m^{-2} s^{-1}.

In addition, the hydrocarbon content and intracellular lipids content also has been confirmed as hydrocarbon content kept relative constant but intracellular lipids content increased significantly at high specific light supply rate condition (**Figure 5-8**). It is worth to note that the residual nitrogen concentration decreased at the high specific light supply rate condition, where

the intracellular lipids overproduced. This is contrary to the previous results obtained in continuous culture with fixed light intensity at photobioreactor surface but changing dilution rate that intracellular lipids overproduced at high light supply rate but nitrogen sufficient condition. Then, it seems the overproduction of intracellular lipids is not influenced by nitrogen condition, but affected by specific light supply rate. The intracellular lipids content increased at high specific light rate whatever the residual sufficient or limited nitrogen condition. Condition of high light intensity was also shown to give the benefit of higher lipids content in another *B. braunii* strain KMITL 2 (Ruangsomboon, 2012). This kind of conversion in microalgae could be the means to convert excess light to chemical energy, in order to avoid photooxidative damage (Solovchenko et al., 2008). Further investigations on kinetics of photoadaptation in *B. braunii* cultures and high irradiances effects on hydrocarbon and total lipids production could be meaningful.

5.4. Conclusion

In order to reveal the characteristics of photoaclimatation process and how irradiance affect hydrocarbon and intracellular lipids production, kinetics investigation was carried out in continuous culture of a *B. braunii* race A strain SAG 30.81 by exposure from 150 μmol photon m^{-2} s^{-1} to 900 μmol photon m^{-2} s^{-1}. High irradiance could stimulate the growth and improve the productivity of biomass, hydrocarbon and lipids. Photoadaptation process was shown as a decrease in a short time of Fv/Fm and the content of chlorophyll and carotenoids exposed to high irradiance, but gradually to be recovered. Total fatty acids and oleic acid proportion and productivity were also increased by exposure to high light. Then, supplying high irradiance to the culture of *B. braunii* could be appropriate for the exploration in this hydrocarbon-rich microalga for biodiesel fuel application.

The characteristic of biomass and hydrocarbon yields obtained in previous chapter has been confirmed in present study at fixed the dilution rate with increasing the light supply at photobioreactor surface. As a result, cultivating microalgae at very high light intensity is

unfavorable because of inefficient light utilization resulting in low biomass and hydrocarbon yields.

In addition, when the hydrocarbon content was confirmed to be relatively independent of the specific light supply, the intracellular lipids content increased significantly at high specific light supply rate condition. The overproduction of intracellular lipids is not influenced by nitrogen condition, but seemed more affected by specific light supply rate. This kind of conversion in microalgae could be the means to convert excess light to chemical energy, in order to avoid photooxidative damage.

CHAPTER VI: A comparative study between *B.braunii* race A and race B at steady states in chemostats

6.1. Introduction

The production of hydrocarbons by the colonial microalga *Botryococccus braunii* is a potential application as biodiesel or biojetfuel. The strains of the species *B. braunii* are classified into four races (A, B, L and S) according to the chemical structures of the liquid hydrocarbons they accumulated in an extracellular matrix (Jin et al., 2015). Race A strains produce alkadienes and alkatrienes; race B strains produce triterpenoid hydrocarbons and race L strains produce a single tetraterpenoid hydrocarbon. Recently, new strains named as race S have been also proposed, which can produce much shorter carbon chains than race A and even-numbered carbon atoms (Kawachi et al., 2012).

Race A and race B strains are more abundant in nature and generally exhibit higher hydrocarbons contents than others. Hydrocarbon content in race A strain could be in the range from 0.1% to 61 %, whereas race B strain from 9.1 % to 39 % (Jin et al., 2015). Their respective hydrocarbons biosynthesis are also related to different pathways, elongation from oleic acid and then converting to hydrocarbons in race A, while triterpene synthesis pathway via MEP and then methylating for higher hydrocarbons in race B (Ioki et al., 2012a). The aforementioned studies suggested great variability in hydrocarbon yields and biosynthesis of *B. braunii* strains among different races (Jin et al., 2015). Some of these could probably be attributed to different photo-bioreactors, growth conditions, and nutrients and light supplies. Thus, a direct comparative study between race A and race B strains could be meaningful to properly assess their potential for commercial exploitation.

Some studies have already contributed to compare different races of *B. braunii* (Eroglu et al., 2011; Li et al., 2013). Eroglu et al (2011) used three different experimental approaches, including density equilibrium, spectrophotometric analysis of hydrocarbon extracts, and gravimetric quantitation of eluted hydrocarbons to compare hydrocarbon productivities of three race A strains and two race B strains (Eroglu et al., 2011). Li et al compared extractable liquid biofuel content, bioenergy content and hydrocarbon content across 16 strains *B. braunii* (mainly A and B races) by direct combustion of algal biomass using thermogravimetric analysis (TGA), pressure differential scanning calorimetry (PDSC) and gas chromatography/mass spectrometry

(GC-MS) (Li et al., 2013). Note that most of previous comparative studies on *B. braunii* were carried out in batch cultures in flasks, where secondary growth and stress effects can often mask subtle physiological differences and trends (Hoskisson, 2005). In addition, the environmental factors could not be well controlled in batch culture. For example, pH could exert a critical influence on hydrocarbon production by our preliminary results, while it seems that none of these studies were conducted with pH control.

The aim of the present study was to compare *B. braunii* race A and race B in chemostat cultures with full environmental factors control. The comparative study is based on By experimental data acquired at steady−states at fixed dilution rate. These data are mainly related with production of biomass, hydrocarbon and lipids and analyzed for a race A and a race B strain. Other data are related to photosynthetic activities, nutrients conversion yields, specific light supply and fatty acids profiles. To our knowledge, this is the first time that continuous cultures system has been used for obtaining data in steady−states to compare *B. braunii* race A and race B strains.

6.2. Experiment design and setup

Experiments were designed as maintaining continuous cultures in torus photobioreactor as shown in **Figure 6-1.** Experiments were conducted with the strain *B. braunii* Kützing race A strain SAG 30.81 and *B. braunii* race B strain BOT 22.

B. braunii race A strain is SAG 30.81 obtained from the Culture Collection of Algae at Gottingen University (Sammlung von Algenkulturen, SAG); race B strain is Bot 22 obtained from University of Tsukuba, Japan. This strain SAG 30.81 is classified as race A strain as identified by characteristic hydrocarbons by GC-MS (Dayananda et al., 2005, 2006; Ranga Rao et al., 2007). BOT 22 is identified as race B strain by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy as mainly producing C_{34} botryococcene (Ishimatsu et al., 2012).

A torus photobioreactor with 1.4L volume was used in present continuous cultures, as described in previous study (Pruvost et al., 2006). Temperature was controlled by air conditioning at 23 ± 1 ºC and illuminated continuously at the photobioreactor surface with a

photon flux density (PFD) of 150 µmol photon m^{-2} s^{-1}. Gas flow rate was 450 mL min^{-1}. Speed of the axial flow impellers was 200 rpm and pH was controlled at 6.5 by CO_2 injection (pH−controller Mettler Toledo M200). The continuous cultures were fed with 3N6P−AF−6 medium at fixed dilution rate (D, d^{-1}) 0.23 d^{-1}.

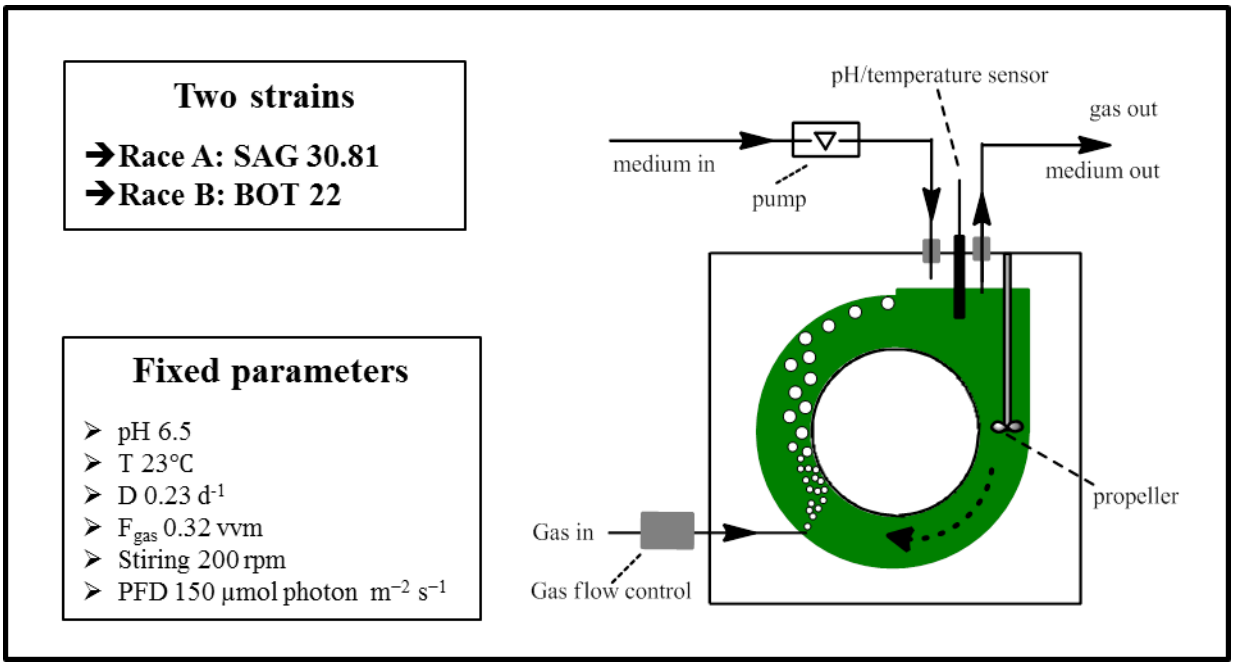


Figure 6-1. Experiment design and setup of *B. braunii* race A and race B strain in the continuous cultures in torus photobioreactor.

In continuous culture, the biomass concentration in photobioreactor, the specific growth rate (µ) of microalga and the dilution rate are related to the equation as flows:

$$\frac{dX}{dt} = \mu \cdot X - D \cdot X$$

Then, at steady state, $\mu = D$. Then, the comparative study of race A and race B strain conducted at the same cell specific growth rate.

6.3. Results and discussion

In order to highlight the control of culture environment factors in steady−state, a continuous culture of *B. braunii* was started in a 1.4 L photobioreactor at fixed dilution rate 0.23 d^{-1}. Environmental conditions were kept in constant during all the continuous cultivation, including temperature (23 °C), pH (6.5), stirring (200 rpm), aeration rate (0.32 vvm) and a continuouslight intensity of 150 µmol photon m^{-2} s^{-1} at the surface of photobioreactor.

6.3.1. Biomass, hydrocarbon and lipids production

The green alga *B. braunii* is generally regarded as a slow-growing microalga, except that a few investigations were shown this species could grow at specific rates up to 0.5 d^{-1} in batch cultures (Yoshimura et al., 2013). The dilution rate of the continuous cultures was fixed at 0.23 d^{-1}, because this dilution rate identified as the opitimal dilution rate for biomass producing in Chaper IV and the specific growth rates of *B. braunii* cultures are usually in the range 0.1 d^{-1} to 0.3 d^{-1}. The dilution rate is equated to specific growth rate at steady state in chemostat cultures, i.e. race A and race B strains were maintained at the same specific growth rate 0.23 d^{-1}. Then, the effect of the specific growth rate on hydrocarbon and lipids production seemed to be excluded.

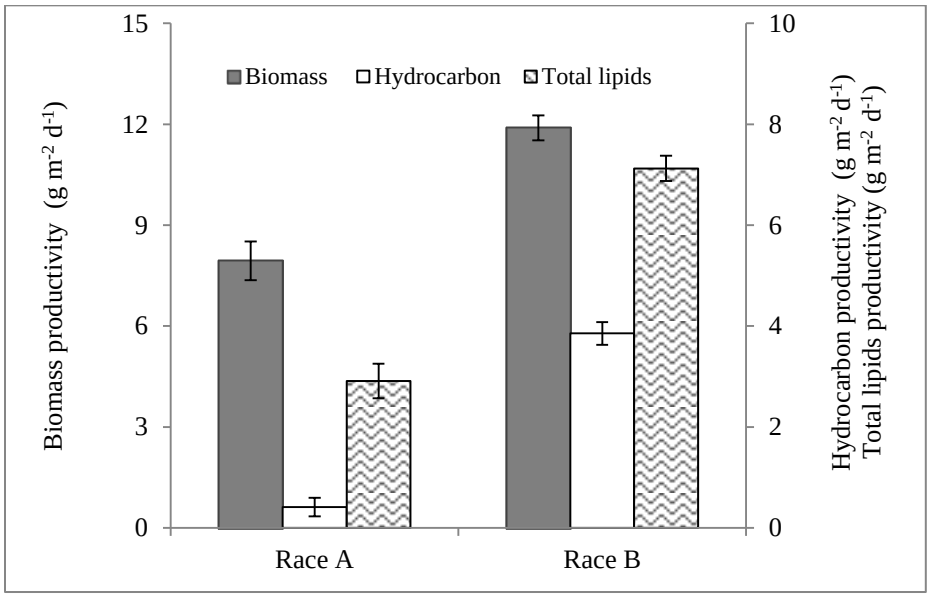


Figure 6-2. Biomass, hydrocarbon and total lipids productivities of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in steady-state continuous cultures.

As shown in **Figure 6-2**, *B. braunii* race A SAG 30.81presents the lower biomass and hydrocarbon productivities than race B strain BOT 22. In addition to hydrocarbon, total lipids of microalgae are also the crucial factors in making algal biofuels economically viable (Griffiths and Harrison, 2009). The productivity of total lipids in race A culture was also significantly lower than that in race B. The difference of productivity could be due to the variety of ability for hydrocarbon and lipids accumulation among different *B. braunii* races.

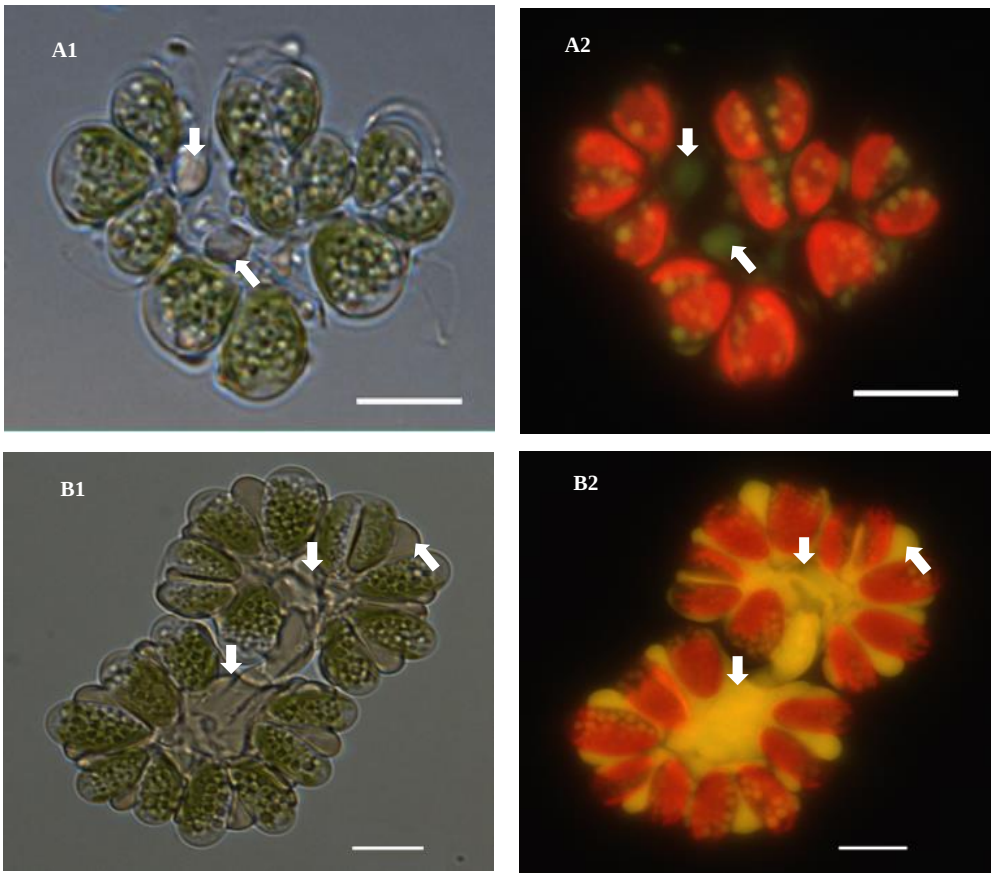


Figure 6-3. Microscope of colonies of *B. braunii* race A strain SAG 30.81 (A1 and A2) and race B strain BOT 22 (B1 and B2) with the Nile Red staining in steady-state continuous cultures; the cells were stained with Nile Red and observed by bright field (A1 and B1) and fluorescence microscopy with UV light and filters to show chlorophyll fluorescence (red) and hydrocarbons (yellow) (A2 and B2); the white arrow indicate extracellular hydrocarbon produced by cells; scale bar, 10 µm.

