UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

Maestría y Doctorado en Ciencias Bioquímicas

Efecto de la relación C/N y la velocidad de crecimiento sobre la composición macromolecular y el perfil proteómico de *Neochloris oleoabundans* en condiciones heterotróficas

TESIS

QUE PARA OPTAR POR EL GRADO DE:

Doctor en Ciencias

P R E S E N T A:

M. en C. Daniela Morales Sánchez

TUTOR PRINCIPAL:
Dr. Alfredo Martínez Jiménez
Instituto de Biotecnología – Universidad Nacional Autónoma de México

MIEMBROS DEL COMITÉ TUTOR:
Dra. Esperanza Martínez Romero
Centro de Ciencias Genómicas – Universidad Nacional Autónoma de México
Dr. Sergio Revah Moiseev
Universidad Autónoma Metropolitana – Cuajimalpa

México, D.F. Enero 2014

El presente trabajo se realizó bajo la dirección del Dr. Alfredo Martínez Jiménez en el Departamento de Ingeniería Celular y Biocatálisis y la Unidad de Escalamiento y Planta Piloto del Instituto de Biotecnología de la Universidad Nacional Autónoma de México.

Para la realización del mismo se contó con el financiamiento del Consejo Nacional de Ciencia y Tecnología (CONACyT) a través de la beca CONACyT No. De Registro 170123 y de la Universidad Nacional Autónoma de México a través del proyecto DGAPA/PAPIIT/UNAM IT200312.

“Soy el amo de mi destino, soy el capitán de mi alma”

Nelson Mandela (Madiba)

Con mucho amor y cariño, dedico este logro a:

Mis papás **Doña Coqui** y **Don Javi**, por su apoyo incondicional en cada paso de mi vida, los acertados y los no tanto. Por ser ejemplo de fortaleza y ayudarme a levantarme cada vez que me caigo.

Mis hermanos **Gabi**, **Ale** y **Javi**, por estar siempre conmigo en las buenas y malas. Por los buenos ratos de peleas, juegos, chistes y los muy elaborados pero buenos apodos.

A mis pequeñas terroristas **Ámbar**, **Jade** y **Maya** y a mis negritos **Gali**, **Gabriel**, **Gael** y **Gala** (mis **Ga4AmJaMy)** por inspirarme a ser mejor persona para ellos y por experimentar con la paciencia de su tía.

AGRADECIMIENTOS

A mi tutor, el Dr. Alfredo Martínez Jiménez

A mi co-tutor, el Dr. John Kyndt

A los miembros del comité tutoral:
Dr. Sergio Revah Moiseev
Dr. Rafael Vázquez Duhalt
Dra. Esperanza Martínez Romero

A los miembros del jurado:
Dra. María Luisa Villareal Ortega
Dr. Gerardo Corzo Burguete
Dr. Leobardo Serrano Carreón
Dr. José Luis Reyes Taboada
Dr. John Kyndt

A la Unidad de Escalamiento y Planta Piloto
Q. F. B. Myriam Ortíz
I. Q. Verónica Albíter
Sr. Arturo Escobar

A la C. D. Mercedes Enzaldo Cruz del laboratorio 4 del IBT/UNAM

De la Unidad de Escalamiento y Planta Piloto del IBT, al M. en C. Raunel Tinoco Valencia por su apoyo con el análisis del perfil de los ácidos grasos, al Dr. Leobardo Serrano Carreón y el Biol. Mario Caro Bermúdez por su apoyo técnico en las fermentaciones.

Al Dr. José Utrilla Carreri de la Universidad de California, San Diego, EUA, por su apoyo técnico en la determinación del transporte de glucosa transmembranal.

A Andrea Hunt y George Tsaprailis del Instituto BIO5 de la Universidad de Arizona por su apoyo técnico con los experimentos de proteómica.

Al grupo de la Dra. Kimberly Ogden de la Universidad de Arizona por su apoyo durante mi estancia en dicha universidad.

A mis BFE:
Anilú y **Pancho** gracias por su compañía, su amistad y por que juntos somos la escoria del IBT. Gracias por los momentos de cine, de juegos, de comidas, por las alegrías y lágrimas compartidas y por ser parte de mi vida.

A mis amigos:
Cris La Flaca, Cris La Común, Rau, Mars, Tabi, Martha, Poncho, Abraham, Chio, Pablo, Blanca, Liz, Karen, Pau, Checho, Karina, Cessna, Ale, Estefa, Mitsue, Sara, Sele, Juanito, Jaz, Remi y Carmen. De Arizona a Los Griffins, SarahMarie, Kelsey, Marissa y JoAnne.

A mi familia:
Mi abuelita Mechita, mis hermanos David y Bere, mi madrina Consuelito, mis tíos Pepe y Mary, mis primos Chela, Luis Fernando, Erika y César con sus retoños.

Especially, I thank **John** for changing my academic and personal life, love you my dear friend.

ÍNDICE

INDICE DE FIGURAS

Figuras del manuscrito: **Producción de biodiesel a partir de microalgas:**

Figuras del manuscrito: **Heterotrophic growth of *Neochloris oleoabundans* using glucose as carbon source:**

Figuras del manuscrito: **Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid and carbohydrate production (accumulation):**

Figuras del manuscrito: **Lipid accumulation triggered by pyruvate dehydrogenase pool accumulation and high pentose phosphate pathway activation was revealed through proteomic studies in heterotrophic N-limited cultures of *Neochloris oleoabundans:***

Figuras de la tesis:

X.3 Artículos de divulgación de la ciencia:

INDICE DE TABLAS

Tablas del manuscrito: **Producción de biodiesel a partir de microalgas:**

Tablas del manuscrito: **Heterotrophic growth of *Neochloris oleoabundans* using glucose as carbon source:**

Tablas del manuscrito: **Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid and carbohydrate production (accumulation):**

PRESENTACIÓN DE LA TESIS

En esta sección se describe la forma en la que está estructurada la presente tesis. Es importante indicar que todas las secciones están escritas en español e inglés debido a que los integrantes del jurado de la defensa de la tesis están conformados por profesores de habla hispana y uno de habla inglesa.

En el capitulo I se presenta el resumen general del trabajo. En el capitulo II se presenta una introducción y, a manera de antecedentes, en el capitulo III se presenta un capitulo de un libro publicado en español, relacionado con la producción de biodiesel a partir de microalgas. La justificación y la hipótesis para la realización de este trabajo se plantean en los capitulos IV y V, respectivamente. En el capítulo VI se presentan el objetivo general y los particulares que se establecieron para el desarrollo experimental.

En el capítulo VII se presentan las publicaciones originadas a partir de esta investigación. Se presentan los datos experimentales obtenidos en este trabajo, mostrados en detalle en tres manuscritos, uno publicado y dos sometidos. Con el fin de facilitar la lectura y la secuencia de la tesis, en el capitulo VIII, el de resumen de resultados, se presentan de manera sintetizada los principales resultados reportados en los manuscritos antes mencionados, y se hace una discusión general acerca de los mismos.

En el capítulo IX se presentan las principales conclusiones y aportaciones y en el X las sugerencias para futuras investigaciones a partir de ideas generadas en el presente trabajo. Del capitulo XI al XVIII se presentan las secciones correspondientes en inglés.

En el capítulo XIX se muestra la bibliografía citada en el capítulo I y el XII (introducción, en español e inglés, respectivamente), se excluyen las que son presentadas en los manuscritos.

Finalmente en el capítulo XX, el de apéndices, se presentan dos notas de divulgación científica publicadas en un periódico local de Cuernavaca, Morelos y los trabajos presentados en congresos nacionales (1) e internacionales (4).

I. RESUMEN

En este trabajo se evaluó la capacidad de *Neochloris oleoabundans* para crecer en condiciones heterotróficas y se estudio el efecto de la relación C/N y la velocidad de crecimiento sobre la composición macromolecular (proteínas, carbohidratos y lípidos), los parámetros cinéticos y estequiométricos, el perfil de ácidos grasos y el perfil proteómico de esta microalga. Cultivos en matraces, con glucosa, fructosa, sacarosa, xilosa, arabinosa, glicerol, ácido acético, celobiosa, lactosa y medio mineral (BBM), demostraron que *N. oleoabundans* es capaz de crecer en estricta oscuridad utilizando glucosa o celobiosa como únicas fuentes de carbono. Bajo una relación C/N balanceada de 17 y utilizando cultivos lote en bioreactores de 4-litros con 3 g_{GLC}/L de glucosa y 0.5 g_{NIT}/L de nitrato de sodio, se obtuvo una masa celular máxima en base seca (DCW) de 1.72 g_{DCW}/L, siendo las proteínas el componente mayoritario de la célula (43.7% w/w; 30.9% de carbohidratos y 24.0% de lípidos totales). A una relación C/N de 278 (50 g_{GLC}/L de glucosa y 0.5 g_{NIT}/L de nitrato de sodio) se logró obtener 9.2 g_{DCW}/L de masa celular máxima. Esas condiciones (limitación de nitrógeno), promovieron la acumulación preferencial de lípidos (51.7% w/w), resultando en una alta productividad (0.53 g_{LIP}/L/día), con un contenido de 14.4% de proteínas y 33.3% de carbohidratos. Cultivos alimentados a una relación C/N inicial de 278 y adiciones de nitrato a pulsos permitieron obtener una masa celular máxima de 14.2 g_{DCW}/L y la acumulación preferencial de carbohidratos (54.2% w/w), con un contenido de 11.6% de proteínas y 33.7% de lípidos.

Asimismo, se estudió el efecto de la velocidad de crecimiento en la composición macromolecular de *N. oleoabundans* en un sistema que consistió de tres etapas. La primera etapa, un cultivo lote con suficiencia de nutrientes, permitió acumular proteínas. En la segunda etapa se controló la velocidad de crecimiento a dos diferentes tasas de dilución, 0.042 y 0.035 h^{-1}, utilizando un perfil exponencial de alimentación de nitrato de sodio. Después de la etapa de alimentación, los cultivos entraron en una condición de ausencia de nitrógeno. En consecuencia, el número de células no incrementó pero la masa celular se

incrementó entre 1.6 y 2 veces a ambas tasas de dilución, siendo el cultivo a 0.035 h^{-1} en el que se obtuvo la mayor productividad (2.04 g_{DCW}/L/día). A 0.042 h^{-1} la acumulación preferencial de lípidos fue del 53.8% (w/w), con una productividad de biomasa de 1.9 g_{DCW}/L/día. El contenido de proteínas y carbohidratos fue de 17.9 y 25.1% (w/w), respectivamente. Sin embargo, a la menor tasa de dilución (0.035 h^{-1}) el contenido lipídidos fue de 45.5% y el contenido de carbohidratos de 37.3%.