The race B strains generally demonstrated higher hydrocarbon content than race A, except there was a report once showing extremely high hydrocarbon content in race A strain reaching 61 % dw. Eroglu et al (Eroglu et al., 2011) compare hydrocarbon content of three race A strains and

two race B strains and find race A strains showing a hydrocarbon content just from 10 % to 14%, while race B strain equivalent to 20 % and 30 %. Li et al (Li et al., 2013) compare hydrocarbon content across 16 strains of *B. braunii* revealing that hydrocarbon content is only in the range 7.81% to 19.29 % in race A, but 13.77 % to 24.97 % in race B strains. In this study, hydrocarbon content in present race A strain SAG 30.81 was 5.2 % dw, significantly less than that in race B strain BOT 22 with a high value up to 32.4 % dw.

Fluorescence microscopic observations of Nile Red-stained colonies showed that race B strain BOT 22 indeed accumulated more hydrocarbon than race A strain SAG 30.81 in their colonies (**Figure 6-3**). Therefore, our results in steady state of chemostat cultures with the same specific growth rate confirmed previous study in batch mode.

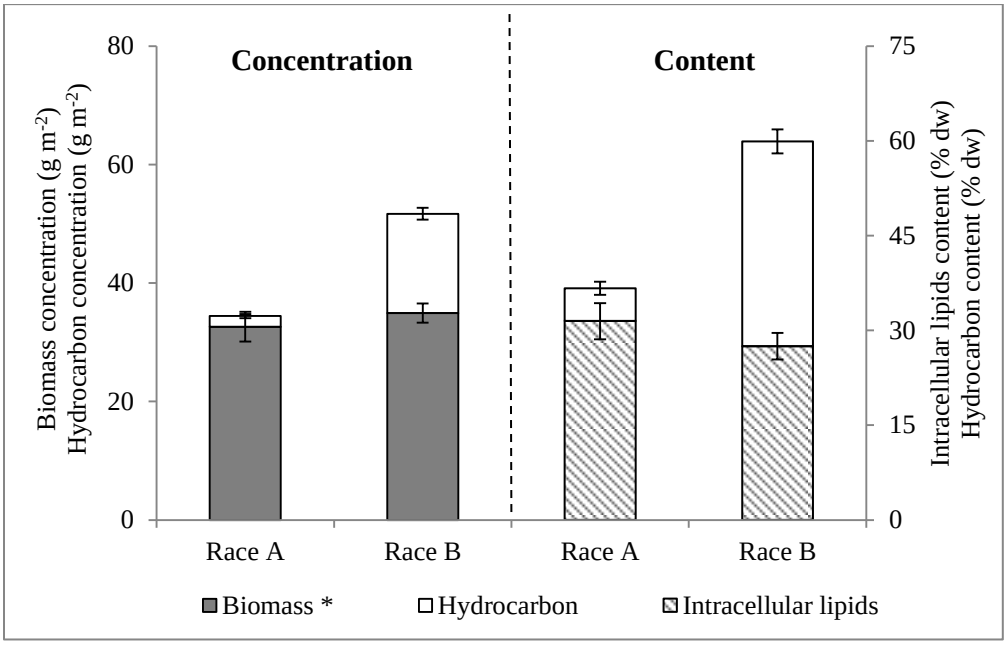


Figure 6-4. Biomass*, hydrocarbon concentration in PBR (left) and intracellular lipids and hydrocarbon contents in biomass (right) of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in steady-state continuous cultures; Biomass*=Biomass-Hydrocarbon.

The cell of this colonial microalga *B. braunii* is essentially surrounded by a complex extracellular matrix, in which liquid hydrocarbon are accumulated. Hydrocarbon is mainly related to the extracellular fraction, which evaluated to about 95 % of total hydrocarbon (Jin et al., 2015). We can use these characteristics to assume that the liquid hydrocarbons as extracellular products

could be dissociated from the cellular material and the solid polymer matrix. Then, a new term "Biomass*" used as the representative of these cellular material and solid polymer matrix, considering that their weight could be estimated by the substraction of the hydrocarbon weight from the total algal biomass dry weights. In the same way, we could also calculate intracellular lipids content by subtracting the hydrocarbon content from that of the total lipids. Then, as shown in **Figure 6-4**, the biomass* concentration and intracellular lipids content seem to be almost in the same level between race A and race B strains, even though the biomass (biomss*+hydrocarbon) and total lipids content are significantly different. Hence, the solid matrix could be modeled as similar to an *in situ* solid-phase for hydrophobic adsorption as used in biphasic bioreactor to trap either useful or toxic products during fermentation (Phillips et al., 2013).

According to this hypothesis, calculations highlight that the overproduction of biomass and total lipids in race B strains could be ascribed to the increase in hydrocarbon accumulation in the *B. braunii* colonies.

6.3.2. Pigments composition and photosynthetic activity

Pigments content and composition in the biomass are indicators of the physiological state of the culture. Contents of chlorophyll and carotenoids and ratios of Chl *a*/Chl *b* and Car/Chl of *B. braunii* are known to depend on strains, but race B strains seem to have a relative high carotenoids content in range 0.21% to 0.54 % than that in race A strains from 0.1% to 0.19 % (Eroglu et al., 2011). The pigments content and composition of race A strain SAG 30.81 and race B strain BOT 22 were obtained (**Table 6-1**). The higher pigments contents in race B could be due to the lower SLSR in race B cultures, as shown in **Figure 6-5**.

The ratio of Chl *a*/Chl *b* has no significant difference between race A and race B strains, while pigments content in race B was slight higher than that in race A, especially on the chlorophyll, leading to a higher ratio of Car/Chl in race A strain. As shown in **Table 6-1**, the ratios of Chl *a* / Chl *b* and Car / Chl were not significantly affected by dilution rate in present cultures, indicating that cell cultures of *B. braunii* were not stressed.

Table 6-1
Steady state pigments composition and photosynthetic activity of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in continuous cultures.

	Total Chl content (% dw)	Total Car content (% dw)	Chl *a* / Chl *b*	Car / Chl	Fv / Fm
Race A	1.73±0.08	0.31±0.02	2.1±0.18	0.18±0.02	0.73±0.02
Race B	2.32±0.17	0.34±0.02	2.0±0.12	0.14±0.01	0.74±0.02

Means ± SD. Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*; Car = carotenoids; Chl = chlorophylls, including Chl *a* and Chl *b*; Fv/Fm = maximum photochemical quantum yield of PS II.

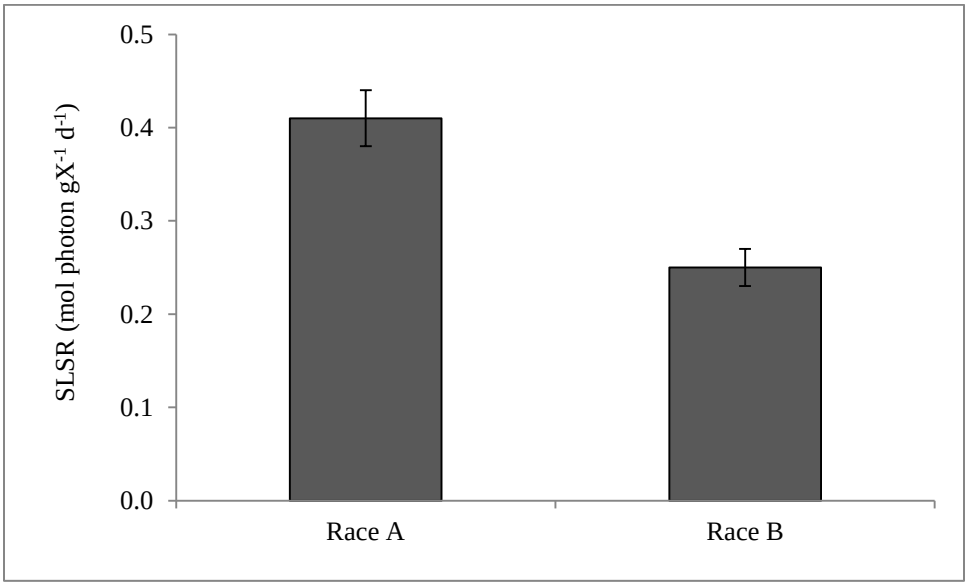


Figure 6-5. SLSR of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in steady-state continuous cultures.

Fv/Fm, maximum photochemical quantum yield of PS II, is frequently used to characterize the photosynthesis activity in algae (Claquin et al., 2008). *B. braunii* generally demonstrated a high value of Fv/Fm, e.g. values of Fv/Fm in race A strain *B. braunii* CCAP 807/2 cultures could be higher than 0.5 (Moheimani et al., 2013), and values about 0.7 were obtained in race B strain *B. braunii* Bot 144 cultures (Baba et al., 2012b). In present studies, values higher than 0.7 could be obtained in both race A and race B cultures at steady state and

these values were in the same level without significant difference ($P < 0.05$). This information confirmed that the race A and race B cells were conducted in a relative high growth rate condition (0.23 d^{-1}) without stress.

6.3.3. Biomass and hydrocarbon yields on light energy

The biomass and hydrocarbon yields on light energy were calculated and plotted in **Figure 6-6.**

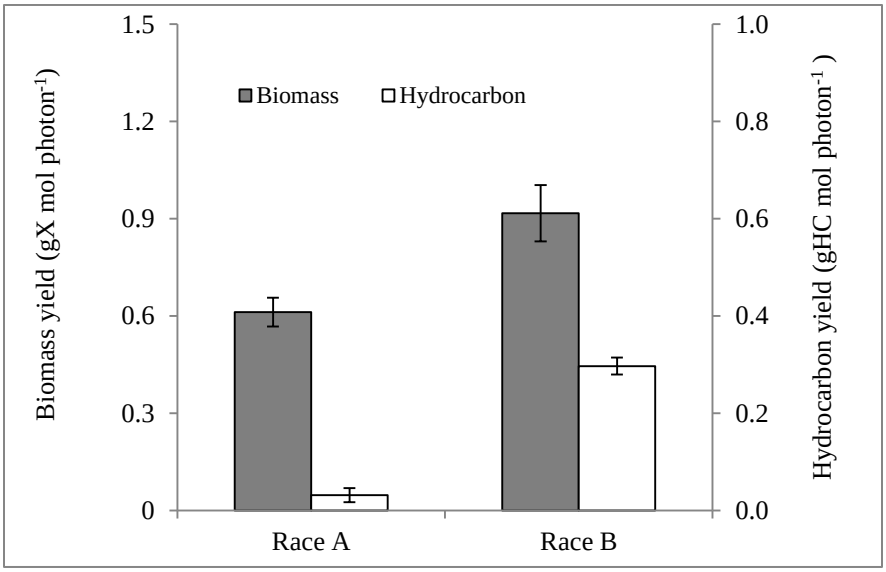


Figure 6-6. Yields of biomass and hydrocarbon on light energy *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in steady-state continuous cultures.

Comparison of biomass and hydrocarbon yields on light energy between race A and race B strains allows to better understand the impact of light on *B. braunii* physiology, since these photosynthetic organisms can convert light energy into biomass and metabolic products. Present cultures in PBR were continuously supplied with the same light 150 µmol photon m^{-2} s^{-1} at the surface of photobioreactor during race A and race B cultivation. The biomass and hydrocarbon yields on light energy in race A culture were 0.61 gX mol photon $^{-1}$ and 0.032 gHC mol photon $^{-1}$, respectively. However, in race B culture, biomass and hydrocarbon yields significant higher than that in race A, reaching 0.92 gX mol photon $^{-1}$ and 0.297 gHC mol photon $^{-1}$, respectively. Then, race B strain BOT 22 reflected efficient light energy utilization for biomass and hydrocarbon production comparing to race A strain SAG 30.81.

6.3.4. Nitrogen and phosphorus consumptions and yields

The ability to efficiently convert available nutrient into cell biomass and target products is a desirable characteristic for microalgae to be used in bioproduction. Thus, an evaluation of nutrient consumption rate and the biomass and hydrocarbon yields on nutrient between race A and race B strain are keys to determine the suitability of candidates. Nitrogen and phosphorus are among the main components of different cultures media used for growing microalgae, as well as the most effective factors to regulate the secondary metabolism in microalgal cells (Cheng et al., 2014). Then, the specific consumption rate of nitrogen and phosphorus were calculated during steady−states of the continuous culture, as well as their yields for biomass and hydrocarbon production (**Table 6-2**).

Table 6-2
Steady state specific consumption rate, biomass and hydrocarbon yield of nitrogen and phosphorus of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in continuous cultures.

	Specific N consumption rate ($mgN\ gX^{-1}\ d^{-1}$)	Biomass yield on N ($gX\ gN^{-1}$)	Hydrocarbon yield on N ($gHC\ gN^{-1}$)	Specific P consumption rate ($mgP\ gX^{-1}\ d^{-1}$)	Biomass yield on P ($gX\ gN^{-1}$)	Hydrocarbon yield on P ($gHC\ gN^{-1}$)
Race A	10.2 ±0.9	22.6±1.9	1.2±0.1	3.3 ±0.3	70.7±6.3	3.8±0.3
Race B	5.9± 0.5	38.9±3.4	12.6±1.1	0.73± 0.04	315.2±17.3	102.1±5.6

Means ± SD. N = nitrogen; P = phosphorus.

Race A strain SAG 30.81 showed a higher consumption rate of nitrogen and phosphorus than race B strain BOT 22. Surprisingly, for the biomass and hydrocarbon yields on nitrogen or phosphorus, BOT 22 illustrated the superiority than SAG 30.81, especially the hydrocarbon yields, a more than ten times disparity. As a result, race B strain BOT 22 is the desirable candidate by taking consideration to economical cost and output, since it demanded less nitrogen and phosphorus for biomass and hydrocarbon production than race A strain SAG 30.81.

6.3.5. Hydrocarbons profiles

B. braunii race B strain BOT 22 has been well identified to produce a high purity $C_{34}H_{58}$ botryococcene by nuclear magnetic resonance (NMR) (Ishimatsu et al., 2012). However, in previous investigations, hydrocarbons in the race A strain *B. braunii* SAG 30.81 are only classified roughly by GC as carbon chain length < C_{20}, between C_{20} and C_{30}, and > C_{30}. Indeed, the carbon chain length of the alkadienes and alkatrienes hydrocarbons in race A strains is generally the odd numbers in the range C_{23} to C_{33} (Jin et al., 2015), but the predominant hydrocarbon could be strain dependent, such as C_{25} hydrocarbon mainly produced in race A strain Overjuyo 7, C_{29} hydrocarbon in race A strain UTEX 572 and C_{31} hydrocarbon in race A strain Lingoult (Li et al., 2013). In order to characterize the detailed hydrocarbons profiles in present SAG 30.81 strain, the hydrocarbon extract was subjected to GC-FID and GC-MS analysis. In the SAG 30.81 hydrocarbon extract, there were main two peaks identified by gas chromatography (**Figure 6-7a**), accounting about 80 % of the total hydrocarbon. In order to characterize the carbon lenth of hydrocarbons in this race A strain, the n-alkane standard mixture (C_{10}–C_{40}, all even) standard mark solution were applied to GC-FID). Then, the carbon chain length of main hydrocarbons in SAG 30.81 was identified as C_{29} and C_{31}, comparing to the standard mark of alkane C_{10} to C_{40} (**Figure 6-7b**).

Hydrocarbons in race A strains could be alkadienes (C_nH_{2n-2}) with 2 double-bonds or alkatrienes (C_nH_{2n-4}) with 3 double-bonds. It was unsure these C_{29} and C_{31} hydrocarbons were alkadienes or alkatrienes only by GC-FID. Then, we further analyzed the hydrocarbons in race A strain SAG 30.81 by GC-MS. The mass spectra of the main two components were shown in **Figure 6-8**. These two main hydrocarbons were observed at m/z. 404.51 (**Figure 6-8a**) and 432.54 (**Figure 6-8b**). In present study, we also confirmed the main hydrocarbon in race B is $C_{34}H_{58}$ (**Figure 6-8c**).

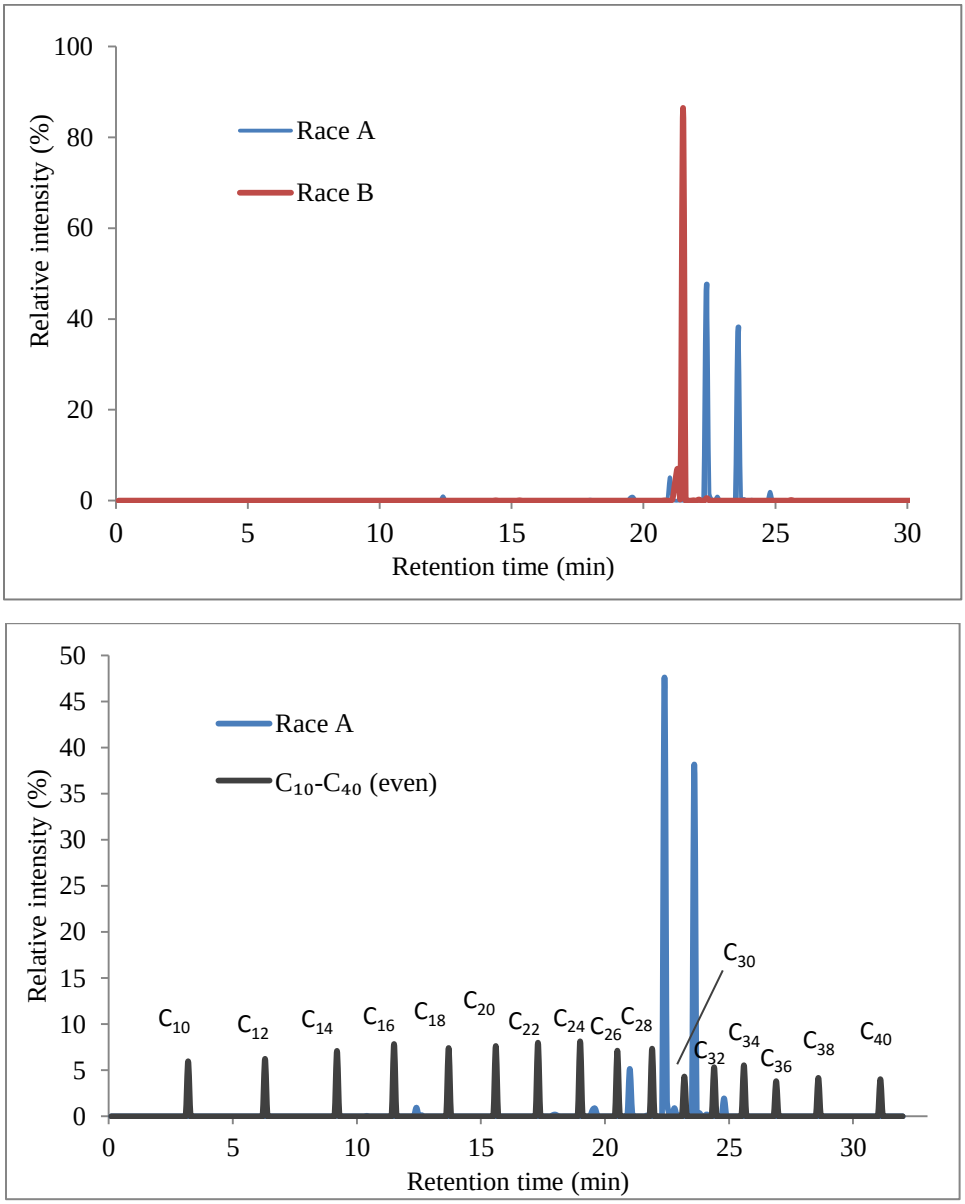


Figure 6-7. GC-FID of (a) the main hydrocarbons in *B. braunii* race A strain SAG 30.80 in steady-state continuous cultures, (b) the mixture of the main hydrocarbons in *B. braunii* race A strain SAG 30.80 and the standard mark of C_{10} to C_{40}.

To this end, nonacosadiene ($C_{29}H_{56}$) and hentriacontadiene ($C_{31}H_{60}$) were determined as the predominant composition in the race A strain SAG 30.81 hydrocarbons extract. Previous nuclear magnetic resonance (NMR) investigations in *B. braunii* race A strains clarify the two unsaturated double bonds in race A alkadienes are located at the C_9-C_{10} and the end of carbon positions (Metzger and Casadevall, 1989; Banerjee et al., 2002; Metzger and Largeau, 2005).

Therefore, the chemical structures of the main hydrocarbons in present *B. braunii* race A strain SAG 30.81 strain could be deduced, as shown in **Figure 6-9**.

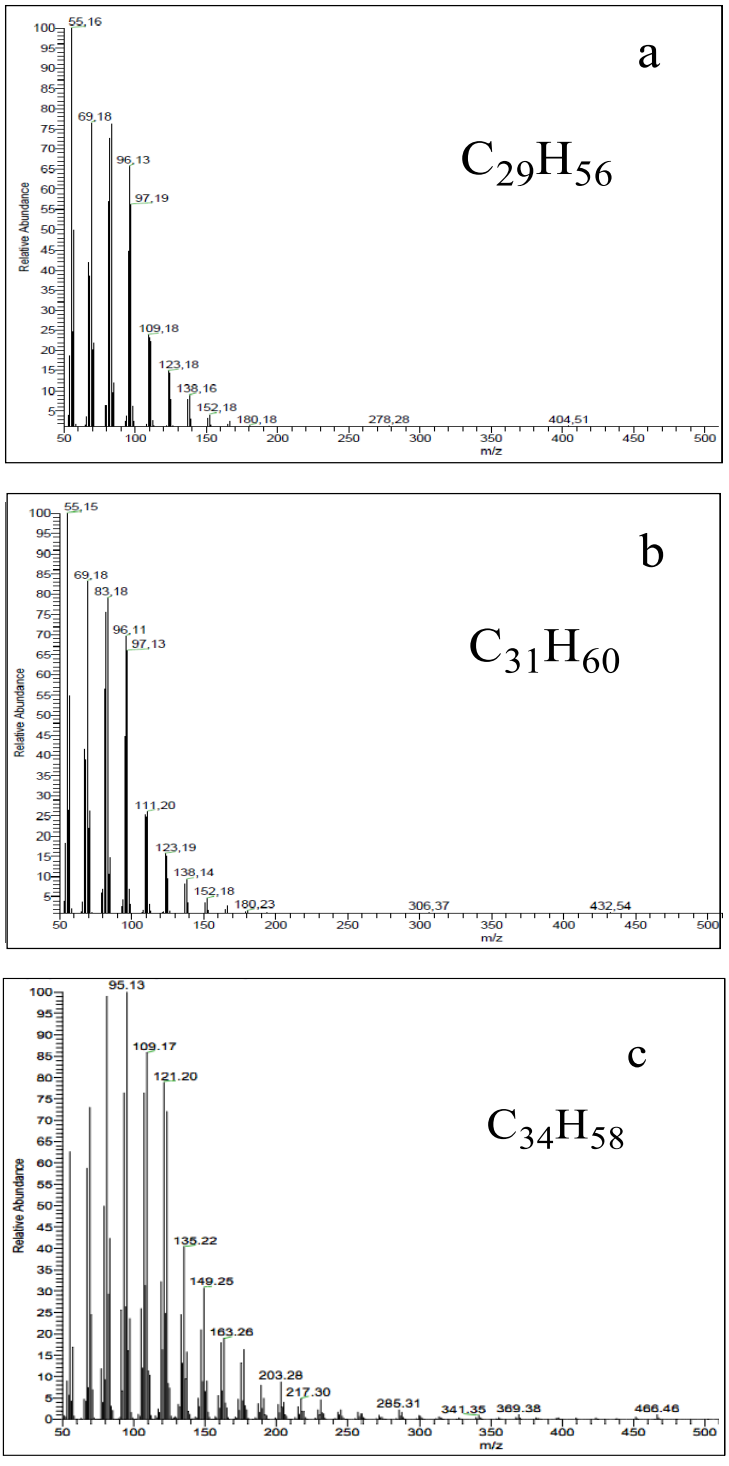


Figure 6-8. GC-MS spectrums of the main hydrocarbons in *B. braunii* race A strain SAG 30.80 (a ,b) and race B BOT 22 (c) in steady-state continuous cultures.

Although there were several previous studies focused on the strain SAG 30.81, to our knowledge, this is the first time to propose the detailed information of its predominant hydrocarbons profile and chemical structures.

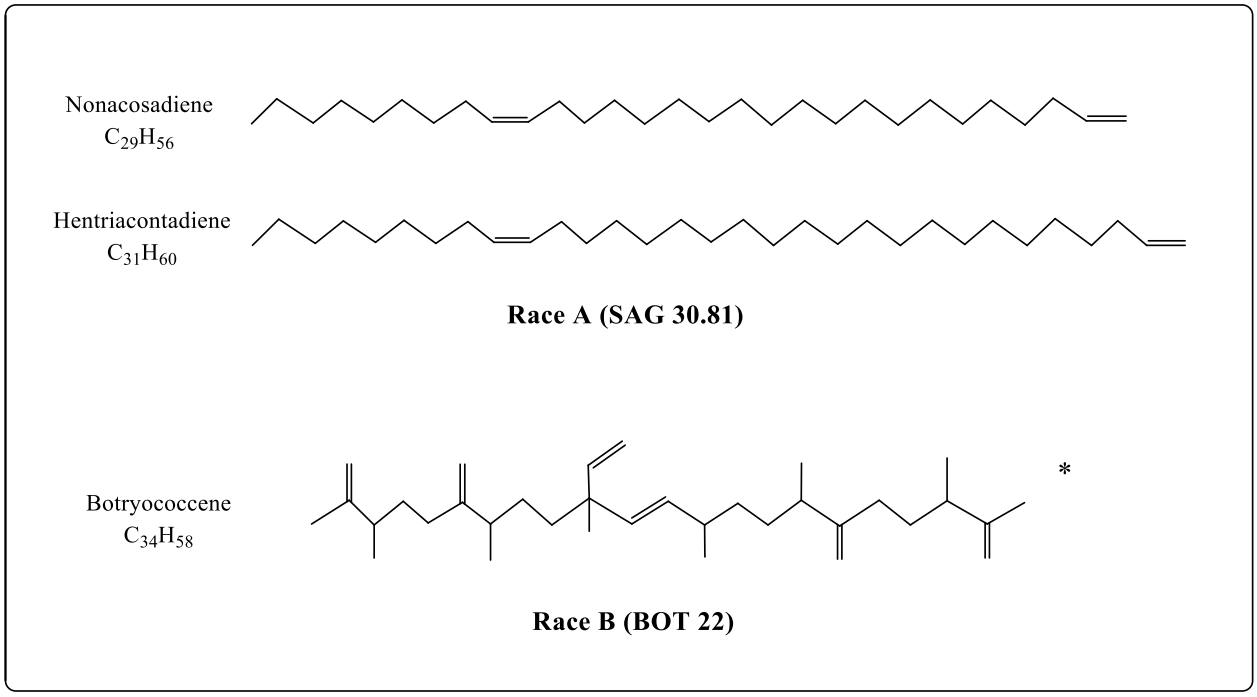


Figure 6-9. Predominant hydrocarbons produced in *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in continuous cultures; *, the hydrocarbon information of BOT 22 was adapted from (Ishimatsu et al., 2012).

6.3.6. Fatty acids productivity and profiles

The fatty acid profiles, indicated the presence of C13:0, C16:0, C16:1(n-7), C18:1(n-9c), C18:2(n-6c), C 18:3(n-3) in both race A and race B strains (**Figure 6-10**).

However, there were also some differences, such as polyunsaturated C22:5(n-3) was only detected in race B strain, very long chain fatty acids C22:0 and C24:1(n-9) were only found in race A strain. These diversities could be due to different hydrocarbon biosynthesis pathways in this hydrocarbon-rich species. In race A, hydrocarbons are converted from very long chain fatty

acids, which are elongated from oleic acid. In race B, higher hydrocarbons methylated from botryococcene $C_{30,}$ which is biosynthesized through triterpene synthesis pathway via MEP (Baba et al., 2012a; Ioki et al., 2012a, 2012b, 2012c). Hence, fatty acids are the precursors of the non-isoprenoid hydrocarbons in race A strain, but not the same case in race B. Then, the total fatty acids content in race A biomass is significantly higher than that in race B strain, and oleic acid constituted 61.3 % of total fatty acids in race A strain, while only 37.4 % in race B strain.

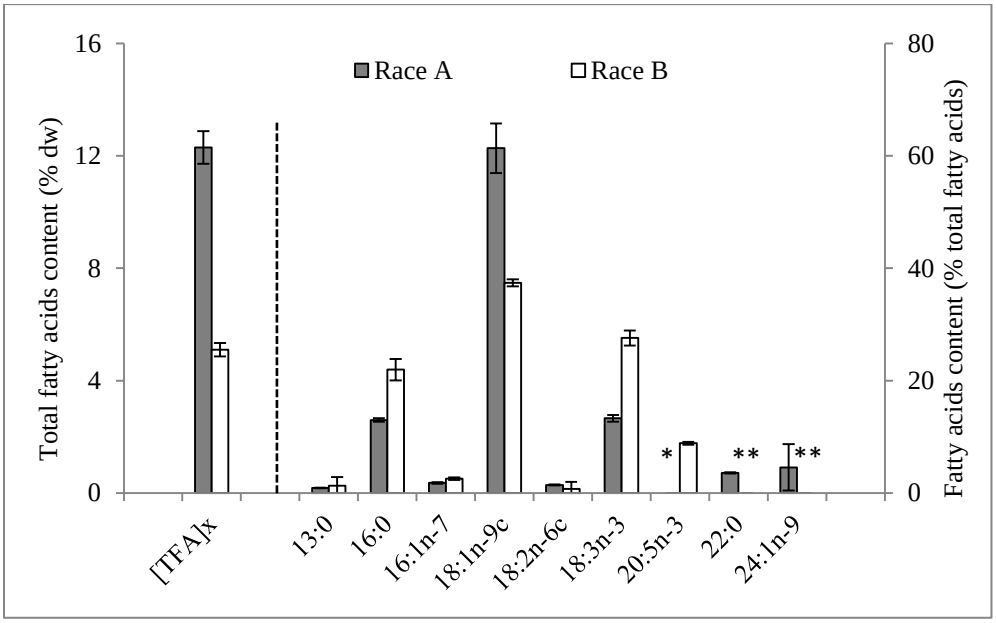


Figure 6-10. Total fatty acids content in biomass ($[TFA]_X$, % dw) and fatty acids profile (% total fatty acids) of *B. braunii* race A strain SAG 30.81 and race B strain BOT 22 in steady-state continuous cultures; *, not detected in race A; **, not detected in race B.