Se llevaron a cabo estudios de proteómica para comparar la síntesis de proteínas bajo dos diferentes condiciones: de acumulación preferencial de carbohidratos (cultivo alimentado a una relación C/N inicial de 278 y adiciones de pulsos de nitrato) y de acumulación preferencial de lípidos (cultivo lote a una relación C/N 278). En la primera condición, las enzimas UDP-glucosa pirofosforilasa y almidón sintetasa fueron altamente sintetizadas para contender con períodos cortos de limitación de nitrógeno. En la segunda condición, las etapas iniciales en la vía de síntesis de lípidos se encontraron altamente activas. Para suministrar precursores a esta vía, la enzima piruvato deshidrogenasa y las reacciones del metabolismo central que dirigen el flujo de carbono hacia la síntesis de piruvato y ATP resultaron altamente sintetizadas. Además, las enzimas glucosa 6-fosfato deshidrogenasa y 6-fosfogluconato deshidrogenasa de la vía de las pentosas fostato, fueron altamente sintetizadas para suministrar poder reductor en forma de NADPH a la síntesis de lípidos y a la asimilación de nitrógeno inorgánico. La enzima ADP-glucosa pirofosforilasa se sintetizó en mayor abundancia, sugiriendo que bajo condiciones de inanición prolongada de nitrógeno, esta enzima actúa hidrolizando las cadenas de almidón para canalizar parte del carbono hacia la síntesis de lípidos.

II. INTRODUCCIÓN

Recientemente ha retoomado un gran auge la investigación con microalgas para obtener compuestos químicos de alto valor comercial como pigmentos, antioxidantes, β-carotenos, polisacáridos, triglicéridos, ácidos grasos ω-3 y ω-6, vitaminas y biomasa, la cual puede ser ampliamente utilizada como producto a granel en diferentes sectores industriales (farmacéuticos, cosméticos, nutracéuticos, alimentos funcionales, biocombustibles). Además, algunos alginatos y carragenanos pueden ser producidos por algas (especialmente macroalgas) y utilizarse como agentes modificadores de viscosidad en alimentos y fármacos [1-3]. También se ha empleado el estrés nutricional, principalmente mediante la limitación e inanición por la fuente de nitrógeno, para incrementar los materiales de reserva en forma de carbohídratos o lípidos; los cuales pueden ser empleados para la manufactura de biocombustibles o bien para obtener "aceites" con calidad y composición similar a la de aceites comestibles. Como consecuencia, varios procesos han sido desarrollados para obtener estos compuestos a escala comercial y muchos de ellos están basados en cultivos fototróficos utilizando CO_2 como fuente de carbono [4,5].

Aunque la mayoría de las microalgas crecen de manera fototrófica, algunas de ellas son capaces de crecer heterotróficamente (en oscuridad), utilizando sustratos orgánicos como única fuente de carbono y energía [4]. El crecimiento heterotrófico de las microalgas depende del tipo de cepa y de las condiciones de cultivo. El consumo de la fuente de carbono depende del transporte o difusión a través de la membrana y de los procesos enzimáticos requeridos para su incorporación al metabolismo central de carbono [6]. Comparado con el crecimiento fototrófico, los cultivos heterotróficos eliminan el requerimiento de luz, pueden incrementar significativamente las velocidades de crecimiento, la cantidad biomasa formada (dry cell weigth por sus siglas en ingles, DCW) y por tanto la productividad másica [7,8]; además, la operación y

mantenimiento de los bioreactores heterotróficos microalgales son relativamente simples y pueden ser operados bajo condiciones axénicas estrictas, tal y como se hace para inumerables cultivos a escala indistrial con bacterias, levaduras y hongos. También, la biomasa obtenida bajo estas condiciones es mayor debido a que la densidad energética de la fuente de carbono (generalmente glucosa) es mayor en comparación con el dióxido de carbono [9]. Los cultivos heterotróficos ofrecen la posibilidad de obtener cultivos con alta densidad celular, lo cual disminuye los costos de la cosecha celular [10,11]. Los cultivos lote pueden ser modificados para obtener cultivos con alta densidad celular a través de la adición de nutrientes o cultivos alimentados [12]. Un cultivo alimentado es, esencialmente, un cultivo lote suplementado de manera continua o intermitente con un nutriente durante el cultivo [4]. Las velocidades específicas de crecimiento pueden ser controladas a la máxima velocidad específica de crecimiento (o cerca de ella) por la adición continua y exponencial del sustrato. Esto permite mantener una concentración limitante del sustrato en el medio durante la alimentación [13].

Los cultivos heterotróficos también tienen algunas limitaciones: las especies de microalgas que pueden crecer de manera heterotrófica son limitadas; los costos de energía y de la adición de una fuente de carbono orgánica son relativamente altos; y los metabolitos inducidos por luz pueden no ser producidos [9, 14]. Una de las ventajas más notables de los cultivos fototróficos es que bajo esas condiciones las microalgas fijan el dióxido de carbono y producen oxígeno, contribuyendo a la reducción de las emisiones de carbono a la atmósfera [15], mientras que los cultivos heterotróficos utilizan una fuente de carbono orgánico, consumen oxígeno y generan CO_2. Además, los cultivos fototróficos permiten el uso de agua no potable y tierras no arables, asimismo si se diseñan de forma adecuada no desplazan ningún cultivo para alimentación. Las microalgas que pueden crecer de manera heterotrófica utilizan los mismos componentes de un medio utilizado en condiciones fototróficas, pero con una fuente de carbono orgánica, en lugar de utilizar un flujo continuo de dióxido de

carbono y luz. Sin embargo, el costo de la fuente de carbono orgánico –que es relativamente alto– y la producción de CO_2 en los cultivo heterotróficos son las mayores preocupaciones a nivel comercial y ambiental [15]. El costo de la glucosa (obtenida a partir del almidón que es producido por plantas cultivadas bajo condiciones fototróficas, p. ej. el maíz) se encuentra alrededor de los 0.6 dólares americanos por kilogramo, mientras que el uso del dióxido de carbono proveniente de los efluentes de gases puede generar algunos bonos ya que se reduce su emisión hacia la atmosfera [15]; aunque es necesario realizar pasos adicionales de limpieza de estos gases. No obstante, existen otras fuentes de carbono más accesibles en precio y cantidad, tales como la sacarosa excedente de los ingenios, el glicerol como subproducto de la creciente industria del biodiesel y los azúcares (pentosas y hexosas) provenientes de la hidrólisis de una amplia gama de residuos agroindustriales.

Las microalgas idóneas para el cultivo heterotrófico deben tener las siguientes habilidades fisiológicas: capacidad para dividirse y metabolizar los substratos sin luz, facultad para crecer en un medio de cultivo fácilmente esterilizable, facilidad para adaptarse rápidamente a cambios en el ambiente y contender con el estrés hidrodinámico generado en los bioreactores de tanque agitado y en los equipos periféricos [4,6,14]. Algunas especies de microalgas, incluyendo *Chlorella protothecoides, Galdieria sulphuraria, Nitzchia laevis* y *Crypthecodinium cohnii,* han sido estudiadas bajo condiciones de crecimiento heterotrófico para alcanzar altas cantidades de masa celular en base seca (DCW) y ácidos grasos, o altas productividades de químicos de alto valor comercial [7,16-18].

Neochloris oleoabundans es una microalga de interés biotecnológico debido a que tiene la capacidad de acumular lípidos bajo condiciones fototróficas y de estrés nutricional, los cuales pueden ser utilizados para diferentes propósitos [19]. Desde el punto de vista de la producción comercial de biocombustibles a partir de lípidos −como el biodiesel− y de productos de alto valor −como los pigmentos− es deseable obtener altas productividades de biomasa microalgal y

de metabolitos de interés. Esto podría lograrse a través de cultivos heterotróficos diseñados para este propósito, utilizando estrategias de cultivo, tales como la manipulación de la relación C/N en el medio y/o de la velocidad de crecimiento a través de cultivos alimentados.

Uno de los obstáculos que limitan el conocimiento del metabolismo microalgal es la falta de información de la secuencia del genoma de diferentes especies de microalgas [20]. El rápido desarrollo de herramientas genómicas y de biología de sistemas, tales como la transcriptómica, proteómica y metabolómica, se han convertido esenciales para el entendimiento de las respuestas y adaptación de diferentes tipos de células a cambios en su ambiente físico [20]. La aplicación de estos enfoques, como la proteómica, ayudará a entender con mayor profundidad, las vías metabólicas, el motivo por el cual son sintetizadas algunas proteínas claves de las vías del metabolismo central y las vías de síntesis de metabolitos de reserva de energía –como los lípidos y carbohidratos– cuando se encuentran bajo ciertas condiciones de cultivo.

III. ANTECEDENTES:

Artículo de revisión: Producción de biodiésel a partir de microalgas
En: *Uso de la biodiversidad para bioenergía y biocombustibles en las zonas áridas de México*. Eds. Castellanos E. y Esqueda M.
Universidad de Sonora 2012. Capítulo 5, pp 114-155.

Morales Sánchez D., Trejo Hernández M. R., Vázquez Duhalt R. y Martínez Jiménez A.

PRODUCCIÓN DE BIODIÉSEL A PARTIR DE MICROALGAS

Daniela Morales Sánchez[1], María del Refugio Trejo Hernández[2], Rafael Vázquez Duhalt[1] y Alfredo Martínez Jiménez[1].

[1] Instituto de Biotecnología – Universidad Nacional Autónoma de México. Av. Universidad 2001, Col. Chamilpa. C.P. 62210. Cuernavaca, Morelos. Apdo. Postal 510-3.
[2] Centro de Investigación en Biotecnología - Universidad Autónoma del Estado de Morelos. Av. Universidad 1001. Col. Chamilpa. C. P. 62209. Cuernavaca, Morelos.
dmorales@ibt.unam.mx, mtrejo@uaem.mx, vazqduh@ibt.unam.mx, alfredo@ibt.unam.mx

RESUMEN

Actualmente se ha identificado el potencial de los lípidos microalgales para la producción de biodiésel. Esta es una alternativa que podría satisfacer o reemplazar la demanda global de petrodiesel. Esta tecnología es prometedora dadas las ventajas que ofrece en contraste con las plantas oleaginosas, tales como: mayor eficiencia fotosintética; eficacia superior en la asimilación de nutrientes; y periodos cortos de producción sostenida durante todo el año, a causa de los breves tiempos de duplicación de las microalgas. Los cultivos microalgales son independientes de la estacionalidad inherente a los cultivos agrícolas y de la fertilidad del suelo. También su condición posibilita prescindir de herbicidas y pesticidas. Una característica muy importante del cultivo de microalgas es que permite emplear territorios no arables, incluyendo áreas marginales no aptas para agricultura y/o alguna otra actividad económica (ej. desiertos y zonas semidesérticas) generando un valor agregado y que también contribuiría a la creación de empleos. En este sentido, las zonas áridas del norte de México representan una opción promisoria para la instalación de sistemas de cultivo con microalgas. Este capítulo presenta un panorama general de la producción de biodiésel y de los sistemas de producción de lípidos utilizando microalgas. Se describen varios aspectos asociados con la producción y fuentes de obtención del biodiésel, con la selección de microalgas, el lugar y los modos de producción, proporcionando un esquema del estado actual del desarrollo de los modos de cultivo. Asimismo, se presentan las ventajas que ofrecería ubicar estos sistemas de cultivo en las zonas áridas de México.