Then, the differences in fatty acids productivities and profiles seem to match with the respective hydrocarbons biosynthesis pathways in race A and race B strains.

6.4. Conclusion

Cultivation in chemostat cultures of *B. braunii* race A and race B strains within the same photobioreactor configuration confirmed that the race B strain BOT 22 has a higher biomass, hydrocarbon, and lipids productivities than the race A SAG 30.81 strain..

These overproductions of biomass and total lipids in race B strains could be ascribed to the higher accumulation of hydrocarbon within the race B colonies. Race B cells also presented higher biomass and hydrocarbon yields on light energy, nitrogen and phosphorus than race A. No much difference on pigments composition and photosynthetic activity, except chlorophyll content in race B slight higher than that in race A.

The total fatty acids and oleic acid contents in race A were significantly higher than that in race B strain, showing a new piece of evidence confirmed their respective hydrocarbons biosynthesis pathways. The predominant hydrocarbons in race A strain SAG 30.81 were determined as nonacosadiene ($C_{29}H_{56}$) and hentriacontadiene ($C_{31}H_{60}$) and their chemical structures were illustrated.

General conclusions and perspectives

Conclusions

Given the decreasing of fossil fuels reserves and intensifying of global warming effects, microalgae have attracted a great attention for providing chemicals and biofuels through biological mitigation of carbon dioxide (CO_2) (Wang et al., 2008). In addition to biodiesel transesterified from microalgal lipids (Serrano-Ruiz and Dumesic, 2011), hydrocarbon, the main components of fossil fuels, could also be produced by some microorganisms, among them the microalga *Botryococcus braunii* (Jin et al., 2015). The expected advantage of bio−hydrocarbon are their high caloric values (40.4 MJ Kg^{-1} of *B. braunii* oil, 38.4 MJ Kg^{-1} of *Chlorella vulgaris* oil) (Jin et al., 2015) and their ability to produce drop-in biofuels which could be easily added into the existing fuel infrastructure without any changes of engines, pumps or distribution networks to accommodate the transportation sector (Serrano-Ruiz and Dumesic, 2011).

In this context, the main objectives of this study were to investigate environmental effects on biomass and hydrocarbon production of *B. braunii* strains by using the continuous cultures technique in controlled photobioreactor, as well as the physiological responses to these environmental factors, particularly in terms of pH, light intensity and the availability of nutrients, such as dissolved inorganic carbon, nitrogen, and phosphorus. Also, a comparative study carried out between a race A strain and a race B strain. Below is the general conclusion of the studies conducted during my Ph.D. study.

1. Biomass and hydrocarbon productivities of *B. braunii* SAG 30.81 are pH-dependent in the range 5.5 to 8.0. At pH 5.5, the inhibition of the PSII activities could be related to a reduction of the hydrocarbon production. Maximal hydrocarbon productivities were obtained at pH 6.5.

2. The pH effect on hydrocarbon production was shown to be mainly related to the residual dissolved $CO_{2(aq)}$ concentration.in the supernatant of the cultures at sussesive steady states.

3. *B. braunii* SAG 30.81 could bear very high photons flux densities up 900 mol photon m^{-2} s^{-1} with photoadaptation, even though a slight decrease of the photosynthetic activity was observed during the transient phase.

4. High irradiance could stimulate the growth of *B. braunii* SAG 30.81 and improve the productivity of biomass, hydrocarbon and lipids, as well as the total fatty acids and oleic acid.

5. The overproduction of intracellular lipids occurred at the highest specific light supply rate tested whatever nitrogen conditions. This characteristic could be attributed to a means to convert excess light to chemical energy, in order to avoid photooxidative damage

7. In contrast to triacylglycerol accumulation in most of oleaginous microalgae at limited growth rate with nitrogen limitation, hydrocarbon content in *B. braunii* SAG 30.81was relative constant irrespective of growth rates, light intensity and nitrogen conditions.

8. Hydrocarbon productivity was correlated linearly to biomass productivity and hydrocarbon specific production rate was in linear correlation to specific growth rate of *B. braunii* SAG 30.81. Maintaining optimal environmental conditions enabling high specific growth rate could be meaningful for efficient hydrocarbon production in *B. braunii* culture.

9. No much difference on pigments composition and photosynthetic activity between *B. braunii* race A SAG 30.81 and race B strain BOT 22, except chlorophyll content in race B slight higher than that in race A.

10. *B. braunii* race B strain BOT 22 had a higher biomass, hydrocarbon, lipids productivities than race A strain SAG 30.81, as well as higher biomass and hydrocarbon yields on light energy, nitrogen and phosphorus. Then, strain selection could be meaningful for biofuels production by *B. braunii*.

11. These overproductions of biomass and total lipids in *B. braunii* race B strain BOT 22 could be ascribed to the rich accumulation of hydrocarbon in the race B colonies, which could be modeled as similar to an *in situ* solid-phase for hydrophobic adsorption. Further investigation on extracellular matrix structure and non destructive extraction of hydrocarbon from colonies could be sustainable for production.

12. The total fatty acids and oleic acid contents in race A were significantly higher than that in race B strain, showing a new piece of evidence confirmed their respective hydrocarbons biosynthesis pathways.

13. The predominant hydrocarbons in race A strain SAG 30.81 were determined as nonacosadiene ($C_{29}H_{56}$) and hentriacontadiene ($C_{31}H_{60}$).

Perspectives

In this thesis, we have attempted to investigate environmental effects on biomass, hydrocarbon production and physiological responses of *B. braunii* strains in the continuous cultures. However, there are still large possibilities to improve and to continue this work in the future. Here are the suggestions for further studies.

1. Continuous culture for omics applications

In present work, we find that pH could have an effect on hydrocarbon biosynthesis, that hydrocarbon content is higher at pH 6.5 than that at pH 7.5. Then, further work could maintain steady state at pH 6.5 & pH 7.5 changing HC content to identify the limiting step for hydrocarbon biosynthesis. Such as using ESTs analysis to identify which genes are overproduced at pH 6.5 or to understand which hydrocarbon intermediates are changed by carbon flux analysis.

2. Photosynthetic active radiation availability

B. braunii SAG 30.81 could bear very high light intensity with photoadaptation, even though a slight decrease of the photosynthetic activity in a short time. Then, we could increase the biomass hydrocarbon by increasing photosynthetic active radiation availability using thin layer or film PBR. Further work could be done relating to light transfer inside culture but also colonies. Note that photoinhibtion is common to microalga. Then, study of light absorption and irradiance distribution in the culture, and that at which light intensity level could lead to photoinbition of *B. braunii* is still needed to be investigated.

3. Investigation of temperature influence.

Temperature is one of the most important factors for growth and metabolic flux responses of microalgal cell, especially for outdoor mass culture. However, with the limitation of time and

equipment, we do not test the temperature effects on biomass and hydrocarbon production of *B. braunii*. Then, further studies could be carried out in this aspect.

4. Fast method for hydrocarbon quantification.

Raw hexane extracts of *B. braunii* were generally used as a relative fast method for hydrocarbon measurement. However, it seems hydrocarbons contents could be overestimated by this method. Then, quantification of hydrocarbon in *B. braunii* is necessary to further purified by silica chromatography, which is time consuming and could be involved in toxic organic solvents. Investigation of Fast method for hydrocarbon quantification, especially cell in vivo measurement method could be desirable.

5. Predictive model of biomass and hydrocarbon production.

There are already some predictive models for other microalgae (Pottier et al., 2005), while, almost no these kinds of studies carried out in *Botryococcus*. Efficient model could be useful for further prediction and optimization of the biomass and hydrocarbon production. For that, it is necessary to determine and to model the optical properties of *Botryococcus* colonies.

6. Characterization of extracellular matrix

According to the basic characteristic of hydrocarbon extracellular production, how to break retaining wall but non-cell-destructive hydrocarbon extraction is meaningful. Generally, chemical treatment could be toxic for cells. Then, physical shear stress could be the second choice. However, what kind of physical shear stress is suitable for this microalgae with physiological adaptation: no decrease Fv/Fm and capacity of hydrocarbon production are needed to be investigated. In another aspect, metabolism manipulation by control the matrix associated genes expression could be carried out. Maybe in one day, we could change the orgianal growth associated hydrocarbon production to mix-growth associated or to Non-growth associated. Growth associated, or not growth associated: that is the question.

Nomenclature

A	light incident area (m^2)
Chl	chlorophylls concentration (mg L^{-1})
$[Chl]_X$	chlorophylls content in biomass (% dw)
Chl *a*	chlorophyll *a* concentration (mg L^{-1})
Chl *b*	chlorophyll *b* concentration (mg L^{-1})
Car	carotenoids concentrations (mg L^{-1})
$[Car]_X$	carotenoids content in biomass (% dw)
D	dilution rate (d^{-1})
D_{opt}	optimal dilution rate (d^{-1})
ETR	electron transport rate (μmol electrons m^{-2} s^{-1})
ETRmax	maximal electron transport rate (μmol electrons m^{-2} s^{-1})
F	flow rate of feeding medium (L d^{-1})
Fv/Fm	maximum photochemical quantum yield of PS II
HC	hydrocarbon concentration (g L^{-1} or g m^{-2})
[HC]x	hydrocarbon content in biomass (% dw)
[IL]x	intracellular lipids content in biomass (% dw)
[N]x	nitrogen content of biomass (% dw)
PFD	photon flux density (μmol photon m^{-2} s^{-1} or μmol m^{-2} s^{-1})
Px	biomass productivity (g L^{-1} d^{-1} or g m^{-2} d^{-1})
P_{HC}	hydrocarbon productivity (g L^{-1} d^{-1} or g m^{-2} d^{-1})
$r_{S/X}$	specific nutrient uptake rate (mgS gX^{-1} d^{-1})
R_{XS}	nutrients requirement for biomass production (mgS gX^{-1})
SLS	specific light supply rate (mol photon gX^{-1} d^{-1})
T	temperature (ºC)
[TL]x	total lipids content in biomass (% dw)
V	volume (L)
X	biomass concentration (g L^{-1} or g m^{-2})
$Y_{X/S}$	biomass yield from nutrient (gX gS^{-1})

Greek letters

μ	specific growth rate (d^{-1})
μ_{max}	maximal specific growth rate (d^{-1})
λ	wavelength (nm)

Abbreviation

ACP	acyl-carrier-protein
ANOVA	one-way analysis of variance
BF3	boron trifluoride
BHT	butylated hydroxytoluene
BSS	*Botryococcus* squalene synthase
CA	carbonic anhydrase enzyme
CoA	coenzyme A
CTP	cytidine triphosphate
DIC	dissolved inorganic carbon
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DOXP	1-deoxy-D-xylulose 5-phosphate
DXS	1-deoxy-D-xylulose-5-phosphate synthase
ECM	extracellular matrix
EST	expression sequence tags
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
FAMEs	fatty acid methyl esters
FAS	fatty acid synthase
FPP	farnesyl pyrophosphate
FPS	farnesyl pyrophosphate synthase
GA-3-P	glyceraldehyde 3-phosphate
GC-FID	gas chromatography coupled to a flame ionization detector
GC-MS	gas chromatography coupled to a mass spectrometer

HC	hydrocarbon
HHV	high heating values
IDI	isopentenyl-diphosphate isomerase
LED	light-emitting diode
IL	intracellular lipids
IPP	isopentenyl pyrophosphate
IspD	4-diphosphocytidyl-2C-methyl-D-erythritol cytidylyltransferase
IspE	4-diphosphocytidyl-2C-methyl-D-erythritol kinase
IspF	2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase
IspG	1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase
IspH	4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase
K_0	chemical equilibrium parameter of $CO_{2(aq)}$, H_2O and $H_2\ CO_3$
K_1	chemical equilibrium parameter of $[HCO_3^-]$, $[H^+]$ and $[H_2\ CO_3]$
K_2	chemical equilibrium parameter of $[CO_3^{2-}]$, $[H^+]$ and $[HCO_3^-]$
MEP	2-C-methyl-D-erythritol-4-phosphate
MVA	mevalonate
MVI	mevalonate independent
PAR	photosynthetically active radiations (400-700 nm)
PBR	photobioreactor
PCR	polymerase chain reaction
PSII	photosystem II
PSPP	presqualene diphosphate
SSL	squalene synthase-like
SAM	s-adenosyl methionine
TL	total lipids
TLC	thin layer chromatography
TMT	triterpene methyltransferase
VLCF acids	very long chain fatty acids
VLCF aldehyde	very long chain fatty aldehydes

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References

Achitouv, E., Metzger, P., Rager, M.-N., Largeau, C., 2004. C_{31}-C_{34} methylated squalenes from a Bolivian strain of *Botryococcus braunii*. Phytochemistry 65, 3159–3165.

Afi, L., Metzger, P., Largeau, C., Connan, J., Berkaloff, C., Rousseau, B., 1996. Bacterial degradation of green microalgae: incubation of *Chlorella emersonii* and *Chlorella vulgaris* with *Pseudomonas oleovorans* and *Flavobacterium aquatile*. Org. Geochem., Proceedings of the 17th International Meeting on Organic Geochemistry 25, 117–130.

Aksu, Z., 2005. Application of biosorption for the removal of organic pollutants: a review. Process Biochem. 40, 997–1026.

Allard, B., Templier, J., 2000. Comparison of neutral lipid profile of various trilaminar outer cell wall (TLS)-containing microalgae with emphasis on algaenan occurrence. Phytochemistry 54, 369–380.

Amin, S., 2009. Review on biofuel oil and gas production processes from microalgae. Energy Convers. Manag. 50, 1834–1840.

An, J.-Y., Sim, S.-J., Lee, J.S., Kim, B.W., 2003. Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*. J. Appl. Phycol. 15, 185–191.

Ashokkumar, V., Agila, E., Sivakumar, P., Salam, Z., Rengasamy, R., Ani, F.N., 2014. Optimization and characterization of biodiesel production from microalgae *Botryococcus* grown at semi-continuous system. Energy Convers. Manag. 88, 936–946.

Ashokkumar, V., Rengasamy, R., 2012. Mass culture of *Botryococcus braunii* Kutz. under open raceway pond for biofuel production. Bioresour. Technol. 104, 394–399.

Atobe, S., Saga, K., Hasegawa, F., Furuhashi, K., Tashiro, Y., Suzuki, T., Okada, S., Imou, K., 2015. Effect of amphiphilic polysaccharides released from *Botryococcus braunii* Showa on hydrocarbon recovery. Algal Res. 10, 172–176.

Baba, M., Ioki, M., Nakajima, N., Shiraiwa, Y., Watanabe, M.M., 2012a. Transcriptome analysis of an oil-rich race A strain of *Botryococcus braunii* (BOT-88-2) by *de novo* assembly of pyrosequencing cDNA reads. Bioresour. Technol. 109, 282–286.

Baba, M., Kikuta, F., Suzuki, I., Watanabe, M.M., Shiraiwa, Y., 2012b. Wavelength specificity of growth, photosynthesis, and hydrocarbon production in the oil-producing green alga *Botryococcus braunii*. Bioresour. Technol. 109, 266–270.

Balkos, K.D., Colman, B., 2007. Mechanism of CO_2 acquisition in an acid-tolerant *Chlamydomonas*. Plant Cell Environ. 30, 745–752.

Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U.C., 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit. Rev. Biotechnol. 22, 245–279.

Bartley, M.L., Boeing, W.J., Dungan, B.N., Holguin, F.O., Schaub, T., 2014. pH effects on growth and lipid accumulation of the biofuel microalgae *Nannochloropsis salina* and invading organisms. J. Appl. Phycol. 26, 1431–1437.