INTRODUCCIÓN

Debido al agotamiento de los combustibles fósiles, el aumento de la demanda energética mundial, el incremento del precio del petróleo y las consecuencias ambientales producidas por los gases de combustión (principalmente los de efecto invernadero), la producción de fuentes alternas de combustibles se ha convertido en una imperiosa necesidad. En este contexto, los biocombustibles, que son caracterizados por ser renovables, biodegradables, amigables con el medio ambiente y que pueden ser producidos con carácter sostenible, están recibiendo cada vez un mayor interés (Martínez-Jiménez 2009, Xiong et al. 2008). Los biocombustibles provienen de diferentes fuentes, incluyendo forestales, agrícola y acuícola que han sido investigadas como materias primas potenciales para la producción de bioetanol, biohidrógeno, biogás y biodiésel (Li et al. 2008a).

Definición, propiedades y producción actual de biodiésel

La American Society for Testing and Materials (ASTM) en su norma 6751 03a define al biodiésel como un combustible compuesto por monoalquil-ésteres de alcoholes de cadena corta con ácidos grasos de cadena larga obtenidos a partir de biomasa renovable (Xiong et al. 2008), que puede ser utilizado en motores de encendido por compresión (Robles-Medina et al. 2009) y que puede reemplazar al diesel derivado del petróleo como combustible (Mata et al. 2010). Comparado con el petrodiesel, el biodiésel ofrece varias ventajas ya que es una fuente de energía renovable y biodegradable (se degrada cuatro veces más rápido que el diesel fósil) y produce menos emisiones indeseables (menores emisiones de CO, hidrocarburos aromáticos policíclicos, partículas de hollín, metales y

óxidos de azufre y nitrógeno) durante su combustión a causa de su estado oxigenado, siendo éstas por ende menos nocivas (Garibay et al. 2009). El contenido de energía y las propiedades fisicoquímicas del biodiésel son similares al diesel convencional (Tabla 1), permitiendo su uso solo o mezclado con el petrodiesel, en cualquier motor diesel sin que requiera de alguna modificación en el sistema de encendido o en el inyector de combustible. Asimismo, posee mejores propiedades lubricantes que aumentan el rendimiento y prolongan la vida útil del motor. Puede ser transportado, almacenado y manejado utilizando la misma infraestructura, mecanismo y procedimientos empleados usualmente para el diesel convencional, debido a que no produce vapores explosivos y tiene un elevado punto de inflamación (cerca de 150 ºC) (Robles-Medina et al. 2009, Garibay et al. 2009).

Tabla 1. Propiedades del Petrodiesel y Biodiesel (Fuente: http:// www.wearcheckiberica.es/documentacion/doctecnica/combustibles.pdf).

Parámetro	Norma	Unidades	Petrodiesel	Biodiesel
Densidad (15ºC)	EN ISO 121185	g/cm^3	0.820 - 0.845	0.860 - 0.900
Viscosidad cinemática (40ºC)	EN ISO 3104	cSt	2.0 - 4.5	3.5 - 5.0
Punto de Inflamación	EN 22719 ISO/CD 3679	ºC	55 mín.	101 mín.
Azufre	EN ISO 14 596	ppm	350 máx.	10 máx.
Residuo Carbonoso (10%)	EN ISO 10370	%	0.30 máx.	0.30 máx.
Contaminación total	EN ISO 12662	ppm	24 máx	24 máx.
Agua	EN ISO 12937	ppm	200 máx.	500 máx.
Corrosión al cobre	EN ISO 2160	-	Clase 1	Clase 1
Cenizas Sulfatadas	EN ISO 6245 ISO 3987	%	0.01 máx	0.02 máx
Estabilidad Oxidación	EN ISO 12205 prEN 14112	mg/l	25 máx	6
Número de Cetanos	EN ISO 5165	-	51	51
Indice de Cetano	EN ISO 4264	-	46	-

Las metodologías existentes para la sustitución de petrodiesel son: uso directo de los aceites o mezclados con diesel fósil, pirólisis, microemulsiones y transesterificación. La aplicación de los productos de las tres primeras alternativas en motores diesel es

impráctica e insatisfactoria, debido a que ocasionan problemas como la obstrucción de los inyectores, la formación de depósitos de carbono, la combustión incompleta, el golpeteo en el motor, el desgaste excesivo del mismo, poca lubricidad y, en el caso específico de la pirólisis, la eliminación de los beneficios ambientales inherentes al uso de combustibles oxigenados. Además, la pirólisis y las microemulsiones presentan altos costos de proceso y bajos rendimientos (Garibay et al. 2009, Robles-Medina et al. 2009).

El método más usual para la producción de biodiésel es mediante transesterificación. Este método consiste en la reacción entre triacilglicéridos (contenidos en los aceites) y un aceptor acilo. El grupo acilo del aceptor puede ser un ácido carboxílico (acidólisis), un alcohol (alcohólisis) o algún otro éster (interesterificación). La transesterificación consiste en reacciones consecutivas reversibles (Freedman et al. 1986). Los triglicéridos son convertidos a diglicéridos, monoglicéridos y finalmente esteres de ácidos grasos y glicerol, tal como se muestra en las siguientes reacciones:

1. Triacilglicérido (TG) + R'OH $\underset{k_{-1}}{\overset{k_1}{\leftrightarrows}}$ Diacilglicérido (DG)+ $R'COOR_1$
2. Diacilglicérido (DG) + R'OH $\underset{k_{-2}}{\overset{k_3}{\leftrightarrows}}$ Monoacilglicérido (MG) + $R'COOR_2$
3. Monoacilglicérido (MG) R'OH $\underset{k_{-3}}{\overset{k_3}{\leftrightarrows}}$ Glicerol + $R'COOR_3$

El mecanismo de reacción de la transesterificación catalizada por álcali ha sido descrito en tres etapas. La primera etapa es un ataque al grupo carbonilo del átomo de carbono de la molécula de triglicérido por el anión del alcohol (ión metóxido) para formar un

intermediario tetraédrico. En la segunda etapa, el intermediario tetraédrico reacciona con un alcohol (metanol, etanol) para regenerar el anión del alcohol (ión metóxido). En la tercera etapa, se produce el rearreglo del intermediario tetraédrico que resulta en la formación de un ácido graso y un diglicérido. Un mol de éster es liberada en cada etapa.

Esta reacción produce glicerol y alquil estéres de ácidos grasos, los cuales son conocidos como biodiésel (Chisti 2007, Al-Zuhair 2007, Liu y Zhao 2007, Sharma et al. 2008, Robles-Medina et al. 2009, Garibay et al 2009). Diferentes autores han reportado la influencia de varios factores en la producción de biodiésel a través de reacciones de transesterificación.

a) Contenido de agua y ácidos grasos libres. En la materia prima el contenido de agua y de ácidos grasos libres son factores que afectan la conversión de los triglicéridos. La materia prima debe estar anhidra y tener valores de acidez menores a 1. La presencia de agua causa la formación de jabón, lo que conduce a un consumo del catalizador y reduce la eficiencia catalítica. Además de incrementar la viscosidad también se dificulta la separación del glicerol. La acidez elevada conduce igualmente a un requerimiento superior de catalizador para neutralizar a los ácidos grasos libres. Los valores de contenido de agua y de acidez deben ser menores a 0.06 y 0.5% respectivamente, para alcanzar la máxima conversión (Ma et al. 1998).
b) Relación molar. Entre las variables más importantes que afectan el rendimiento de la producción de esteres es la relación molar alcohol/triglicérido. De acuerdo con la reacción de transesterificación se requieren 3 moles de alcohol y 1 mol de triglicérido para producir 3 moles de esteres de ácidos grasos y 1 mol de glicerol. Esta relación molar se ha asociado con el tipo de catalizador utilizado. Rendimientos superiores al 90% se han asociado con relaciones molares de 6:1 a 30:1 utilizando catalizadores alcalinos y con diferentes aceites vegetales. Los

catalizadores ácidos en general requieren de relaciones molares de 15:1 a 30:1. (Tanaka et al. 1981; Freedman et al. 1986). La relación molar más utilizada en los procesos de producción de biodiésel es 6:1.

c) Tipo de catalizador. Los catalizadores se clasifican en alcalinos, ácidos y enzimas. Los catalizadores alcalinos son más eficientes que los ácidos, no obstante, los catalizadores ácidos son conveniente cuando la materia prima presenta un alto contenido de ácidos grasos libres y agua. En la actualidad, los procesos de transesterificación se llevan a cabo utilizando NaOH debido a su bajo costo (Freedman et al. 1984; Freedman et al. 1986). La cantidad recomendada de base es de 0.1 a 1% p/p de aceites o grasas. Las enzimas usualmente utilizadas son las lipasas, sin embargo su aplicación requiere de condiciones muy controladas para mantener la actividad de las mismas. Lo anterior conlleva bajos rendimientos y tiempos de reacción elevados que comparados con los obtenidos durante las reacciones de transesterificación con catalizadores alcalinos son desfavorables.

d) Temperatura de la reacción. La reacción de transesterificación puede ocurrir a diferentes temperaturas dependiendo del tipo de aceite usado. Freedman eta al. (1986) demostró que en la transestrificación del aceite refinado de soya con metanol (6:1) y 1% de NaOH como catalizador, se obtienen rendimientos de 64, 87 y 97% a 32, 45 y 60 ºC, respectivamente. Las altas temperaturas incrementan la velocidad de la reacción y reducen el tiempo del proceso.

Por otra parte, los aceites utilizados para la producción de biodiésel deben ser caracterizados de acuerdo a sus propiedades físicas (densidad, viscosidad, punto de fusión, índice de refracción, etc.) y químicas (acidez, índice de yodo, peróxido, saponificación, etc.) ya que esos parámetros inciden en la calidad del biodiésel (Chisti 2007, Schenk et al. 2008, Robles-Medina et al. 2009). Además, deben cumplir con las normas que regulan sus propiedades y aseguran su calidad. Los estándares usualmente

utilizados como referencia son la norma ASTM D6751 03a en EUA y las normas europeas EN 14213 (biodiésel para calefacción) y EN 14214 (biodiésel para uso vehicular). El cumplimiento de tales disposiciones precisa de biodiésel enriquecido en ácidos grasos de cadena larga con elevado grado de saturación (preferentemente los ácidos palmitoleico (16:1), oleico (18:1) y mirístico (14:0)) (Chisti 2007, Schenk et al. 2008, Garibay et al. 2009, Robles-Medina et al. 2010).

El biodiésel, es uno de los combustibles renovables más promisorios y como tal ha alcanzado un reconocimiento mundial importante. De acuerdo al reporte del Banco Mundial (2008), 6,500 millones de litros de biodiésel se produjeron a nivel mundial en el 2006, 75% por la Unión Europea y 13% por los EUA. Sin embargo, la contribución actual del biodiésel al consumo global de combustibles para transportación es solamente del 0.14%, pero se espera que esta contribución aumente cinco veces en el 2020 en los países desarrollados (Dorval et al. 2009). El elevado costo del biodiésel, el cual se debe principalmente a los altos costos de las materias primas, es el principal obstáculo para su amplia comercialización. Por consiguiente, se han realizado numerosos esfuerzos por investigar la manera de minimizar los costos de las materias primas (Li et al. 2007).

Fuentes de obtención de biodiésel y potencial de producción a partir de microalgas

Los aceites de origen vegetal actualmente son la fuente convencional para la producción de biodiésel (Xiong et al. 2008), razón por la cual el uso de cultivos de alto contenido oleaginoso ha sido estudiado exhaustivamente. Los principales materiales oleaginosos derivan de la palma, colza y soya, además del girasol, coco, cacahuate, oliva y mostaza, entre otros (Garibay et al. 2009). Sin embargo, una de las principales desventajas del uso

de cultivos vegetales tradicionales para la producción de biodiésel es la porción de tierra que se utiliza en el cultivo, lo cual desplaza a los cultivos destinados para alimentación (Chisti 2007). En consecuencia se ha planteado el uso de aceites no comestibles procedentes de cultivos en tierras marginales, tales como *Jatropha curcas* (piñón), *Calophyllum inophyllum* (tamanu), *Pongamia pinnata* (haya de la India, karanja), *Madhuca indica, Swida wilsoniana, Ricinus communis* (higuerilla) y *Vernicia fordii* (tung). Estos cultivos en principio no requieren de terrenos fértiles, ya que pueden proliferar en suelos semiáridos, pobres en nutrientes, con altos niveles de radiación y baja precipitación pluvial (Fairless 2007, Liu y Zhao 2007, Sharma et al. 2008, Song et al. 2008, Garibay et al. 2009). El elevado costo de la materia prima, que contribuye del 50 al 90% del precio de producción del biodiésel, ha obstaculizado la comercialización del biocombustible a gran escala, motivo por el cual se ha propuesto el uso de aceites de desecho y de grasas animales. Esta alternativa que no ha sido viable a causa de los gastos adicionales necesarios para el refinamiento y la transesterificación del material, pero principalmente a la logística en la colecta y acopio del material, así como a la limitada disponibilidad de estos desechos (Al-Zuhair 2007, Liu y Zhao 2007, Song et al. 2008, Meng et al. 2009, Garibay et al. 2009). Asimismo, la obtención de biodiésel a partir de plantas oleaginosas (comestibles y no comestibles) está limitada por varios inconvenientes, tales como los largos períodos de producción (varios meses o años) inherentes a la tecnología agrícola, el rendimiento lipídico restringido (menor al 5% del peso seco total), la dependencia con las condiciones climáticas, la ubicación geográfica, la fertilidad de los suelos y la variedad cultivada. No obstante, los principales obstáculos

son la extensa superficie requerida y el enorme volumen de agua necesario para el riego (Garibay et al. 2009). En el 2008 la demanda de petrodiesel en México fue de 23,630 millones de litros (Indicadores Petroleros, 2008), el reemplazo de esta demanda con biodiésel de origen vegetal, incluso con aquél derivado de cultivos de elevada productividad lipídica como la palma, requeriría de extensas regiones fértiles (Garibay et al. 2009). Este escenario podría cambiar drásticamente si se utilizan cultivos con microalgas para la producción de biodiésel. Aproximadamente el 1% de la superficie total de México puede ser suficiente para producir biodiésel a partir de microalgas para reemplazar el 100% de las necesidades de petrodiesel del país (Tabla 2**,** Garibay et al. 2009). Además, si los cultivos de microalgas se realizan en zonas áridas o semiáridas, ya sea costeras o tierra adentro, se produciría un valor agregado para estas regiones y contribuiría a la creación de empleos (Robles-Medina et al. 2009) en regiones como el norte de México.

Tabla 2. Comparación de distintas fuentes de insumos para la producción de biodiesel (teórico) en México. Se indican las proporciones de superficie fértil y total del país necesarias para reemplazar con biodiesel el 100% de la demanda de petrodiesel en México. Las fracciones de superficie total sólo se señalan para materias primas que no precisan de suelos fértiles (fuente: Garibay et al. 2009).

Materia prima	Productividad de Biodiesel (L/ha*año)	Superficie equivalente requerida (ha x 10^6)	Equivalente de la superficie fértil requerida (%)	Equivalente de la superficie total (no fértil) requerida (%)
Palma	5,950	3.97	16.14	--
Jatropha	1,892	12.49	50.75	6.43
Colza	1,190	19.86	80.69	--
Girasol	952	24.82	100.9	--
Soya	446	52.99	215.3	--
Microalgas[a]	12,000	1.97	8.00	1.01
Microalgas[b]	20,000	1.18	4.80	0.61

[a] Rendimiento conservador de productividad de biodiesel microalgal acorde con Schenk et al. (2008), Garibay et al. (2009) y Garibay (2010).
[b] Productividad de biodiesel microalgal asequible a través de la tecnología actualmente disponible, acorde con Wijffels (2008).

Las microalgas como fuente de materia prima para la producción de biocombustibles

¿Que son las microalgas?

Las microalgas son microorganismos fotosintéticos, procariontes o eucariontes. Las cianobacterias son ejemplos de procariotas (*Cyanophyceae*), mientras que las eucariotas, incluyen a las algas verdes (*Chlorophyta*), las diatomeas (*Bacillariophyta*), algas cafés (*Phaeophyceae*), algas rojas (*Rhodophyceae*), etc. (Mata et al. 2010). Las microalgas están presentes en todos los ecosistemas existentes de la tierra, tanto acuáticos como terrestres, representando a una gran variedad de especies viviendo en un amplio intervalo de condiciones ambientales. Se estiman más de 50,000 especies existentes, de las cuales únicamente se han estudiado y analizado alrededor de 30,000 especies (Mata et al. 2010). Algunos criterios de clasificación de las microalgas se basan en el color, ciclo vital y estructura celular, entre otros. La Tabla 3 muestra algunas características de varias categorías de microalgas (Martínez-Jiménez 2008).

Tabla 3. Características relevantes de algunas categorías de microalgas (Fuente: Martínez Jiménez 2008, http://www.dictus.uson.mx/bio3/bio1.html).

CATEGORÍA	COMENTARIOS
Chlorophyceae (Algas verdes)	Especies abundantes, esp. aguas dulces. Existen como células individuales o colonias. Reserva de C: almidón principalmente.
Bacillariophyceae (Diatomeas)	Abundantes en océanos (aguas dulces y residuales). Paredes celulares con Si. Reservas de C: lípidos y polímeros de CH's.
Chrysophyceae (Algas doradas)	Predominan en agua dulce. Sistemas complejos de pigmentación. Reservas de C: lípidos y CH's.
OTRAS CATEGORÍAS: Xantophyceae (algas verde-amarillas), Rhodophyceae (algas rojas), Phaeophyceae (algas cafés), Dinophyceae (dinoflageladas), Prasinophyceae y Eustigmatophyceae (plancton).	

Cultivo de microalgas, perspectiva histórica

Previo al establecimiento del Programa de Especies Acuáticas del Departamento de Energía de los EUA (DOE), se habían conducido pocos trabajos en relación a la producción de biocombustibles a partir de lípidos provenientes de las microalgas. Históricamente, las algas han sido vistas como una excelente fuente de proteínas y han sido cultivadas, principalmente, para la alimentación. Desde el punto de vista ingenieril, en el Instituto de Investigaciones de Stanford (1948-1950) se realizaron las primeras investigaciones para la producción de microalgas a gran escala. En 1951 Arthur D. Little (tomado de Hu et al. 2008) logró importantes avances a través de la construcción y operación de una planta piloto para la producción de *Chlorella* en el Instituto Carnegie. Se considera que, gracias a estos estudios, actualmente se tienen conocimientos acerca del crecimiento, fisiología y bioquímica de las microalgas y el surgimiento de los conceptos para los cultivos de microalgas aplicados a la producción de biocombustibles.

El uso de microalgas como fuente de energía

El concepto del uso de microalgas como fuente de combustibles fue propuesto por Meier en 1955 para la producción de gas metano. Esta idea fue desarrollada más tarde por Oswald y Golueke en 1960, quienes introdujeron el análisis conceptual de ingeniería tecno-económica para digerir la biomasa microalgal crecida en grandes estanques tipo circuito (raceway ponds) para producir gas metano. En 1970, debido al rápido incremento del costo de combustibles convencionales, empezó a tener un renovado interés la posibilidad del uso de microalgas como fuente de combustibles. El análisis y

Tabla 4. Resumen de investigaciones orientadas a la producción de microalgas y lípidos desde 1986 a 2009. *Adaptado del trabajo presentado por Mayfield (2008) en el "Workshop on algal Oil for Jet Fuel Production". NR: No reportado