Bazaes, J., Sepulveda, C., Acién, F.G., Morales, J., Gonzales, L., Rivas, M., Riquelme, C., 2012. Outdoor pilot-scale production of *Botryococcus braunii* in panel reactors. J. Appl. Phycol. 24, 1353–1360.

Benvenuti, G., Lamers, P.P., Breuer, G., Bosma, R., Cerar, A., Wijffels, R.H., Barbosa, M.J., 2016. Microalgal TAG production strategies: why batch beats repeated-batch. Biotechnol. Biofuels 9, 64.

Bergstrom, J.D., Bostedor, R.G., Masarachia, P.J., Reszka, a a, Rodan, G., 2000. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. Arch. Biochem. Biophys. 373, 231–241.

Berrios, H., Zapata, M., Rivas, M., 2016. A method for genetic transformation of *Botryococcus braunii* using a cellulase pretreatment. J. Appl. Phycol. 28, 201–208.

Bertheas, O., Metzger, P., Largeau, C., 1999. A high molecular weight complex lipid, aliphatic polyaldehyde tetraterpenediol polyacetal from *Botryococcus braunii* (L race). Phytochemistry 50, 85–96.

Biller, P., Riley, R., Ross, A.B., 2011. Catalytic hydrothermal processing of microalgae: decomposition and upgrading of lipids. Bioresour. Technol. 102, 4841–4848.

Bognar, A.L., Paliyath, G., Rogers, L., Kolattukudy, P.E., 1984. Biosynthesis of alkanes by particulate and solubilized enzyme preparations from pea leaves (*Pisum sativum*). Arch. Biochem. Biophys. 235, 8–17.

Brown, A.C., Knights, B.A., Conway, E., 1969. Hydrocarbon content and its relationship to physiological state in the green alga *Botryococcus braunii*. Phytochemistry 8, 543–547.

Buchanan, R.A., Cull, I.M., Otey, F.H., Russell, C.R., 1978. Hydrocarbon-and rubber-producing crops. Econ. Bot. 32, 146–153.

Bukoreshtliev, N.V., Hodneland, E., Eichler, T.W., Eifart, P., Rustom, A., Gerdes, H.-H., Bukoreshtliev, N.V., Hodneland, E., Eichler, T.W., Eifart, P., Rustom, A., Gerdes, H.-H., 2012. Partitioning and exocytosis of secretory granules during division of PC12 cells. Int. J. Cell Biol. 2012, e805295.

Buono, S., Langellotti, A.L., Martello, A., Bimonte, M., Tito, A., Carola, A., Apone, F., Colucci, G., Fogliano, V., 2012. Biological activities of dermatological interest by the water extract of the microalga *Botryococcus braunii*. Arch. Dermatol. Res. 304, 755–764.

Burgoyne, R.D., Morgan, A., 2003. Secretory Granule Exocytosis. Physiol. Rev. 83, 581–632.

Cabanelas, I.T.D., Marques, S.S.I., de Souza, C.O., Druzian, J.I., Nascimento, I.A., 2015. *Botryococcus*, what to do with it? Effect of nutrient concentration on biorefinery potential. Algal Res. 11, 43–49.

Carvalho, A.P., Silva, S.O., Baptista, J.M., Malcata, F.X., 2011. Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. Appl. Microbiol. Biotechnol. 89, 1275–1288.

Casadevall, E., Dif, D., Largeau, C., Gudin, C., Chaumont, D., Desanti, O., 1985. Studies on batch and continuous cultures of *Botryococcus braunii*: hydrocarbon production in relation to physiological state, cell ultrastructure, and phosphate nutrition. Biotechnol. Bioeng. 27, 286–295.

Chandrappa, D., Lakshmanan, V., Neelwarne, B., Aswathanarayana, R.G., 2010. Assessment of genetic polymorphism among green microalgae *Botryococcus* of distinct origin by RAPD. J. Appl. Phycol. 4, 65–69.

Chan, D., Vogel, H., 2010. Current understanding of fatty acid biosynthesis and the acyl carrier protein. Biochem J 430, 1–19.

Cheng, P., Ji, B., Gao, L., Zhang, W., Wang, J., Liu, T., 2013. The growth, lipid and hydrocarbon production of *Botryococcus braunii* with attached cultivation. Bioresour. Technol. 138, 95–100.

Cheng, P., Wang, J., Liu, T., 2014. Effects of nitrogen source and nitrogen supply model on the growth and hydrocarbon accumulation of immobilized biofilm cultivation of *B. braunii*. Bioresour. Technol. 166, 527–533.

Chen, Y., Wu, Y., Ding, R., Zhang, P., Liu, J., Yang, M., Zhang, P., 2015. Catalytic hydrothermal liquefaction of *D. tertiolecta* for the production of bio-oil over different acid/base catalysts. AIChE J. doi:DOI 10.1002/aic.14740

Chen, Y., Zhang, L., Xu, C., Vaidyanathan, S., 2016. Dissolved inorganic carbon speciation in aquatic environments and its application to monitor algal carbon uptake. Sci. Total Environ. 541, 1282–1295.

Chirac, C., Casadevall, E., Largeau, C., Metzger, P., 1985. Bacterial influence upon growth and hydrocarbon production of the green alga *Botryococcus braunii*. J. Phycol. 21, 380–387.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Choi, G.G., Kim, B.H., Ahn, C.Y., Oh, H.M., 2011. Effect of nitrogen limitation on oleic acid biosynthesis in *Botryococcus braunii*. J. Appl. Phycol. 23, 1031–1037.

Choi, S.P., Bahn, S.-H., Sim, S.J., 2013. Improvement of hydrocarbon recovery by spouting solvent into culture of *Botryococcus braunii*. Bioprocess Biosyst. Eng. 36, 1977–1985.

Claquin, P., Probert, I., Lefebvre, S., Véron, B., 2008. Effects of temperature on photosynthetic parameters and TEP production in eight species of marine microalgae. Aquat. Microb. Ecol. 51, 1–11.

Clark, D.R., Merrett, M.J., Flynn, K.J., 1999. Utilization of dissolved inorganic carbon (DIC) and the response of the marine flagellate *Isochrysis galbana* to carbon or nitrogen stress. New Phytol. 144, 463–470.

Cosgrove, J., Borowitzka, M., 2006. Applying pulse amplitude modulation (PAM) fluorometry to microalgae suspensions: stirring potentially impacts fluorescence. Photosynth. Res. 88, 343–350.

Cramer, J.A., Morris, R.E., Giordano, B., Rose-Pehrsson, S.L., 2009. Partial least-squares predictions of nonpetroleum-derived fuel content and resultant properties when blended with petroleum-derived fuels. Energy Fuels 23, 894–902.

Danckwerts, P.V., Kennedy, A.M., 1958. The kinetics of absorption of carbon dioxide into neutral and alkaline solutions. Chem. Eng. Sci. 8, 201–215.

Davis Jr., W., 2014. The ATP-binding cassette transporter-2 (ABCA2) regulates esterification of plasma membrane cholesterol by modulation of sphingolipid metabolism. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1841, 168–179.

Dayananda, C., Sarada, R., Bhattacharya, S., Ravishankar, G.A., 2005. Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*. Process Biochem. 40, 3125–3131.

Dayananda, C., Sarada, R., Kumar, V., Ravishankar, G.A., 2007a. Isolation and characterization of hydrocarbon producing green alga *Botryococcus braunii* from Indian freshwater bodies. Electron. J. Biotechnol. 10, 78–91.

Dayananda, C., Sarada, R., Shamala, T.R., Ravishankar, G.A., 2006. Influence of nitrogen sources on growth, hydrocarbon and fatty acid production by *Botryococcus braunii*. Asian J. Plant Sci. 5, 799–804.

Dayananda, C., Sarada, R., Usharani, M., Shamala, T., Ravishankar, G., 2007b. Autotrophic cultivation of *Botryococcus braunii* for the production of hydrocarbons and exopolysaccharides in various media. Biomass Bioenergy 31, 87–93.

de-Bashan, L.E., Antoun, H., Bashan, Y., 2005. Cultivation factors and population size control the uptake of nitrogen by the microalgae *Chlorella vulgaris* when interacting with the microalgae growth-promoting bacterium *Azospirillum brasilense*. FEMS Microbiol. Ecol. 54, 197–203.

Demura, M., Ioki, M., Kawachi, M., Nakajima, N., Watanabe, M.M., 2014. Desiccation tolerance of *Botryococcus braunii* (Trebouxiophyceae, Chlorophyta) and extreme temperature tolerance of dehydrated cells. J. Appl. Phycol. 26, 49–53.

Dennis, M., Kolattukudy, P.E., 1992. A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. Proc. Natl. Acad. Sci. 89, 5306–5310.

Dennis, M.W., Kolattukudy, P.E., 1991. Alkane biosynthesis by decarbonylation of aldehyde catalyzed by a microsomal preparation from *Botryococcus braunii*. Arch. Biochem. Biophys. 287, 268–275.

Derber, C., Coudron, P., Tarr, C., Gladney, L., Turnsek, M., Shankaran, S., Wong, E., 2011. *Vibrio furnissii*: an unusual cause of bacteremia and skin lesions after ingestion of seafood. J. Clin. Microbiol. 49, 2348–2349.

Drapier, D., Dubacq, J.-P., Trémolières, A., Mazliak, P., 1982. Cooperative pathway for lipid biosynthesis in young pea leaves: oleate exportation from chloroplasts and subsequent integration into complex lipids of added microsomes. Plant Cell Physiol 23, 125–135.

Ducharme, N.A., Bickel, P.E., 2008. Lipid droplets in lipogenesis and lipolysis. Endocrinology 149, 942–949.

Dunford, J.E., Thompson, K., Coxon, F.P., Luckman, S.P., Hahn, F.M., Poulter, C.D., Ebetino, F.H., Rogers, M.J., 2001. Structure-activity relationships for inhibition of farnesyl diphosphate synthase *in vitro* and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J. Pharmacol. Exp. Ther. 296, 235–242.

Ehimen, E.A., Connaughton, S., Sun, Z., Carrington, G.C., 2009. Energy recovery from lipid extracted, transesterified and glycerol codigested microalgae biomass. GCB Bioenergy 1, 371–381.

Eroglu, E., Melis, A., 2010. Extracellular terpenoid hydrocarbon extraction and quantitation from the green microalgae *Botryococcus braunii* var. Showa. Bioresour. Technol. 101, 2359–2366.

Eroglu, E., Okada, S., Melis, A., 2011. Hydrocarbon productivities in different *Botryococcus* strains: comparative methods in product quantification. J. Appl. Phycol. 23, 763–775.

Fang, J.-Y., Chiu, H.-C., Wu, J.-T., Chiang, Y.-R., Hsu, S.-H., 2004. Fatty acids in *Botryococcus braunii* accelerate topical delivery of flurbiprofen into and across skin. Int. J. Pharm. 276, 163–173.

Fang, L., Sun, D., Xu, Z., He, J., Qi, S., Chen, X., Chew, W., Liu, J., 2015. Transcriptomic analysis of a moderately growing subisolate *Botryococcus braunii* 779 (Chlorophyta) in response to nitrogen deprivation. Biotechnol. Biofuels 8, 130.

Feng, D., Chen, Z., Xue, S., Zhang, W., 2011. Increased lipid production of the marine oleaginous microalgae *Isochrysis zhangjiangensis* (Chrysophyta) by nitrogen supplement. Bioresour. Technol. 102, 6710–6716.

Francisco, É.C., Neves, D.B., Jacob-Lopes, E., Franco, T.T., 2010. Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. J. Chem. Technol. Biotechnol. 85, 395–403.

Frenz, J., Largeau, C., Casadevall, E., 1989. Hydrocarbon recovery by extraction with a biocompatible solvent from free and immobilized cultures of *Botryococcus braunii*. Enzyme Microb. Technol. 11, 717–724.

Friedl, A., Padouvas, E., Rotter, H., Varmuza, K., 2005. Prediction of heating values of biomass fuel from elemental composition. Anal. Chim. Acta, Papers Presented at the 9th

International Conference on Chemometrics in Analytical Chemistry Papers Presented at the 9th International Conference on Chemometrics in Analytical chemistry 544, 191–198.

Furuhashi, K., Saga, K., Okada, S., Imou, K., 2013. Seawater-cultured *Botryococcus braunii* for efficient hydrocarbon extraction. PLoS ONE 8, e66483.

Gallio, M., Sturgill, G., Rather, P., Kylsten, P., 2002. A conserved mechanism for extracellular signaling in eukaryotes and prokaryotes. Proc. Natl. Acad. Sci. 99, 12208–12213.

Garciano, L.O., Tran, N.H., Kannangara, G.S.K., Milev, A.S., Wilson, M.A., McKirdy, D.M., Hall, P.A., 2012. Pyrolysis of a naturally dried *Botryococcus braunii* Residue. Energy Fuels 26, 3874–3881.

Garnier, M., Carrier, G., Rogniaux, H., Nicolau, E., Bougaran, G., Saint-Jean, B., Cadoret, J.P., 2014. Comparative proteomics reveals proteins impacted by nitrogen deprivation in wild-type and high lipid-accumulating mutant strains of *Tisochrysis lutea*. J. Proteomics, Special Issue: Proteomics of non-model organisms 105, 107–120.

Gelin, F., de Leeuw, J.W., Sinninghe Damsté, J.S., Derenne, S., Largeau, C., Metzger, P., 1994. The similarity of chemical structures of soluble aliphatic polyaldehyde and insoluble algaenan in the green microalga *Botryococcus braunii* race A as revealed by analytical pyrolysis. Org. Geochem. 21, 423–435.

Gelin, F., Volkman, J.K., Largeau, C., Derenne, S., Sinninghe Damsté, J.S., De Leeuw, J.W., 1999. Distribution of aliphatic, nonhydrolyzable biopolymers in marine microalgae. Org. Geochem. 30, 147–159.

Ge, Y., Liu, J., Tian, G., 2011. Growth characteristics of *Botryococcus braunii* 765 under high CO_2 concentration in photobioreactor. Bioresour. Technol. 102, 130–134.

Gołębiowski, M., Boguś, M.I., Paszkiewicz, M., Stepnowski, P., 2011. Cuticular lipids of insects as potential biofungicides: methods of lipid composition analysis. Anal. Bioanal. Chem. 399, 3177–3191.

Griehl, C., Kleinert, C., Griehl, C., Bieler, S., 2015. Design of a continuous milking bioreactor for non-destructive hydrocarbon extraction from *Botryococcus braunii*. J. Appl. Phycol. 27, 1–11.

Griffiths, M.J., Harrison, S.T.L., 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J. Appl. Phycol. 21, 493–507.

Grima, E.M., Camacho, F.G., Pérez, J.A.S., Sevilla, J.M.F., Fernández, F.G.A., Gómez, A.C., 1994. A mathematical model of microalgal growth in light-limited chemostat culture. J. Chem. Technol. Biotechnol. 61, 167–173.

Grima, E.M., Pérez, J.A.S., Garc Camacho, F.Í., Medina, A.R., 1993. Gas-liquid transfer of atmospheric CO_2 in microalgal cultures. J. Chem. Technol. Biotechnol. 56, 329–337.

Guarnieri, M.T., Pienkos, P.T., 2014. Algal omics: unlocking bioproduct diversity in algae cell factories. Photosynth. Res. 123, 255–263.

Hampel, D., Mosandl, A., Wüst, M., 2005. Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. Phytochemistry 66, 305–311.

Hejazi, M.A., Holwerda, E., Wijffels, R.H., 2004. Milking microalga *Dunaliella salina* for β-carotene production in two-phase bioreactors. Biotechnol. Bioeng. 85, 475–481.

Hidalgo, P., Ciudad, G., Mittelbach, M., Navia, R., 2015. Biodiesel production by direct conversion of *Botryococcus braunii* lipids: Reaction kinetics modelling and optimization. Fuel 153, 544–551.

Hidalgo, P., Ciudad, G., Navia, R., 2016. Evaluation of different solvent mixtures in esterifiable lipids extraction from microalgae *Botryococcus braunii* for biodiesel production. Bioresour. Technol. 201, 360–364.

Hifney, A.F., Abdel-Basset, R., 2014. Photosynthesis, respiration and carotenoid contents in the green alga *Botryococcus braunii* at elevated nutrient levels. J. Biol. Earth Sci. 4, B191–B198.

Hillen, L.W., Pollard, G., Wake, L.V., White, N., 1982. Hydrocracking of the oils of *Botryococcus braunii* to transport fuels. Biotechnol. Bioeng. 24, 193–205.

Hirose, M., Mukaida, F., Okada, S., Noguchi, T., 2013. Active hydrocarbon biosynthesis and accumulation in a green alga, *Botryococcus braunii* (race A). Eukaryot. Cell 12, 1132–1141.

Hlousek-Radojcic, A., Evenson, K., 1998. Fatty acid elongation is independent of acyl-Coenzyme A synthetase activities in leek and *Brassica napus*. Plant Physiol. 73402, 251–258.

Hoskisson, P.A., 2005. Continuous culture – making a comeback. Microbiology 151, 3153–3159.

Hou, L., Park, H., Okada, S., Ohama, T., 2014. Release of single cells from the colonial oil-producing alga *Botryococcus braunii* by chemical treatments. Protoplasma 251, 191–199.

Hsu, J.-P., Hsu, W.-C., Tsao, H.-K., 1993. Diffusion-enhanced bioreactions: a hypothetical mechanism for plant cell aggregation. Bull. Math. Biol. 55, 869–889.

Huang, X., Wang, C., Zhang, J., Wiesenberg, G.L.B., Zhang, Z., Xie, S., 2011. Comparison of free lipid compositions between roots and leaves of plants in the Dajiuhu Peatland, central China. Geochem. J. 45, 365–373.

Huang, Z., Dale Poulter, C., 1989. Tetramethylsqualene, a triterpene from *Botryococcus braunii* var. showa. Phytochemistry 28, 1467–1470.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. Cell Mol. Biol. 54, 621–639.

Illman, A.M., Scragg, A.H., Shales, S.W., 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol. 27, 631–635.

Inoue, H., Korenaga, T., Sagami, H., Koyama, T., Ogura, K., 1994. Phosphorylation of farnesol by a cell-free system from *Botryococcus braunii*. Biochem. Biophys. Res. Commun. 200, 1036–1041.

Inoue, H., Korenaga, T., Sagami, H., Koyama, T., Sugiyama, H., Ogura, K., 1993. Formation of farnesal and 3-hydroxy-2,3-dihydrofarnesal from farnesol by protoplasts of *Botryococcus braunii*. Biochem. Biophys. Res. Commun. 196, 1401–1405.

Inoue, H., Sagami, H., Koyama, T., Ogura, K., 1995. Properties of farnesol phosphokinase of *Botryococcus braunii*. Phytochemistry 40, 377–381.

Ioki, M., Baba, M., Bidadi, H., Suzuki, I., Shiraiwa, Y., Watanabe, M.M., Nakajima, N., 2012a. Modes of hydrocarbon oil biosynthesis revealed by comparative gene expression analysis for race A and race B strains of *Botryococcus braunii*. Bioresour. Technol. 109, 271–276.

Ioki, M., Baba, M., Nakajima, N., Shiraiwa, Y., Watanabe, M.M., 2012b. Transcriptome analysis of an oil-rich race B strain of *Botryococcus braunii* (BOT-70) by *de novo* assembly of 5′-end sequences of full-length cDNA clones. Bioresour. Technol. 109, 277–281.

Ioki, M., Baba, M., Nakajima, N., Shiraiwa, Y., Watanabe, M.M., 2012c. Transcriptome analysis of an oil-rich race B strain of *Botryococcus braunii* (BOT-22) by *de novo* assembly of pyrosequencing cDNA reads. Bioresour. Technol. 109, 292–296.

Ishimatsu, A., Matsuura, H., Sano, T., Kaya, K., Watanabe, M.M., 2012. Biosynthesis of isoprene units in the C_{34} Botryococcene molecule produced by *Botryococcus braunii* strain Bot-22. Procedia Environ. Sci. 15, 56–65.

Jin, J., Dupré, C., Yoneda, K., Watanabe, M.M., Legrand, J., Grizeau, D., 2015. Characteristics of extracellular hydrocarbon-rich microalga *Botryococcus braunii* for biofuels production: Recent advances and opportunities. Process Biochem. doi:http://dx.doi.org/10.1016/j.procbio.2015.11.026

Johnson, K.J., Morris, R.E., Rose-Pehrsson, S.L., 2006. Evaluating the predictive powers of spectroscopy and chromatography for fuel quality assessment. Energy Fuels 20, 727–733.

Jones, J.G., 1969. Studies on lipids of soil micro-organisms with particular reference to hydrocarbons. J. Gen. Microbiol. 59, 145–152.

Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J., Rolland, N., 2010. Chloroplast proteomics highlights the subcellular compartmentation of lipid metabolism. Prog. Lipid Res. 49, 128–158.

Kalacheva, G.S., Zhila, N.O., Volova, T.G., 2002. Lipid and hydrocarbon compositions of a collection strain and a wild sample of the green microalga *Botryococcus*. Aquat. Ecol. 36, 317–331.

Kavitha, C., Ashokkumar, V., Chinnasamy, S., Bhaskar, S., Rengasamy, R., 2014. Pretreatment of lipid extracted *Botryococcus braunii* spent biomass for bio ethanol production. Int. J. Curr. Biotechnol. 2, 11–18.

Kawachi, M., Tanoi, T., Demura, M., Kaya, K., Watanabe, M.M., 2012. Relationship between hydrocarbons and molecular phylogeny of *Botryococcus braunii*. Algal Res. 1, 114–119.

Kaya, K., Nakazawa, A., Matsuura, H., Honda, D., Inouye, I., Watanabe, M.M., 2011. Thraustochytrid *Aurantiochytrium* sp. 18W-13a accummulates high amounts of squalene. Biosci. Biotechnol. Biochem. 75, 2246–2248.

Khan, N.E., Myers, J.A., Tuerk, A.L., Curtis, W.R., 2014. A process economic assessment of hydrocarbon biofuels production using chemoautotrophic organisms. Bioresour. Technol. 172, 201–211.

Khatri, W., Hendrix, R., Niehaus, T., Chappell, J., Curtis, W.R., 2014. Hydrocarbon production in high density *Botryococcus braunii* race B continuous culture. Biotechnol. Bioeng. 111, 493–503.

Kleinegris, D.M.M., Janssen, M., Brandenburg, W.A., Wijffels, R.H., 2011. Two-phase systems: potential for *in situ* extraction of microalgal products. Biotechnol. Adv., Marine Biotechnology in Europe European Science Foundation-COST Conference: "Marine Biotechnology: Future Challenges" 29, 502–507.

Kliphuis, A.M.J., Klok, A.J., Martens, D.E., Lamers, P.P., Janssen, M., Wijffels, R.H., 2012. Metabolic modeling of *Chlamydomonas reinhardtii*: energy requirements for photoautotrophic growth and maintenance. J. Appl. Phycol. 24, 253–266.

Knothe, G., 2008. Designer biodiesel : optimizing fatty ester composition to improve fuel properties. Energy Fuels 22, 1358–1364.

Kodner, R.B., Summons, R.E., Knoll, A.H., 2009. Phylogenetic investigation of the aliphatic, non-hydrolyzable biopolymer algaenan, with a focus on green algae. Org. Geochem. 40, 854–862.

Kojima, E., Zhang, K., 1999. Growth and hydrocarbon production of microalga *Botryococcus braunii* in bubble column photobioreactors. J. Biosci. Bioeng. 87, 811–815.

Kong WeiBao, Song Hao, Hua ShaoFeng, Yang Hong, Yang Qi, Xia ChunGu, 2012. Enhancement of biomass and hydrocarbon productivities of *Botryococcus braunii* by mixotrophic cultivation and its application in brewery wastewater treatment. Afr. J. Microbiol. Res. 6, 1489–1496.

Kotajima, T., Shiraiwa, Y., Suzuki, I., 2014. Functional screening of a novel Δ15 fatty acid desaturase from the coccolithophorid *Emiliania huxleyi*. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1841, 1451–1458.

Koussa, J., Chaiboonchoe, A., Salehi-Ashtiani, K., 2014. Computational approaches for microalgal biofuel optimization: a review. BioMed Res. Int. 2014, e649453.

Kumaar, L.K., Kumar, G.R., 2015. Transcritome analysis of *Botryococcus braunii* Race A and Race B to identify the enzymes involved in biodiesel biosynthesis. J. Chem. Pharm. Res. 7, 147–152.

Kunst, L., Samuels, L., 2009. Plant cuticles shine: advances in wax biosynthesis and export. Curr. Opin. Plant Biol. 12, 721–727.

Kützing, F.T., 1849. , in: Species Algarum. p. 892.

Ladygina, N., Dedyukhina, E.G., Vainshtein, M.B., 2006. A review on microbial synthesis of hydrocarbons. Process Biochem. 41, 1001–1014.

Largeau, C., 1995. Formation of refractory organic matter from biological precursors, in: The Role of Nonliving Organic Matter in the Earth's Carbon Cycle. John Wiley and Sons, pp. 275–292.

Largeau, C., Casadevall, E., Berkaloff, C., Dhamelincourt, P., 1980. Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. Phytochemistry 19, 1043–1051.

Lee, E., Pruvost, J., He, X., Munipalli, R., Pilon, L., 2014. Design tool and guidelines for outdoor photobioreactors. Chem. Eng. Sci. 106, 18–29.

Lichtenthaler, H.K., Babani, F., 2004. Light adaptation and senescence of the photosynthetic apparatus- changes in pigment composition, chlorophyll fluorescence parameters and photosynthetic activity, in: Chlorophyll a Fluorescence: A Signature of Photosynthesis. Springer, pp. 713–736.

Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy, in: Current Protocols in Food Analytical Chemistry. John Wiley & Sons, Inc., pp. F431–438.

Lindberg, P., Park, S., Melis, A., 2010. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. Metab. Eng. 12, 70–79.

Liu, J., Ge, Y., Cheng, H., Wu, L., Tian, G., 2013. Aerated swine lagoon wastewater: a promising alternative medium for *Botryococcus braunii* cultivation in open system. Bioresour. Technol. 139, 190–194.

Liu, T., Li, Y., Liu, F., Wang, C., 2016. The enhanced lipid accumulation in oleaginous microalga by the potential continuous nitrogen-limitation (CNL) strategy. Bioresour. Technol. 203, 150–159.

Liu, Y.-Q., Lim, L.R.X., Wang, J., Yan, R., Mahakhant, A., 2012. Investigation on pyrolysis of microalgae *Botryococcus braunii* and *Hapalosiphon* sp. Ind. Eng. Chem. Res. 51, 10320–10326.

Li, Y., Lian, S., Tong, D., Song, R., Yang, W., Fan, Y., Qing, R., Hu, C., 2011. One-step production of biodiesel from *Nannochloropsis* sp. on solid base Mg–Zr catalyst. Appl. Energy, Special Issue of Energy from algae: Current status and future trends 88, 3313–3317.

Li, Y., Moore, R.B., Qin, J.G., Scott, A., Ball, A.S., 2013. Extractable liquid, its energy and hydrocarbon content in the green alga *Botryococcus braunii*. Biomass Bioenergy 52, 103–112.

Los, D.A., Murata, N., 1998. Structure and expression of fatty acid desaturases. Biochim. Biophys. Acta BBA-Lipids Lipid Metab. 1394, 3–15.

Lupi, F.M., Fernandes, H.M.L., Sá-Correia, I., Novais, J.M., 1991. Temperature profiles of cellular growth and exopolysaccharide synthesis by *Botryococcus braunii* Kütz. UC 58. J. Appl. Phycol. 3, 35–42.

Mallick, N., Mandal, S., Singh, A.K., Bishai, M., Dash, A., 2012. Green microalga *Chlorella vulgaris* as a potential feedstock for biodiesel. J. Chem. Technol. Biotechnol. 87, 137–145.

Marsh, E.N.G., Waugh, M.W., 2013. Aldehyde decarbonylases: enigmatic enzymes of hydrocarbon biosynthesis. ACS Catal. 3, 2515–2521.

Martínez Sancho, M., Jiménez Castillo, J.M., Yousfi, F. El, 1999. Photoautotrophic consumption of phosphorus by *Scenedesmus obliquus* in a continuous culture. Influence of light intensity. Process Biochem. 34, 811–818.

Masters, M.J., 1971. The occurrence of *Chytridium marylandicum* on *Botryococcus braunii* in School Bay of the Delta Marsh. Can. J. Bot. 49, 1479–1485.

Mather, I.H., Keenan, T.W., 1998. Origin and secretion of milk lipids. J. Mammary Gland Biol. Neoplasia 3, 259–273.

Matsushima, D., Jenke-Kodama, H., Sato, Y., Fukunaga, Y., Sumimoto, K., Kuzuyama, T., Matsunaga, S., Okada, S., 2012. The single cellular green microalga *Botryococcus braunii* race B possesses three distinct 1-deoxy-d-xylulose 5-phosphate synthases. Plant Sci. 185–186, 309–320.

Matsuura, H., Watanabe, M.M., Kaya, K., 2011. Echinenone production of a dark red-coloured strain of *Botryococcus braunii*. J. Appl. Phycol. 24, 973–977.

Melis, A., 2012. Photosynthesis-to-fuels: from sunlight to hydrogen, isoprene, and botryococcene production. Energy Env. Sci 5, 5531–5539.

Mendes, L.B.B., Vermelho, A.B., 2013. Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnol. Biofuels 6, 152.

Metzger, P., Berkaloff, C., Casadevall, E., Couté, A., 1985. Alkadiene- and botryococcene-producing races of wild strains of *Botryococcus braunii*. Phytochemistry 24, 2305–2312.

Metzger, P., Casadevall, E., 1989. Aldehydes, very long chain alkenylphenols, epoxides and other lipids from an alkadiene-producing strain of *Botryococcus braunii*. Phytochemistry 28, 2097–2104.

Metzger, P., Casadevall, E., 1987. Lycopadiene, a tetraterpenoid hydrocarbon from new strains of the green alga *Botryococcus braunii*. Tetrahedron Lett. 28, 3931–3934.

Metzger, P., David, M., Casadevall, E., 1987. Biosynthesis of triterpenoid hydrocarbons in the B-race of the green alga *Botryococcus braunii*. Sites of production and nature of the methylating agent. Phytochemistry 26, 129–134.

Metzger, P., Largeau, C., 2005. *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. Appl. Microbiol. Biotechnol. 66, 486–496.

Metzger, P., Pouet, Y., Bischoff, R., Casadevall, E., 1993. An aliphatic polyaldehyde from *Botryococcus braunii* (A race). Phytochemistry, The International Journal of Plant Biochemistry 32, 875–883.

Metzger, P., Pouet, Y., Summons, R., 1997. Chemotaxonomic evidence for the similarity between *Botryococcus braunii* L race and *Botryococcus neglectus*. Phytochemistry 44, 1071–1075.

Metzger, P., Rager, M.-N., Fosse, C., 2008. Braunicetals: Acetals from condensation of macrocyclic aldehydes and terpene diols in *Botryococcus braunii*. Phytochemistry 69, 2380–2386.

Metzger, P., Rager, M.-N., Largeau, C., 2007. Polyacetals based on polymethylsqualene diols, precursors of algaenan in *Botryococcus braunii* race B. Org. Geochem. 38, 566–581.

Millero, F.J., Roy, R.N., 1997. A chemical equilibrium model for the carbonate system in natural waters. Croat. Chem. Acta 70, 1–38.