Microalga	Biomasa (g/l)	Productividad área (g/m^2*día)	Productividad lípidos (g/l*día)	Observaciones	Referencias
Arhrospira (espirulina)	NR	7 - 25	NR	215 días de cultivo abierto en Fotobioreactor (FBR) (externo). *	Torzillo et al. (1986)
Tetraselmis suecica	NR	20 - 70	NR	Cultivos en estanque tipo circuito (open ponds). Duración menor a 1 mes, resultado de 1 día. *	Laws et al. (1986)
Arthrospira (espirulina)	NR	5.44	NR	FBR helicoidal con luz artificial. *	Watanabe et al. (1995)
Resumen del programa de biomasa acuática (ABP) obtenido de 1978 a 1996	NR	3 - 40	NR	Cultivos en estanque tipo circuito experimental. *	Beneman y Oswald (1996)
Phaeodactylum tricornutum	NR	61 - 73	NR	FBR con velocidades de dilución maximizadas. *	Acien Fernández et al. (1998)
Scenedesmus obliquus	NR	48	NR	Cultivo en estanque tipo circuito de 20 m^2. Resultado no publicados. *	Grobbelaar (2000)
Arthrospira (espirulina)	2.37	25.4	NR	FBR tubular, luz solar, 146 L	Carlozzi (2000)
Chlorella vulgaris	NR	36.1 - 41.6	NR	FBR tubular, 700 m^3. *	Moore (2001)
Phaeodactylum sp.	4.1	25.3	NR	FBR tubular, luz solar, 200 L	Molina Grima et al. (2001)
Nitzchia laevis	30	NR	0.04	Cultivo heterotrófico en perfusión con glucosa.	Wen y Chen (2001)
Chlorella vulgaris	NR	0.57 - 0.97	NR	FBR helicoidal con luz artificial. *	Scragg et al. (2002)
Chlorella sp., Arthrospira sp. Dunaliella sp.	NR	3 - 8	NR	Cultivos en estanque tipo circuito a nivel comercial. *	Jiménez et al. (2003)
Arhrospira (espirulina)	NR	2 - 15	NR	Cultivos en estanque tipo circuito en 450 m^2. *	Jiménez et al. (2003)
Crypthecodinium cohnii	83	NR	1.2	Cultivo heterotrófico en lote alimentado con glucosa.	de Swaaf et al. (2003 a y b)
Dunaliella salina	NR	2	NR	FBR abierto escala pequeña	García-González et al., (2005)
Chlorella sp.	NR	22.8	NR	FBR panel plano, luz solar, 400 L	Doucha et al. (2005)
Tetraselmis sp.	1.7	38.2	NR	FBR columna, luz solar, 120 L.	Zitelli et al. 2006
Arhrospira (espirulina)	NR		NR	FBR tubular, luz artificial, 5.5 L	Converti et al. (2006)
Chlorococcum	1.2	14.9	NR	Diseño alternativo de reactor tipo parábola solar 70 L	Sato et al. (2006)
Chlorococcum	1.2	11	NR	Diseño alternativo de reactor tipo domo solar 130 L	Sato et al. (2006)
Hematococcus pluvialus	NR	10 - 15	NR	Cultivos en estanque tipo circuito. *	Huntley y Redalje (2007)
Nannochloropsis sp. F&M-M24	0.4	NR	0.11	Cultivos en FBR abiertos tipo panel del pared verde (Green Wall Panel) de 110 L	Rodolfi et al. (2008)
Chlorococcum sp. UMACC 112 *Scenedesmus sp.* DM *Chlorella sorokiniana* IAM-212 *Pavlova lutheri* CS182 *Nannochloropsis sp.* F&M-M26 F&M-M24 F&M-28	0.28 0.26 0.23 0.14 0.21 0.18 0.17	NR	0.053 0.053 0.044 0.05 0.061 0.054 0.061	Cultivos en matraces de 250 ml con iluminación artificial continua y CO_2	Rodolfi et al. (2008)
Chlorella protothecoides	51.2	NR	3.6	Cultivo heterotrófico en lote alimentado con glucosa.	Xiong et al. (2008)
Nannochloropsis sp. *Neochloris oleabundans*	2.5 2	NR	NR	Cultivos en bolsas de polietileno con burbujeo de aire y luz artificial.	Gouveia et al. (2009)
Nannochloropsis oculata NCTU-3	NR	NR	0.12	Cultivos en sistema semicontinuo con FBR cilíndrico de 800 ml.	Chiu et al. (2009)
Chlorella protothecoides	17	NR	1.7	Cultivo heterotrófico en lote con fructosa y glucosa.	Cheng et al. (2009)

diseño de ingeniería de este concepto se efectuó en 1978 por Benemann y sus colaboradores, quienes concluyeron que en estos sistemas biológicos podrían producir biocombustibles y competir con los precios de los combustibles fósiles convencionales (Hu et al. 2008).

Las microalgas pueden proveer diferentes tipos de biocombustibles renovables. Estos incluyen el metano producido por la digestión anaeróbica de la biomasa microalgal (Spolaore et al. 2006); biodiésel derivado de los lípidos microalgales (Roessler et al. 1994, Sawayama et al. 1995, Dunahay et al. 1996, Sheehan et al. 1998, Banerjee et al. 2002, Gavrilescu y Chisti 2005); y biohidrógeno fotobiológicamente producido (Ghirardi et al. 2000, Akkerman et al. 2002, Melis 2002, Fedorov et al. 2005, Kapdan y Kargi 2006). Asimismo, mediante la sacarificación y la fermentación de los carbohidratos microalgales es posible producir etanol, la gasificación de la biomasa produce "gas de síntesis" (mezcla de monóxido de carbono e hidrógeno), los esqueletos hidrocarbonados e isoprenoides rinden gasolina (Rosenberg et al. 2008), y actualmente se trabaja en la obtención de biopetróleo o "biocrudo" y combustibles para la aviación a partir de microalgas (Figura 1).

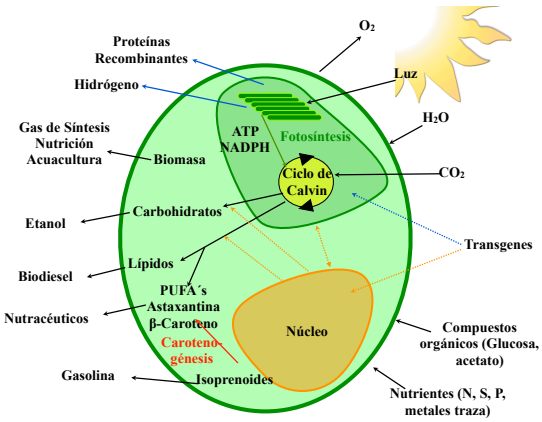


Figura 1. Esquema general de las vías metabólicas importantes en microalgas para la obtención de productos comerciales. Esta representación esquemática muestra las vías celulares simplificadas que están involucradas en la biosíntesis de algunos productos derivados de las microalgas. Aunque el cloroplasto puede actuar como una fábrica de producción de hidrógeno y proteínas (líneas sólidas azules), el núcleo juega un papel fundamental en el control metabólico (líneas discontinúas rojas). Ambos organelos contienen genomas individuales, lo cual ofrece la posibilidad de incorporar transgenes independientes (Rosenberg et al. 2008).

Una característica relevante de los cultivos microalgales es su gran cobertura, es decir, que se pueden alcanzar diferentes objetivos dentro de un mismo proceso, por ejemplo, el tratamiento de aguas residuales, la obtención de biocombustibles y la producción de sustancias químicas finas (Arredondo y Vázquez-Duhalt 1991). La producción comercial exitosa de microalgas ha sido establecida en bajos volúmenes para la obtención de productos de alto valor, tales como ficobiliproteínas, astaxantina, xantofilos, β-carotenos, suplementos nutricionales, antioxidantes, cosméticos, colorantes naturales y ácidos grasos poliinsaturados (PUFA, por sus siglas en inglés) (Rosenberg et al. 2008; Zemke et al. 2008).

Con la finalidad de resumir, desde 1986 a la fecha, los principales trabajos orientados a la producción de microalgas y lípidos, para su potencial uso como biocombustibles, se presenta la Tabla 4. Es interesante observar que, comparado con las productividades de biomasa obtenidas mediante cultivos vegetales tradicionales, como granos de maíz, caña de azúcar y canola (1.91, 23.8 y 0.73 g/m^2*día, respectivamente), entre otros; las productividades logradas con microalgas, en algunos casos, son 3.7 veces mayores (Dismukes et al. 2008). Además, en cuanto a las productividades de biodiésel obtenidas mediante aceites de origen vegetal y aceite proveniente de microalgas, éste último supera de 2 a 44 veces lo logrado con cultivos vegetales tradicionales (Tabla 2) (Garibay et al. 2009). Por las razones anteriores y debido a que la producción de microalgas para la obtención de lípidos es técnicamente viable (Chisti, 2007), actualmente se están estableciendo industrias para producir comercialmente biocombustibles a partir de microalgas. La Tabla 5 muestra varios ejemplos de ellas.

Selección de especies de microalgas y del lugar de operación para la producción de biodiésel

Las investigaciones del programa de especies acuáticas (ASP, Aquatic Species Program) del Laboratorio Nacional de Energía Renovable (NREL, National Renewable Energy Laboratories), en Golden, Colorado, EUA, establecieron una colección de más de 300 cepas de microalgas aisladas desde varios hábitats, comprendiendo Hawai, y del este al oeste de los EUA, los cuales representan toda una diversidad de ambientes acuáticos y tipos de aguas (marinas, dulces, salobres y residuales). Los constituyentes de esta colección fueron caracterizados y seleccionados en base a su robustez, contenido de aceite, velocidad de crecimiento, eficiencia metabólica y tolerancia a condiciones severas (salinidad, pH y temperaturas extremas). La colección fue acotada a las 300 especies más prometedoras, muchas de las cuales fueron algas verdes (Chlorophyceae) y diatomeas (Bacillariophyceae) (Rosenberg et al. 2008, Hu et al. 2008). En general, para la producción de biodiésel se requiere de microalgas de las que se pueda obtener altas productividades de aceite (Chisti 2007).

Actualmente se han realizado muchos esfuerzos que se han centrado en investigar la mejor opción en cuanto al lugar del cultivo de microalgas, ya que en muchos casos, esto representa el paso central que determina la viabilidad económica del proceso. De acuerdo con Maxwell et al. (1985) para la implementación del módulo de cultivo de microalgas, el lugar seleccionado y la evaluación de las fuentes de materia prima tienen que ser desarrollados considerando varios criterios: (i) El suplemento/demanda del agua y sus características fisicoquímicas; (ii) la topografía, geología y propiedad de la tierra en donde se colocará el módulo; (iii) las condiciones ambientales: temperatura, evaporación,

Tabla 5. Compañías que actualmente realizan investigación enfocadas a obtener biocombustibles a partir de microalgas (Adaptado de: Rosenberg et al. 2008 y http://www.seambiotic.com/uploads/algae%20report%2004%202009.pdf).