Moheimani, N.R., Cord-Ruwisch, R., Raes, E., Borowitzka, M.A., 2013. Non-destructive oil extraction from *Botryococcus braunii* (Chlorophyta). J. Appl. Phycol. 25, 1653–1661.

Moheimani, N.R., Matsuura, H., Watanabe, M.M., Borowitzka, M.A., 2014. Non-destructive hydrocarbon extraction from *Botryococcus braunii* BOT-22 (race B). J. Appl. Phycol. 26, 1453–1463.

Molnár, I., Lopez, D., Wisecaver, J.H., Devarenne, T.P., Weiss, T.L., Pellegrini, M., Hackett, J.D., 2012. Bio-crude transcriptomics: gene discovery and metabolic network

reconstruction for the biosynthesis of the terpenome of the hydrocarbon oil-producing green alga, *Botryococcus braunii* race B (Showa). BMC Genomics 13, 576.

Moutel, B., André, M., Kucma, D., Legrand, J., Grizeau, D., Pruvost, J., Gonçalves, O., 2015. Assessing the biofuel production potential of *Botryococcus braunii* strains by sensitive rapid qualitative chemotyping using chemometrically-assisted gas chromatography–mass spectrometry. Algal Res. 11, 33–42.

Murata, K., Liu, Y., Watanabe, M.M., Inaba, M., Takahara, I., 2014. Hydrocracking of algae oil into aviation fuel-range hydrocarbons using a Pt–Re catalyst. Energy Fuels 28, 6999–7006.

Nagano, S., Yamamoto, S., Nagakubo, M., Atsumi, K., Watanabe, M.M., 2012. Physical properties of hydrocarbon oils produced by *Botryococcus braunii*: density, kinematic viscosity, surface tension, and distillation properties. Procedia Environ. Sci., Innovative Researches on Algal Biomass 15, 73–79.

Nakazawa, A., Matsuura, H., Kose, R., Kato, S., Honda, D., Inouye, I., Kaya, K., Watanabe, M.M., 2012. Optimization of culture conditions of the thraustochytrid *Aurantiochytrium* sp. strain 18W-13a for squalene production. Bioresour. Technol., Special Issue: Innovative Researches on Algal Biomass 109, 287–291.

Nedbal, L., Cervený, J., Keren, N., Kaplan, A., 2010. Experimental validation of a nonequilibrium model of CO_2 fluxes between gas, liquid medium, and algae in a flat-panel photobioreactor. J. Ind. Microbiol. Biotechnol. 37, 1319–1326.

Niehaus, T.D., Kinison, S., Okada, S., Yeo, Y., Bell, S.A., Cui, P., Devarenne, T.P., Chappell, J., 2012. Functional identification of triterpene methyltransferases from *Botryococcus braunii* race B. J. Biol. Chem. 287, 8163–8173.

Niehaus, T.D., Okada, S., Devarenne, T.P., Watt, D.S., Sviripa, V., Chappell, J., 2011. Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus braunii*. Proc. Natl. Acad. Sci. 108, 12260–12265.

Niitsu, R., Kanazashi, M., Matsuwaki, I., Ikegami, Y., Tanoi, T., Kawachi, M., Watanabe, M.M., Kato, M., 2012. Changes in the hydrocarbon-synthesizing activity during growth of *Botryococcus braunii* B70. Bioresour. Technol. 109, 297–299.

Noguchi, T., Kakami, F., 1999. Transformation of trans-Golgi network during the cell cycle in a green alga,*Botryococcus braunii*. J. Plant Res. 112, 175–186.

Ohlrogge, J.B., Kuhn, D.N., Stumpf, P.K., 1979. Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc. Natl. Acad. Sci. U. S. A. 76, 1194–8.

Okada, S., Devarenne, T.P., Chappell, J., 2000. Molecular characterization of squalene synthase from the green microalga *Botryococcus braunii*, race B. Arch. Biochem. Biophys. 373, 307–317.

Okada, S., Devarenne, T.P., Murakami, M., Abe, H., Chappell, J., 2004. Characterization of botryococcene synthase enzyme activity, a squalene synthase-like activity from the green microalga *Botryococcus braunii*, Race B. Arch. Biochem. Biophys. 422, 110–118.

Okada, S., Murakami, M., Yamaguchi, K., 1997. Hydrocarbon production by the yayoi, a new strain of the green microalga *Botryococcus braunii*. Appl. Biochem. Biotechnol. 67, 79–86.

Okada, S., Murakami, M., Yamaguchi, K., 1995. Hydrocarbon composition of newly isolated strains of the green microalga *Botryococcus braunii*. J. Appl. Phycol. 7, 555–559.

Ozkan, A., Kinney, K., Katz, L., Berberoglu, H., 2012. Reduction of water and energy requirement of algae cultivation using an algae biofilm photobioreactor. Bioresour. Technol. 114, 542–548.

Papa, R.D., Wu, J.-T., Baldia, S., Cho, C., Cruz, M.A., Saguiguit, A., Aquino, R., 2008. Blooms of the colonial green algae, *Botryococcus braunii* Kützing, in Paoay Lake, Luzon Island, Philippines. Philipp. J. Syst. Biol. 2, 21–31.

Park, M.-O., Heguri, K., Hirata, K., Miyamoto, K., 2005. Production of alternatives to fuel oil from organic waste by the alkane-producing bacterium, *Vibrio furnissii* M1. J. Appl. Microbiol. 98, 324–331.

Peccia, J., Haznedaroglu, B., Gutierrez, J., Zimmerman, J.B., 2013. Nitrogen supply is an important driver of sustainable microalgae biofuel production. Trends Biotechnol. 31, 134–138.

Pereira, S.L., Leonard, A.E., Mukerji, P., 2003. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. Prostaglandins Leukot. Essent. Fatty Acids 68, 97–106.

Phillips, T., Chase, M., Wagner, S., Renzi, C., Powell, M., DeAngelo, J., Michels, P., 2013. Use of *in situ* solid-phase adsorption in microbial natural product fermentation development. J. Ind. Microbiol. Biotechnol. 40, 411–425.

Platt, T., Gallegos, C., Harrison, W., 1980. Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. J. Mar. Res. 38, 687–701.

Pollard, D.J., Woodley, J.M., 2007. Biocatalysis for pharmaceutical intermediates: the future is now. Trends Biotechnol. 25, 66–73.

Pottier, L., Pruvost, J., Deremetz, J., Cornet, J.-F., Legrand, J., Dussap, C. g., 2005. A fully predictive model for one-dimensional light attenuation by *Chlamydomonas reinhardtii* in a torus photobioreactor. Biotechnol. Bioeng. 91, 569–582.

Procházková, G., Brányiková, I., Zachleder, V., Brányik, T., 2014. Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. J. Appl. Phycol. 26, 1359–1377.

Pruvost, J., Pottier, L., Legrand, J., 2006. Numerical investigation of hydrodynamic and mixing conditions in a torus photobioreactor. Chem. Eng. Sci. 61, 4476–4489.

Qin, J.G., Li, Y., 2006. Optimization of the growth environment of *Botryococcus braunii* strain CHN 357. J. Freshw. Ecol. 21, 169–176.

Ranga Rao, A., Ravishankar, G.A., Sarada, R., 2012. Cultivation of green alga *Botryococcus braunii* in raceway, circular ponds under outdoor conditions and its growth, hydrocarbon production. Bioresour. Technol. 123, 528–533.

Ranga Rao, A., Sarada, R., Ravishankar, G.A., 2007. Influence of CO_2 on growth and hydrocarbon production in *Botryococcus braunii*. J. Microbiol. Biotechnol. 17, 414–419.

Rao, A.R., Sarada, R., Baskaran, V., Ravishankar, G.A., 2006. Antioxidant activity of *Botryococcus braunii* Extract Elucidated *in vitro* Models. J. Agric. Food Chem. 54, 4593–4599.

Raven, J.A., 2010. Inorganic carbon acquisition by eukaryotic algae: four current questions. Photosynth. Res. 106, 123–134.

Ribeiro, B.D., Barreto, D.W., Coelho, M.A.Z., 2011. Technological aspects of β-carotene production. Food Bioprocess Technol. 4, 693–701.

Ross, A.B., Biller, P., Kubacki, M.L., Li, H., Lea-Langton, A., Jones, J.M., 2010. Hydrothermal processing of microalgae using alkali and organic acids. Fuel 89, 2234–2243.

Ruangsomboon, S., 2015. Effects of different media and nitrogen sources and levels on growth and lipid of green microalga *Botryococcus braunii* KMITL and its biodiesel properties based on fatty acid composition. Bioresour. Technol. 191, 377–384.

Ruangsomboon, S., 2012. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. Bioresour. Technol. 109, 261–265.

Rutherford, S., Moore, I., 2002. The *Arabidopsis* Rab GTPase family: another enigma variation. Curr. Opin. Plant Biol. 5, 518–528.

R. Wolf, F., K. Nemethy, E., H. Blanding, J., A. Bassham, J., 1985. Biosynthesis of unusual acyclic isoprenoids in the Alga *Botryococcus braunii*. Phytochemistry 24, 733–737.

Saga, K., Hasegawa, F., Miyagi, S., Atobe, S., Okada, S., Imou, K., Osaka, N., Yamagishi, T., 2015. Comparative evaluation of wet and dry processes for recovering hydrocarbon from *Botryococcus braunii*. Appl. Energy 141, 90–95.

Sakamoto, K., Baba, M., Suzuki, I., Watanabe, M.M., Shiraiwa, Y., 2012. Optimization of light for growth, photosynthesis, and hydrocarbon production by the colonial microalga *Botryococcus braunii* BOT-22. Bioresour. Technol. 110, 474–479.

Samorì, C., Torri, C., Samorì, G., Fabbri, D., Galletti, P., Guerrini, F., Pistocchi, R., Tagliavini, E., 2010. Extraction of hydrocarbons from microalga *Botryococcus braunii* with switchable solvents. Bioresour. Technol. 101, 3274–3279.

Satoh, A., Kurano, N., Miyachi, S., 2001. Inhibition of photosynthesis by intracellular carbonic anhydrase in microalgae under excess concentrations of CO_2. Photosynth. Res. 68, 215–224.

Sato, Y., Ito, Y., Okada, S., Murakami, M., Abe, H., 2003. Biosynthesis of the triterpenoids, botryococcenes and tetramethylsqualene in the B race of *Botryococcus braunii* via the non-mevalonate pathway. Tetrahedron Lett. 44, 7035–7037.

Scherholz, M.L., Curtis, W.R., 2013. Achieving pH control in microalgal cultures through fed-batch addition of stoichiometrically-balanced growth media. BMC Biotechnol. 13, 39.

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., Smith, A.G., 2010. Biodiesel from algae: challenges and prospects. Curr. Opin. Biotechnol. 21, 277–286.

Serrano-Ruiz, J.C., Dumesic, J.A., 2011. Catalytic routes for the conversion of biomass into liquid hydrocarbon transportation fuels. Energy Env. Sci 4, 83–99.

Sforza, E., Urbani, S., Bertucco, A., 2014. Evaluation of maintenance energy requirements in the cultivation of *Scenedesmus obliquus*: effect of light intensity and regime. J. Appl. Phycol. 27, 1453–1462.

Shen, Y., Zhang, H., Xu, X., Lin, X., 2014. Biofilm formation and lipid accumulation of attached culture of *Botryococcus braunii*. Bioprocess Biosyst. Eng. 38, 481–488.

Shiho, M., Kawachi, M., Horioka, K., Nishita, Y., Ohashi, K., Kaya, K., Watanabe, M.M., 2012. Business evaluation of a green microalgae *Botryococcus braunii* oil production system. Procedia Environ. Sci., Innovative Researches on Algal Biomass 15, 90–109.

Shimamura, R., Watanabe, S., Sakakura, Y., Shiho, M., Kaya, K., Watanabe, M.M., 2012. Development of *Botryococcus* seed culture system for future mass culture. Procedia Environ. Sci., Innovative Researches on Algal Biomass 15, 80–89.

Sim, S.-J., An, J.-Y., Kim, B.-W., 2001. Two-phase extraction culture of *Botryococcus braunii* producing long-chain unsaturated hydrocarbons. Biotechnol. Lett. 23, 201–205.

Singh, S.P., Singh, D., 2010. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: a review. Renew. Sustain. Energy Rev. 14, 200–216.

Singh, Y., Kumar, H.D., 1992. Lipid and hydrocarbon production by *Botryococcus* spp. under nitrogen limitation and anaerobiosis. World J. Microbiol. Biotechnol. 8, 121–124.

Solovchenko, A.E., Khozin-Goldberg, I., Cohen, Z., Merzlyak, M.N., 2009. Carotenoid-to-chlorophyll ratio as a proxy for assay of total fatty acids and arachidonic acid content in the green microalga *Parietochloris incisa*. J. Appl. Phycol. 21, 361–366.

Solovchenko, A.E., Khozin-Goldberg, I., Didi-Cohen, S., Cohen, Z., Merzlyak, M.N., 2008. Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incisa*. J. Appl. Phycol. 20, 245–251.

Stewart, P.S., 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotechnol. Bioeng. 59, 261–272.

Suzuki, R., Ito, N., Uno, Y., Nishii, I., Kagiwada, S., Okada, S., Noguchi, T., 2013. Transformation of lipid bodies related to hydrocarbon accumulation in a green alga, *Botryococcus braunii* (race B). PloS One 8, e81626.

Suzuki, R., Nishii, I., Noguchi, T., 2015. 3D analysis reveals the structural transformation of the endoplasmic reticulum involved in hydrocarbon secretion in a green alga, *Botryococcus braunii*. Microscopy 64, i131–i131.

Sydney, E.B., da Silva, T.E., Tokarski, A., Novak, A.C., de Carvalho, J.C., Woiciecohwski, A.L., Larroche, C., Soccol, C.R., 2011. Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. Appl. Energy, Special Issue of Energy from algae: Current status and future trends 88, 3291–3294.

Sydney, E.B., Sturm, W., de Carvalho, J.C., Thomaz-Soccol, V., Larroche, C., Pandey, A., Soccol, C.R., 2010a. Potential carbon dioxide fixation by industrially important microalgae. Bioresour. Technol. 101, 5892–5896.

Sydney, E.B., Sturm, W., de Carvalho, J.C., Thomaz-Soccol, V., Larroche, C., Pandey, A., Soccol, C.R., 2010b. Potential carbon dioxide fixation by industrially important microalgae. Bioresour. Technol. 101, 5892–5896.

Talebi, A.F., Mohtashami, S.K., Tabatabaei, M., Tohidfar, M., Bagheri, A., Zeinalabedini, M., Hadavand Mirzaei, H., Mirzajanzadeh, M., Malekzadeh Shafaroudi, S., Bakhtiari, S., 2013. Fatty acids profiling: a selective criterion for screening microalgae strains for biodiesel production. Algal Res. 2, 258–267.

Talukdar, J., Kalita, M.C., Goswami, B.C., 2013. Characterization of the biofuel potential of a newly isolated strain of the microalga *Botryococcus braunii* Kützing from Assam, India. Bioresour. Technol. 149, 268–275.

Talukdar, J., Kalita, M.C., Goswami, B.C., Hong, D.D., Das, H.C., 2014. Liquid hydrocarbon production potential of a novel strain of the mcroalga *Botryococcus braunii*: assessing the reliability of *in situ* hydrocarbon recovery by wet process solvent extraction. Energy Fuels 28, 3747–3758.

Tanabe, Y., Ioki, M., Watanabe, M.M., 2014. The fast-growing strain of hydrocarbon-rich green alga *Botryococcus braunii*, BOT-22, is a vitamin B_{12} autotroph. J. Appl. Phycol. 26, 9–13.

Tanabe, Y., Kato, S., Matsuura, H., Watanabe, M.M., 2012. A *Botryococcus* strain with bacterial ectosymbionts grows fast and produces high amount of hydrocarbons. Procedia Environ. Sci., Innovative Researches on Algal Biomass 15, 22–26.

Tanaka, H., 1982. Oxygen transfer in broths of plant cells at high density. Biotechnol. Bioeng. 24, 425–442.

Tang, H., Chen, M., Simon Ng, K. y., Salley, S.O., 2012. Continuous microalgae cultivation in a photobioreactor. Biotechnol. Bioeng. 109, 2468–2474.

Tanoi, T., Kawachi, M., Watanabe, M.M., 2014. Iron and glucose effects on the morphology of *Botryococcus braunii* with assumption on the colony formation variability. J. Appl. Phycol. 26, 1–8.

Tanoi, T., Kawachi, M., Watanabe, M.M., 2011. Effects of carbon source on growth and morphology of *Botryococcus braunii*. J. Appl. Phycol. 23, 25–33.

Templier, J., Largeau, C., Casadevall, E., 1984. Mechanism of non-isoprenoid hydrocarbon biosynthesis in *Botryococcus braunii*. Phytochemistry 23, 1017–1028.

Templier, J., Largeau, C., Casadevall, E., Berkaloff, C., 1992. Chemical inhibition of resistant biopolymers in outer walls of the A and B races of *Botryococcus braunii*. Phytochemistry 31, 4097–4104.

Templier, J.O.L.L.E., Largeau, C., 1991. Biosynthesis of n-alkatrienes in *Botryococcus braunii*. Phytochemistry 30, 2209–2215.

Thierie, J., 2015. Luedeking-Piret related method for enhancement of butyrate production by a crabtree-positive-like bacterial consortium cultivated in a chemostat. Res. Rev. J. Microbiol. Biotechnol. 4, 52–56.

Thompson, G.A., 1996. Lipids and membrane function in green algae. Biochim. Biophys. Acta 1302, 17–45.

Tikhonov, A.N., 2013. pH-Dependent regulation of electron transport and ATP synthesis in chloroplasts. Photosynth. Res. 116, 511–534.

Trentacoste, E.M., Shrestha, R.P., Smith, S.R., Glé, C., Hartmann, A.C., Hildebrand, M., Gerwick, W.H., 2013. Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. Proc. Natl. Acad. Sci. 110, 19748–19753.

Uno, Y., Nishii, I., Kagiwada, S., Noguchi, T., 2015. Colony sheath formation is accompanied by shell formation and release in the green alga *Botryococcus braunii* (race B). Algal Res. 8, 214–223.

Versteegh, G.J.M., Blokker, P., 2004. Resistant macromolecules of extant and fossil microalgae. Phycol. Res. 52, 325–339.

Villarreal-Rosales, E., Metzger, P., Casadevall, E., 1992. Ether lipid production in relation to growth in *Botryococcus braunii*. Phytochemistry 31, 3021–3027.

Volkman, J.K., 2014. Acyclic isoprenoid biomarkers and evolution of biosynthetic pathways in green microalgae of the genus *Botryococcus*. Org. Geochem. 75, 36–47.

Wang, B., Li, Y., Wu, N., Lan, C.Q., 2008. CO_2 bio-mitigation using microalgae. Appl. Microbiol. Biotechnol. 79, 707–718.

Wang, C.-W., 2015. Lipid droplets, lipophagy, and beyond. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids. doi:http://dx.doi.org/10.1016/ j.bbalip.2015.12.010

Wang, W., Han, F., Li, Y., Wu, Y., Wang, J., Pan, R., Shen, G., 2014. Medium screening and optimization for photoautotrophic culture of *Chlorella pyrenoidosa* with high lipid productivity indoors and outdoors. Bioresour. Technol. 170, 395–403.

Wang, X., Kolattukudy, P.E., 1995. Solubilization and purification of aldehyde-generating fatty acyl-CoA reductase from green alga *Botryococcus braunii*. FEBS Lett. 370, 15–18.

Watanabe, H., Li, D., Nakagawa, Y., Tomishige, K., Kaya, K., Watanabe, M.M., 2014. Characterization of oil-extracted residue biomass of *Botryococcus braunii* as a biofuel feedstock and its pyrolytic behavior. Appl. Energy 132, 475–484.

Watanabe, H., Li, D., Nakagawa, Y., Tomishige, K., Watanabe, M.M., 2015. Catalytic gasification of oil-extracted residue biomass of *Botryococcus braunii*. Bioresour. Technol. doi:10.1016/j.biortech.2015.03.034

Weiss, T.L., Chun, H.J., Okada, S., Vitha, S., Holzenburg, A., Laane, J., Devarenne, T.P., 2010. Raman spectroscopy analysis of botryococcene hydrocarbons from the green microalga *Botryococcus braunii*. J. Biol. Chem. 285, 32458–32466.

Weiss, T.L., Johnston, J.S., Fujisawa, K., Okada, S., Devarenne, T.P., 2011. Genome size and phylogenetic analysis of the A and L races of *Botryococcus braunii*. J. Appl. Phycol. 23, 833–839.

Weiss, T.L., Roth, R., Goodson, C., Vitha, S., Black, I., Azadi, P., Rusch, J., Holzenburg, A., Devarenne, T.P., Goodenough, U., 2012. Colony organization in the green alga *Botryococcus braunii* (race B) is specified by a complex extracellular matrix. Eukaryot. Cell 11, 1424–1440.

Weiss, T.L., Spencer Johnston, J., Fujisawa, K., Sumimoto, K., Okada, S., Chappell, J., Devarenne, T.P., 2010. Phylogenetic placement, genome size and GC content of the liquid-hydrocarbon producing green microalga *Botryococcus braunii* strain Berkeley (Showa) (Chlorophyta). J. Phycol. 46, 534–540.

White, J.D., Somers, T.C., Reddy, G.N., 1992. Degradation and absolute configurational assignment to C_{34}-botryococcene. J. Org. Chem. 57, 4991–4998.

White, S., Anandraj, A., Bux, F., 2011. PAM fluorometry as a tool to assess microalgal nutrient stress and monitor cellular neutral lipids. Bioresour. Technol. 102, 1675–1682.

Wood, A.M., 1979. Chlorophyll *a:b* ratios in marine planktonic algae. J. Phycol. 15, 330–332.

Xu, L., Wang, F., Guo, C., Liu, C.-Z., 2012a. Improved algal oil production from *Botryococcus braunii* by feeding nitrate and phosphate in an airlift bioreactor. Eng. Life Sci. 12, 171–177.

Xu, L., Wang, F., Guo, C., Liu, C.Z., 2012b. Improved algal oil production from *Botryococcus braunii* by feeding nitrate and phosphate in an airlift bioreactor. Eng. Life Sci. 12, 171–177.

Yamamoto, S., Mandokoro, Y., Nagano, S., Nagakubo, M., Atsumi, K., Watanabe, M.M., 2014. Catalytic conversion of *Botryococcus braunii* oil to diesel fuel under mild reaction conditions. J. Appl. Phycol. 26, 55–64.

Yeesang, C., Cheirsilp, B., 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresour. Technol. 102, 3034–3040.

Yen, C.-L.E., Stone, S.J., Koliwad, S., Harris, C., Farese, R.V., 2008. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. J. Lipid Res. 49, 2283–2301.

Ying, K., Al-Mashhadani, M.K.H., Hanotu, J.O., Gilmour, D.J., Zimmerman, W.B., 2013. Enhanced mass transfer in microbubble driven airlift bioreactor for microalgal culture. Engineering 05, 735–743.

Yonezawa, N., Matsuura, H., Shiho, M., Kaya, K., Watanabe, M.M., 2012. Effects of soybean curd wastewater on the growth and hydrocarbon production of *Botryococcus braunii* strain BOT-22. Bioresour. Technol., Special Issue: Innovative Researches on Algal Biomass 109, 304–307.

Yoo, C., Jun, S.-Y., Lee, J.-Y., Ahn, C.-Y., Oh, H.-M., 2010. Selection of microalgae for lipid production under high levels carbon dioxide. Bioresour. Technol. 101 Suppl, S71–S74.

Yoshida, M., Tanabe, Y., Yonezawa, N., Watanabe, M.M., 2012. Energy innovation potential of oleaginous microalgae. Biofuels 3, 761–781.

Yoshimura, T., Okada, S., Honda, M., 2013. Culture of the hydrocarbon producing microalga *Botryococcus braunii* strain Showa: optimal CO_2, salinity, temperature, and irradiance conditions. Bioresour. Technol. 133, 232–239.

Zhang, F., Cheng, L., Gao, W., Xu, X., Zhang, L., Chen, H., 2011. Mechanism of lipid extraction from *Botryococcus braunii* FACHB 357 in a biphasic bioreactor. J. Biotechnol. 154, 281–284.

Zhang, F., Cheng, L.-H., Xu, X.-H., Zhang, L., Chen, H.-L., 2013. Application of membrane dispersion for enhanced lipid milking from *Botryococcus braunii* FACHB 357. J. Biotechnol. 165, 22–29.

Zhang, H., Wang, W., Li, Y., Yang, W., Shen, G., 2011. Mixotrophic cultivation of *Botryococcus braunii*. Biomass Bioenergy 35, 1710–1715.

Zhang, K., Kojima, E., 1998. Effect of light intensity on colony size of microalga *Botryococcus braunii* in bubble column photobioreactors. J. Ferment. Bioeng. 86, 573–576.

Zhao, S., Wang, L., Liu, L., Liang, Y., Sun, Y., Wu, J., 2014. Both the mevalonate and the non-mevalonate pathways are involved in ginsenoside biosynthesis. Plant Cell Rep. 33, 393–400.

Zhila, N.O., Kalacheva, G.S., Volova, T.G., 2005. Effect of nitrogen limitation on the growth and lipid composition of the green alga *Botryococcus braunii* Kutz IPPAS H-252. Russ. J. Plant Physiol. 52, 311–319.

Zijffers, J.-W.F., Schippers, K.J., Zheng, K., Janssen, M., Tramper, J., Wijffels, R.H., 2010. Maximum photosynthetic yield of green microalgae in photobioreactors. Mar. Biotechnol. 12, 708–718.

Zou, J., Bi, G., 2016. Complete mitochondrial genome of a hydrocarbon-producing green alga *Botryococcus braunii* strain Showa. Mitochondrial DNA 27, 2619–2620.

List of publications and presentations

Papers for Journals

1. **JIN J**, DUPRE C, YONEDA K , WATANABE MM, LEGRAND J, GRIZEAU D. Characteristics of extracellular hydrocarbon-rich microalga *Botryococcus braunii* for biofuels production: recent advances and opportunities. Process Biochemistry, 2015, doi:10.1016/j.procbio.2015.11.026

2. **JIN J**, DUPRE C, LEGRAND J, GRIZEAU D. Extracellular hydrocarbons and intracellular lipids accumulations are related to nutrient sufficient conditions in pH controlled chemostat cultures of the microalga *Botryococcus braunii* 30.81. Algal Research. 2016. 17: 244-252.

3. **JIN J**. et al. Extracellular hydrocarbon and intracellular lipids production in relation to specific growth rate the microalgae *Botryococcus braunii* SAG 30.81in continuous cultures. 2016, in writing.

Presentations at congress

1. **JIN J**, DUPRE C, LEGRAND J, GRIZEAU D. Kinetics of biomass and hydrocarbon oils production of microalgae *Botryococcus braunii* in continuous culture. Oral presentation at 7th Asia-Pacific Biotech congress, July 13 -15, 2015, Beijing, China.

2. **JIN J**, DUPRE C, LEGRAND J, GRIZEAU D. Contribution of continuous culture studies for improving *Botryococcus braunii* production: a case study of effect o of pH. Poster presentation at ECCE10+ECAD3+EPIC5, September 27 - October1, 2015 Nice, France

3. **JIN J**, DUTERTRE S, YONEDA K , LEGRAND J, SUZUKI I, WATANABE MM, GRIZEAU D, DUPRE C. Characteristics of lipids body and extracellular matrix in hydrocarbon-rich microalga *Botryococccus braunii* race A and race B strains. Poster presentation at GEN2BIO, Le Congrès Biotech de Biogenouest, March 31 2016, Saint Brieuc France.

4. GRIZEAU D, KANG W, **JIN J**, DUPRE C. Procédés adaptés à l'intensification de productions d'exométabolites par des microalgues et cyanobactéries; contraintes et perspectives. Presentation at Congrès International de Biotechnologie et de Valorisation des Bio-Ressources, Mars 24 -27 2016, Hammamet (Tunisie).

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UNIVERSITE BRETAGNE LOIRE

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Jian JIN

Etude cinétique des productions de biomasse et d'hydrocarbures dans des cultures de la microalgue *Botryococcus braunii* en mode chemostat : une approche physiologique

Kinetic study of biomass and hydrocarbon production in chemostat cultures of the microalga *Botryococcus braunii* : a physiological approach

Résumé

L'espèce *Botryococcus braunii* a la propriété d'accumuler des quantités importantes d'hydrocarbures à longue chaîne. L'objectif est d'étudier les effets de variables opératoires en photobioréacteur sur les productivités en biomasse et hydrocarbures de cultures continues de deux souches de *B. braunii*, l'une de race A et l'autre de race B accumulant respectivement soit des alkadiènes et alkatriènes soit des botryococcènes.

Les deux principaux hydrocarbures contenus dans la biomasse de la souche de race A SAG 30.81, sont identifiés comme étant le nonacosadiene et l'hentriacontadiene. L'analyse d'états stationnaires successifs montre que ses productivités en biomasse et hydrocarbures sont pH-dépendantes avec un optimum à pH 6.5, effet corrélé à la concentration résiduelle en $CO_{2\,(aq)}$ des cultures. Par contre, le taux de croissance spécifique, le flux lumineux et l'approvisionnement en azote ne modifient pas les teneurs en hydrocarbures. Les données répondent au modèle de Luedeking-Piret, confirmant que les cinétiques de production des hydrocarbures sont principalement associées à la croissance des cultures et à une surproduction en lipides intracellulaires, indépendamment du taux d'azote du milieu de culture ou du flux lumineux.

Les productivités en biomasse, hydrocarbures et lipides intracellulaires sont plus élevées avec la souche de race B BOT 22. Les teneurs significativement plus élevées en acides gras totaux et en acide oléique de la souche SAG 30.81 semblent indicatrices des voies différentes de biosynthèse des hydrocarbures des deux souches.

Ces caractéristiques physiologiques sont discutées dans la perspective de la sélection de conditions optimales d'exploitation.

Mots clés

microalgues, *Botryococcus*, hydrocarbures, chemostat, lipides, photobioréacteur

Abstract

The microalgal species *Botryococcus braunii* is attracting particular interest due to its ability to accumulate large amounts of long-chain hydrocarbons in an extracellular matrix. This study aimed to investigate the effects of operative factors on biomass and hydrocarbon production of two strains within the continuous cultures at successive steady-states in a controlled PBR. A comparative study was carried out on a race A strain and a race B strain, accumulating respectively either the alkadienes and alkatrienes or botryococcenes hydrocarbons.

The predominant hydrocarbons in the race A strain SAG 30.81 were determined as nonacosadiene and hentriacontadiene. Its biomass and hydrocarbon productivities were shown to be pH-dependent with an optimum at pH 6.5. This effect was related to the dissolved CO_2 concentration. Data from successive steady-states confirmed, according to the Luedeking-Piret model, that the hydrocarbon production is mainly growth associated, whatever nitrogen conditions. By contrast, the hydrocarbon content was relative constant irrespective of growth rates, light intensity and nitrogen conditions. Intracellular lipids were overproduced even in nitrogen sufficient conditions at high specific light supply. Higher biomass, hydrocarbon and lipids productivities were obtained with the race B strain BOT 22 than with the race A strain SAG 30.81. The total fatty acids and oleic acid contents in race A were significantly higher than in race B, supporting their respective hydrocarbons biosynthesis pathways.

These physiological features are discussed in view of the selection of optimal operative conditions for efficient hydrocarbon production by microalgae.

Key Words

microalgae, *Botryococcus,* hydrocarbons, chemostat, lipids, photobioreactor