Compañía	Localización	Año de creación	Compañía asociada	Página web
Cellana	Hawaii	2007	Shell	http://cellana.twokings.eu/
Green Fuel Technologies	E.U.A.	1999	The Home Depot	http://www.greenfuelsolar.com
LiveFuels, Inc.	E.U.A.	2006	------	http://www.livefuels.com/index.php
PetroAlgae	E.U.A.	2006	------	http://www.petroalgae.com/green-diesel.php
Sapphire Energy	E.U.A.	2007	Departamento de Energía y Departamento de Agricultura de E.U.A.	http://www.sapphireenergy.com/sapphire-renewable-energy/
Solazyme, Inc.	E.U.A.	2003	------	http://www.solazyme.com/
Solix Biofuels	E.U.A.	2006	National Instruments, CEMEX, Targeted Growth Inc., NOOC, Colorado State University, HAZEN, Shangai Alliance Investment, Ltd., VALERO, Los Alamos National Laboratory, Southern Ute Alternative Energy	http://www.solixbiofuels.com/
IGV Institut für Getreideverarbeitung	Alemania	1960	------	http://www.igv-gmbh.com/
Algae Biofuels	E.U.A.	2006	------	
Imperium Renewables	E.U.A.	2004	National Biodiesel Board	http://www.imperiumrenewables.com/about.php
Aquaflow Bionomic	E.U.A.	2007	UOP, Departamento de Energía, E.U.A., Milestone, Trade & Enterprise, Purepower	http://www.aquaflowgroup.com/
Kent SeaTech Corp.	E.U.A.	1972	------	http://kentbioenergy.com/index.html
Diversified Energy	E.U.A.	2005	------	http://www.diversified-energy.com/index.cfm
Alga Technologies	Israel	1999	------	http://www.algatech.com/about.htm
Seambiotic	Israel	2003	Israeli Electric Corporation	http://www.seambiotic.com/
Cyanotech	E.U.A.	1995	------	http://www.cyanotech.com/
FujiChemical	E.U.A., Sweden	2000	NatuReal Corporation, BioReal Inc., BioReal AB, Fuji Health Science Inc., Nishida Museum	http://www.fujichemical.co.jp/english/
Mera Pharma	E.U.A.	2004	------	http://www.merapharma.com/
Amyris Biotech.	E.U.A.	2008	------	http://www.amyrisbiotech.com/
Yamaha Motor	Japón	2007	------	http://www.global.yamaha.com/
NREL	E.U.A.	1991	Departamento de Energía de E.U.A.	http://www.nrel.gov/
Chevron	E.U.A.	2001	The Texas Company (TEXACO)	http://www.chevron.com/
C2B2	E.U.A.	2007	Universidad de Colorado, Universidad Estatal de Colorado, Escuela de Minas de Colorado, NREL, General Motors, Shell.	http://www.c2b2web.org/
A2BE Carbon Capture	E.U.A.	2007	------	http://www.algaeatwork.com/
Inventure Chemical	E.U.A.	2007	Arizona Public Service, Seambiotic, Imperium Renewables,	http://www.inventurechem.com/
Aurora Biofuels Inc.	E.U.A.	2007	------	http://www.aurorabiofuels.com/about-aurora-biofuels.php
Bodega Algae	E.U.A.	2007	NASA, Bigelow Laboratory for Ocean Sciences, MHT.	http://www.bodegaalgae.com/
HR Biopetroleum	E.U.A.	2004	Shell, Cellana, Alexander & Baldwin, Hawaiian electric y Maui Electric Companies, Mera Pharmaceuticals, Universidad de Hawaii.	http://www.hrbp.com/index.html
Community Fuels	U.K.	------	------	http://www.communityfuels.com/
OriginOil	E.U.A.	2007	Idaho National Laboratory, Desmet Ballestra, StrategicFit, Universidad Estatal de California.	http://www.originoil.com/about-us/partners.html
Enhanced Biofuels & Technologies	E.U.A.	2007	The Consensus Busines Group, Alchemie Technology Group, Green World Biotech.	http://www.ebtplc.com/index.htm
General Atomics	E.U.A.	1955	General Atomics Aeronautical Systems, Aircraft Systems Group, Reconnaissance Systems Group, Cotter, Heathgate Resources Pty, Ltd., Nuclear Fuels Corporation, General Atomics Electronic Systems, General Atomics Systems Integration LLC, ConverDyn, Rio Grande Resources Corporation, Spezialtechnik Dresden and Subsidiaries.	http://www.ga.com/affiliates.php
Global Green Solutions	E.U.A.	2006	------	http://www.globalgreensolutionsinc.com/s/CorpStructure.asp
Green Star	E.U.A.	1990	------	http://www.greenstarusa.com/index.html
Texas Clean Fuels	E.U.A.	2006	------	http://www.texascleanfuels.com/index.html
Trident Exploration	E.U.A.	2000	------	http://www.tridentexploration.ca/index.html
GreenShift	Canadá	1984	------	http://www.greenshift.com/
Valcent Products	E.U.A.	1996	------	http://www.valcent.net/s/Home.asp
Algenol Biofuels	E.U.A. México	2008	Gobierno de México	http://www.algenolbiofuels.com/default.html
Subitec	Alemania	2000	Fraunhofer IGB, EnBW, e.on Hanse, High-Tech Gründerfonds, Fraunhofer VENTURE, Ebner Stolz& Mönning Bachem, Hombach, GMB.	http://en.subitec.com/company/
Ifremer	E.U.A., Francia	1984	------	http://www.ifremer.fr/francais/index.php
Biofields	México	2007	Grupo Gondi	http://www.biofields.com/index.php

precipitación pluvial; (iv) el fácil acceso al suministro de las fuentes de carbono y otros nutrientes (Chisti 2007, Schenk et al. 2008, Li et al. 2008a, Robles-Medina et al. 2009, Mata et al. 2010). También es necesario decidir si el módulo de cultivo de microalgas deberá operar en modo lote o continuo, y si el sistema de producción debe operar en sistema abierto al ambiente o cerrado. Esto depende de la especie de microalga seleccionada, las condiciones ambientales pronosticadas, la disponibilidad de nutrientes e incluso la posibilidad de combinar el crecimiento microalgal con una estrategia de control de contaminación de aire de alguna industria, p.ej. para la remoción de CO_2 desde las emisiones del efluente gaseoso o la remoción de nitrógeno o fósforo desde el efluente de aguas residuales (Chisti 2007, Dismukes et al. 2008, Schenk et al. 2008, Li et al. 2008a, Robles-Medina et al. 2009, Mata et al. 2010).

Además del equipo necesario para el crecimiento microalgal, es esencial enfatizar en la selección de las especies y cepas más adecuadas, sus condiciones de cultivo y nutrientes disponibles para su crecimiento. En la mayoría de los casos, la producción de biodiésel depende de las especies y cepas ya disponibles que han mostrado ser adecuadas debido a su contenido de lípidos o productividades (Tabla 4). Sin embargo, en algunos casos este

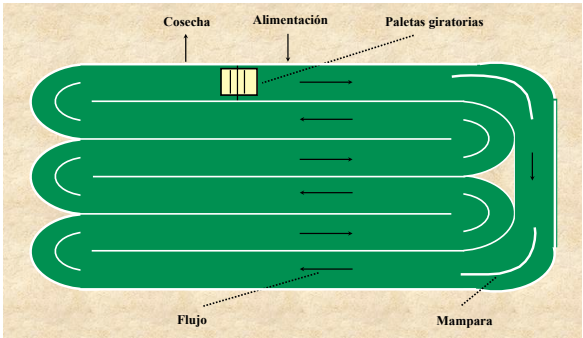


Figura 2. Vista aérea esquemática de un estanque tipo circuito abierto al ambiente (Xu et al. 2009).

enfoque no proporciona una solución adecuada y por consiguiente, se realiza una búsqueda de especies de microalgas más convenientes (Robles-Medina et al. 2009, Mata et al. 2010). Típicamente, las fuentes de microalgas incluyen las colecciones de microalgas existentes, comercialmente disponibles, ya sea en colecciones universitarias o en otras fundaciones internacionales o de compañías específicamente dedicadas al crecimiento de microalgas (Torrey 2008) o su aislamiento de muestras de agua y suelos en diversos ambientes (Mata et al. 2010).

Debido a que las microalgas viven y sobreviven en un amplio intervalo de condiciones ambientales, especialmente bajo escasez de nutrientes y otras condiciones adversas, las muestras de microalgas obtenidas en ambientes severos como aguas termales o aguas residuales industriales, pueden ser utilizadas. De este modo se asegura que si alguna opción viable es encontrada, ésta podría ser robusta y posiblemente mejor adaptada a condiciones específicas (Mata et al. 2010).

El proceso de muestreo y selección de microalgas está bien establecido, aunque se requiere de equipamiento especializado y puede consumir mucho tiempo. Una estrategia de multicriterios tiene que ser considerada en este proceso, tomando en cuenta los siguientes factores (Richmond 2004, Dismukes et al. 2008, Hu et al. 2008, Mata el al. 2010):

- Velocidad de crecimiento, normalmente determinada por la cantidad total de biomasa acumulada por unidad de tiempo y unidad de volumen.
- Contenido de lípidos, por un lado refiriéndose a la cantidad total, y por otro a la distribución de ácidos grasos libres y triacilgliceroles, un factor que es relevante en la producción de biodiésel.

- Resistencia a los cambios en las condiciones ambientales, en particular de temperatura, aporte de nutrientes, intensidad luminosa, competencia con otras especies de microalgas y/o otros microorganismos.
- Disponibilidad de nutrientes, en particular de las fuentes de dióxido de carbono.
- Simplicidad en el procesamiento y separación de la biomasa.
- Posibilidad de obtención de otros compuestos químicos de alto valor.

Estos criterios no solamente consideran a las microalgas como tal, sino que también sus formas de cultivo. De este modo, todos los experimentos deberían imitar, en lo mayormente posible, las condiciones que se tienen en el sitio donde se cultivarán las microalgas, así como el procesamiento de la biomasa, antes de considerar la producción del biodiésel como tal.

Incluso cuando las especies sugeridas en la literatura o aisladas desde el ambiente no son adecuadas, la utilización de la ingeniería genética puede ser una solución. De esta manera, es posible ajustar las características de la microalga y del producto deseado al proceso y el mejoramiento de la productividad, el rendimiento y composición de los lípidos. Sin embargo, el riesgo de contaminación biológica, la legislación restrictiva, la existencia de las opciones viables de microalgas silvestres, y la posibilidad de transferencia genética todavía dificultan la amplia utilización de microalgas genéticamente modificadas.

Es crucial el entendimiento de cómo seleccionar las especies de microalga correcta, obtener las condiciones de foto-cultivo más favorables para cada especie, y construir el módulo de cultivo viable económicamente que pueda aportar correctamente los nutrimentos y condiciones ambientales para el desarrollo óptimo de la célula microalgal,

sin importar el tamaño de las instalaciones o su localización geográfica (Mata et al. 2010).

Acumulación de lípidos en microalgas

Los lípidos forman parte sustancial de la biomasa de microalgas oleaginosas, conteniendo comúnmente de 20 a 69% de su peso seco (Tabla 6). Desde 1940, la limitación de nitrógeno ha sido reportada como la principal influencia en el almacenamiento de lípidos en microalgas. Spoher y Milner (1949) describieron el efecto de varios suministros de nitrógeno en el contenido de lípidos y clorofila de *Chlorella* y algunas diatomeas. Werner (1966) reportó un incremento en la concentración de lípidos intracelulares de una diatomea durante su inanición con silicio. La limitación de fósforo resultó en un incremento en el contenido de lípidos, principalmente triacilglicéridos (TAG), en *Monodus subterraneus* (Khozin-Goldberg y Cohel, 2006). Estudios conducidos por Otsuka (1961) demostraron que la limitación de azufre en *Chlorella sp.* aumentó el contenido de lípidos totales intracelulares. El uso de algas oleaginosas fototróficas, con contenidos del 20 al 50% de lípidos bajo ciertas condiciones de cultivo, genera una opción comercial importante, sin embargo, uno de los principales obstáculos que impiden la producción comercial de biodiésel a partir de estas microalgas es, el todavía relativamente, alto costo de producción, debido a las bajas velocidades de crecimiento, de acumulación de biomasa y de productividad de lípidos (Tabla 4; Li et al. 2008 b y c).

Además de los factores nutricionales, la composición y el contenido de los lípidos en las microalgas también son afectados por otros factores como la luz (Constantopolous y

Tabla 6. Contenido de lípidos de diferentes microalgas (fuente: Li et al. 2008a, Mata et al. 2010).

Microalga	% Contenido lipídico ($g_{Lípidos}/g_{Peso\text{-}seco}$ x 100)	Microalga	% Contenido lipídico ($g_{Lípidos}/g_{Peso\text{-}seco}$ x 100)
Ankistrodesmus sp.	24-31	*Haematococcus pluvialis*	25
Botryococcus braunii	25-75	*Isochrysis sp.*	7.1-33
Chaetoceros muelleri	33.6	*Monodus subterraneus*	39.3
Chaetoceros calcitrans	14.6-16.4/39.8	*Monallanthus salina*	20-22
Chlorella emersonii	25-63	*Nannochloris sp.*	20-56
Chlorella sp.	10-48	*Nannochloropsis oculata*	22.7-29.7
Chlorella pyrenoidosa	2	*Nannochloropsis sp.*	12-53
Chlorella	18-57	*Neochloris oleabundans*	29-65
Chlorella vulgaris	40	*Nitzchia sp.*	16-47
Chlorella emersonii	63	*Nitzschia laevis*	69.1
Chlorella protothecoides	23	*Pavlova salina*	30.9
Chlorella sorokiniana	22	*Pavlova lutheri*	35.5
Chlorella minutissima	57	*Parietochloris insisa*	62
Chlorella vulgaris	56.6	*Phaeodactylum tricornutum*	18-57
Chlorococcum sp.	19.3	*Porphyridium cruentum*	9-18.8/60.7
Crypthecodinium cohnii	20-51.1	*Scenedesmus sp.*	19.6-21.1
Dunaliella salina	6-25	*Skeletonema sp.*	13.3-31-8
Dunaliella primolecta	23.1	*Skeletonema costatum*	13.5-51.3
Dunaliella tertiolecta	16.7-71	*Spirulina platensis*	4-16.6
Dunaliella sp.	17.5-67	*Spirulina maxima*	4-9
Ellipsoidion sp.	27.4	*Thalassiosira pseudomona*	20.6
Euglena gracilis	14-20	*Tetraselmis suecica*	8.5-23

Bloch 1967, Nichols 1965, Pohl y Wagner 1972, Rosenberg y Gouaux 1967), las bajas temperaturas (Ackman et al. 1968) y, en el caso de microalgas con capacidad de crecimiento heterotrófico, las fuentes de carbono orgánico, en donde pueden llegar a un contenido lipídico del 54% ($g_{Lípidos}/g_{Peso\text{-}seco}$ x 100), con una mayor cantidad de biomasa que en los cultivos fototróficos y además, en una composición ideal para la producción de biodiésel (Cheng et al. 2009a).

Sistemas de cultivo de microalgas

A diferencia de los lípidos provenientes de cultivos vegetales, la producción de lípidos microalgales no requiere del uso de tierra fértil. Este hecho representa una oportunidad para el uso de tierras marginales, semiáridas y áridas, entre otras, donde se pueden

establecer cultivos con microalgas, ya sea de modo fototrófico o heterotrófico (Mata et al. 2010).

Las microalgas están adaptadas para acceder a los nutrientes y ambientes que le permitan su crecimiento y supervivencia, así como para incrementar la eficiencia de utilización de esos nutrientes. En general en el caso del crecimiento fototrófico, para el crecimiento de la biomasa (consistiendo el 40-50% de carbono) las microalgas dependen de un suministro suficiente de dióxido de carbono y luz para llevar a cabo la fotosíntesis. No obstante, las células microalgales pueden ajustar o cambiar su estructura interna (ej. aclimatación bioquímica y fisiológica), mientras que externamente pueden excretar una variedad de compuestos para, entre otras cosas, disponer de nutrientes o limitar el crecimiento de otros microorganismos competidores. Las microalgas pueden asumir varios tipos de metabolismo (ej. autotrófico, heterotrófico, mixotrófico, fotoheterotrófico) y son capaces de realizar un cambio metabólico en respuesta a cambios en las condiciones ambientales. Por ejemplo algunos organismos pueden crecer (Lee 2001, Wen y Chen 2003, Chen y Chen 2006, Chisti 2007, Li et al. 2008c, Patil et al. 2008, Mata et al. 2010):

- Fototróficamente, utilizando dióxido de carbono y luz como única fuente de energía, la cual es convertida en energía química a través de reacciones fotosintéticas.
- Heterotróficamente, utilizando compuestos orgánicos (p. ej. Azúcares) como única fuente de carbono y energía.
- Mixotróficamente, realizando fotosíntesis como la principal fuente de energía, aunque es esencial la presencia de compuestos orgánicos y de CO_2. También existe el concepto de crecer Anfitróficamente, el cual es definido como un subtipo de mixotrofía, en donde los organismos son capaces de crecer tanto

autotróficamente como heterotróficamente, dependiendo de la concentración del compuesto orgánico y la intensidad de luz disponible.

- Fotoheterotróficamente, también conocido como fotoorganotróficamente, fotoasimilación o fotometabolismo, donde el metabolismo para que la microalga puede crecer requiere de la presencia de luz para utilizar los compuestos orgánicos como fuente de carbono.

No solamente la fuente de carbono, vitaminas, sales y otros nutrientes son vitales para el crecimiento microalgal, también es esencial establecer los parámetros operacionales del cultivo como el CO_2, el oxígeno, el pH, la temperatura, transferencia de masa, control de temperatura, la intensidad de luz y la remoción de productos, entre otros (Lee 2001, Eriksen 2008, Li et al. 2008c, Robles-Medina et al. 2009, Posten 2009). Cuando se considera el uso de microalgas para la producción de biodiésel, es importante, para la determinación de los rendimientos del cultivo, definir la influencia de esos parámetros operacionales y su interrelación para saber cómo manipularlos durante el cultivo (Mata et al. 2010).

Cultivo fototrófico

Muchas especies de microalgas son fotótrofos obligados y por lo tanto, requieren luz, CO_2, agua y sales inorgánicas para su crecimiento. La temperatura debe permanecer entre 20 y 30 ºC. Para minimizar los costos de producción, la producción de biodiésel debería depender de la luz de sol disponible libremente, a pesar de las variaciones del nivel de luz en el día y durante las estaciones del año (Chisti 2007, Patil et al. 2008). Varias tecnologías de cultivo que son utilizadas para la producción de biomasa microalgal han sido desarrolladas por investigadores y productores comerciales. Las microalgas fototróficas son comúnmente cultivadas en estanques tipo circuito abiertos al ambiente

(Figura 2) y fotobioreactores cerrados (Figura 3) (Lee 2001, Chisti 2007, Eriksen 2008, Li et al. 2008c, Schenk et al. 2008, Rosenberg et al. 2008, Posten 2009, Xu et al. 2009, Mata et al. 2010). El cultivo en estanques tipo circuito abierto son económicamente más favorables, y son los más utilizados ya que asemejan el entorno natural de las microalgas. Los cultivos abiertos comprenden sistemas naturales (lagos, lagunas, estanques), artificiales, de superficie inclinada y estanques tipo circuito. Estos últimos consisten en circuitos de 15 a 30 cm de profundidad, en los cuales una rueda de paletas mantiene un flujo constante del cultivo; las producciones y productividades biomásicas factibles en estos sistemas son bajas, próximas a 1 g/l y 10-25 g/m^2*d, respectivamente. Las ventajas inherentes a los cultivos abiertos radican en su sencillez y su bajo costo de inversión, a causa de la diversidad de materiales útiles para su construcción (concreto, tierra, plástico, etc.) y la factibilidad que ofrecen para su operación y mantenimiento (Garibay et al. 2009). Sin embargo, los costos de producción se incrementan al considerar el uso de tierras, la disponibilidad de agua y, debido a que son abiertos al ambiente, a las condiciones climatológicas. Además, estos sistemas presentan inconvenientes tales como pérdidas de agua por evaporación, transferencia limitada del CO_2 al cultivo por su baja concentración en el aire (0.035% v/v) y su difusión hacia la atmósfera, control limitado de las condiciones de cultivo, amplios períodos de producción (6 a 8 semanas), producciones reducidas de biomasa y penetración limitada de luz. Además tienen problemas de contaminación por hongos, bacterias y protozoarios y compiten con otras especies de microalgas (excepto en cultivos de especies extremófilas) (Lee 2001, Chisti

2007, Eriksen 2008, Li et al. 2008c, Schenk et al. 2008, Rosenberg et al. 2008, Posten 2009, Xu et al. 2009, Garibay et al. 2009, Mata et al. 2010).

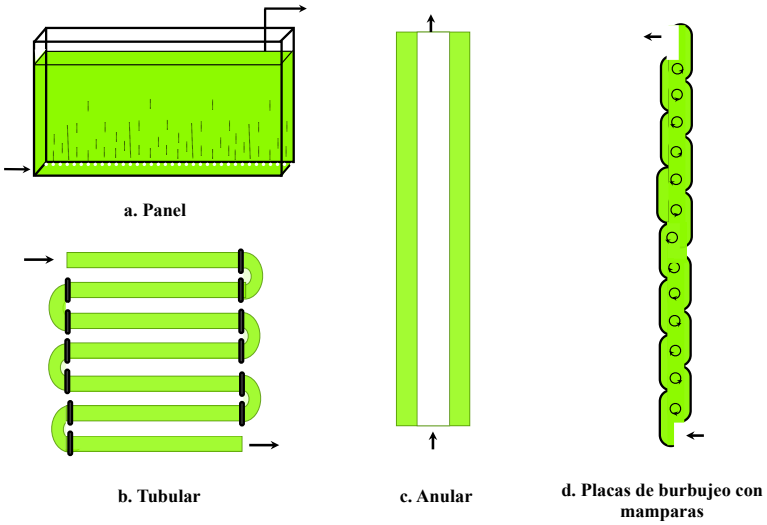


Figura 3. Diseños de fotobioreactores cerrados para la producción de microalgas: a. plano (propuesta clásica); b. tubular (fotobioreactor cerrado más grande actualmente construido); c. anular (actúa como columna de burbujeo, el cilindro interno para evitar partes oscuras e incrementar la relación superficie/volumen); d. de placas de burbujeo con mamparas (estas últimas soportan los efectos de destellos de luz debido al diseño de tubería de flujo controlado). Todos estos diseños son considerados adecuados, pero también económicamente muy costosos para la producción de biodiésel (Schenk et al. 2008).

En contraste con los cultivos abiertos, los fotobioreactores ofrecen un ambiente cerrado que protegen al cultivo de las lluvias y de la invasión de microorganismos no deseados, las condiciones del cultivo son controladas y existe un aumento en la fijación de CO_2, el cual es burbujeado a través del medio de cultivo. Además se requiere del equivalente de menos superficie terrestre, menores periodos de producción (2 a 4 semanas) y se pueden obtener productividades considerablemente superiores (5 a 13 veces en comparación con los estanques). La consideración de factores tales como la luz, la relación CO_2/O_2, la temperatura, los nutrientes, la salinidad, el pH, entre otros, resulta trascendental para el diseño de sistemas cerrados. Las altas productividades inherentes a estos sistemas

precisan de una penetración y distribución favorable de la luz, condición que a su vez requiere de materiales de construcción transparentes y de relaciones superficie/volumen elevadas. Sin embargo, la intensidad de la luz incidente debe ser moderada, de lo contrario se presentan fenómenos de fotoinhibición y fotoblanqueo. Asimismo, la relación CO_2/O_2 debe ser tal que la proporción de O_2 sea mínima y por ende, sean impedidos procesos de fotorespiración y daño fotooxidativo (Chisti 2007, Schenk et al. 2008, Garibay et al. 2009). Este sistema de cultivo es económicamente más costoso comparado con los estanques tipo circuito abierto, debido al precio relativamente alto de la infraestructura (Lee 2001, Chisti 2007, Eriksen 2008, Li et al. 2008c, Schenk et al. 2008, Rosenberg et al. 2008, Posten 2009, Xu et al. 2009, Garibay et al. 2009, Mata et al. 2010). Se han desarrollado varios diseños de fotobioreactores (Figura 3), el diseño tubular parece ser el más satisfactorio para la producción de biomasa microalgal en la escala necesaria para la producción de biodiésel (Mata et al. 2010). Este tipo de configuración es utilizada actualmente para la producción de metabolitos de alto valor, como los farmacéuticos, los cuales pueden ser combinados con la producción de biodiésel para reducir los costos de producción (Patil et al. 2008). Sin embargo, la mayor limitante en estos sistemas de cultivo es que se obtienen bajas densidades celulares y en consecuencia bajas productividades de lípidos, lo que ocasiona el incremento de los costos de producción al dificultarse la obtención de biomasa desde estos cultivos diluidos (Mata et al. 2010).

Cultivo heterotrófico

Algunas microalgas son capaces de crecer heterotróficamente, con uno o más sustratos

como única fuente de carbono y energía. Para este tipo de microalgas, es posible adoptar y modificar tecnologías de fermentación que permitan producir biodiésel a gran escala (Wen y Chen, 2003). Los equipos utilizados en este sistema son bioreactores de tanque agitado, similares a los bioreactores-fermentadores utilizados para muchos otros microorganismos e independientes de luz. El escalamiento en estos sistemas es mucho más simple, incluyendo consideraciones como el tamaño del reactor, el mezclado, la transferencia de masa y la productividad (Eriksen 2008). Además ofrecen la posibilidad de incrementar en forma importante la densidad celular y productividad en cultivos por lotes, permitiendo la recuperación de la biomasa con bajos costos. Los cultivos heterotróficos en lote pueden ser modificados convenientemente para obtener altas densidades celulares mediante cultivos alimentados y para lograr altas productividades de lípidos mediante cultivos continuos o cultivos de perfusión (Wen y Chen, 2003). Además, este tipo de cultivos ofrecen la ventaja de maximizar la concentración de lípidos intracelulares, como es el caso de *Chlorella protothecoides*, la cual, al ser cultivada heterotróficamente logró acumular más del 55% de lípidos ($g_{Lípidos}/g_{Peso\text{-}seco}$), comparado con el 14% que lograba acumular en cultivos fototróficos (Wu y Miao, 2006). Sin embargo, la mayor limitante en este tipo de cultivos es la utilización de fuentes de carbono orgánico que provienen de organismos fotosintéticos (Chisti 2007). No obstante, se han realizado investigaciones para utilizar azúcares que provienen de residuos lignocelulósicos, obteniéndose resultados alentadores. La utilización de este tipo de fuentes de carbono resultan ser viables, tanto técnico como económicamente, debido a su abundancia, capacidad de renovación sustentable y su bajo costo de adquisición

(Martínez Jiménez y Gosset-Lagarda, 2007).

Potencial de las zonas áridas para la producción de biodiésel

Actualmente se ha propuesto el uso de lípidos microalgales para la producción de biodiésel, ya que es una alternativa que asegura satisfacer o reemplazar la demanda global de petrodiesel (Gouveia y Oliveira, 2009, Garibay et al. 2009). En este sentido y enfocándose a la utilización de tierras no aptas para la agricultura como es el caso de las zonas semiáridas y áridas del norte de México (ej. el desierto de Sonora), es interesante notar que los sistemas de cultivo de microalgas pueden ser implementados en estas áreas (Campbell 2008). Algunas microalgas potencialmente pueden utilizar eficientemente el agua de mar proveniente de las costas de Sonora. En contraste con los cultivos tradicionales, los cultivos de microalgas son flexibles ante el tipo y la calidad de agua; dependiendo de la especie pueden prosperar convenientemente en aguas marinas, dulces, salobres y residuales), (Garibay et al. 2009). Esto genera una gran ventaja, ya que generalmente en las zonas áridas costeras el agua dulce y la lluvia son escasas (Fairless 2007, Liu y Zhao 2007, Sharma et al. 2008, Song et al. 2008). Además, si se utilizan otros tipos de agua, diferentes al agua pura o de riego y/o de lluvia, tal como aguas residuales o salobres, potencialmente se podrían disminuir los costos de producción, se aprovecharían residuos, se produciría un valor agregado y se contribuiría a la creación de empleos (Robles-Medina et al. 2009).

Las zonas áridas de México tienen un gran potencial para la producción de biodiésel, para aprovechar los recursos naturales de estas zonas se pueden emplear estanques tipo circuito abierto al ambiente y/o fotobiorreactores cerrados, o una

combinación de estos para generar biomasa microalgal en fotobiorreacores cerrados y favorecer la acumulación de lípidos en los estanques tipo circuito. Es más, si estos sistemas de cultivo se localizan geográficamente en zonas desérticas cercanas al mar, se podría aprovechar el agua proveniente del mar para el cultivo utilizando microalgas marinas. Un aspecto que se debe cuidar es la evaporación del agua en los cultivos y contemplar esquemás de recuperación para reciclarla. Además, en estas zonas se encuentran varias condiciones ambientales favorables para el cultivo de cierto tipo de microalgas, por ej., de acuerdo con Ben-Amotz (2008) la temperatura ideal para algunos cultivos es arriba de los 15 ºC, lo cual es compatible con varias regiones semiáridas del país. La temperatura representa un papel fundamental en la producción de lípidos, ya que este parámetro determina la cantidad total de lípidos contenidos en las microalgas que se puede obtener (Hu et al. 2008), así como el tipo de lípidos sintetizados. Por su parte, la radiación solar es un aspecto de suma relevancia, dado que las microalgas son organismos que requieren de la radiación solar para realizar el proceso de fotosíntesis. Este es un aspecto fundamental en los cultivos fototróficos con microalgas, ya que las productividades que se pueden lograr son limitadas por el suministro de luz (Eriksen 2008), como se describe en las ecuaciones propuestas por Zemke et al. (2008):

$$P_a = \frac{\tau \varepsilon_a \dot{E}_S}{E_a} \qquad \text{Ec. 1}$$

en donde P_a es la velocidad de producción de microalgas, τ es la eficiencia de transmisión de luz al cultivo, ε_a es la eficiencia de conversión de la luz de sol incidente a

biomasa microalgal, $\dot{E}_S$ es la radiación solar y E_a es el contenido energético de las microalgas.

Para determinar los valores de la ecuación 1, es necesario definir cada uno de sus componentes. El contenido energético de las microalgas se define mediante la siguiente ecuación:

$$E_a \approx f_L E_L + f_p E_p + f_c E_c \qquad \text{Ec. 2}$$

donde $E_{L,\ P\ y\ C}$ es el contenido energético de lípidos, proteínas y carbohidratos en las microalgas; $f_{L,\ P\ y\ C}$ es la fracción, en base seca, del contenido de lípidos, proteínas y carbohidratos. Por otro lado, la eficiencia de transmisión de luz al cultivo se define de acuerdo a la siguiente ecuación:

$$\tau = \varepsilon_{opt} \alpha \eta c_{PAR} \qquad \text{Ec. 3}$$

donde ε_{opt} es la fracción de luz que no es reflejada, α es el coeficiente o cantidad de luz absorbida por las microalgas, η es la fracción de superficie que ocupa el medio cultivo respecto al área total del sistema de estanques, c_{PAR} es la fracción de la radiación de la luz solar que es fotosintéticamente activa. Por último, la eficiencia de conversión de la luz solar incidente a biomasa microalgal (ε_a) se define como:

$$\varepsilon_a = \varepsilon_{env} \varepsilon_{ph} u_p (1 - r) \qquad \text{Ec.4}$$

donde ε_{env} son las pérdidas debido a condiciones ambientales subóptimas, ε_{ph} es la eficiencia fotosintética, r es la fracción de energía consumida por la respiración de las

microalgas y u_P es la fracción de fotones capturados utilizados por las microalgas, que a su vez se define mediante la siguiente ecuación:

$$u_p = \left\{ \frac{I_S}{I_I} \left[\ln\left(\frac{I_I}{I_S} \right) + 1 \right] \right\} \qquad I_I \geq I_S \qquad \text{Ec.5}$$

$$u_p = 1 \qquad I_I \leq I_S \qquad \text{Ec. 6}$$

donde I_I se define como la densidad del flujo de fotones incidentes en las microalgas en μmol fotones m^{-2} s^{-1}, I_S es la densidad del flujo de fotones incidentes saturantes para las microalgas en μmol fotones m^{-2} s^{-1}.

Es relevante determinar la productividad máxima teórica que se puede alcanzar en estos sistemas de cultivo en donde utilizan la luz solar para la producción de lípidos. Este parámetro se puede determinar mediante el cálculo de la velocidad de producción de lípidos potencialmente utilizables para biodiésel, se define como

$$P_{CL} = \frac{f_{CL} P_a}{\rho_{CL}} \qquad \text{Ec. 7}$$

donde f_{CL} es la fracción de lípidos microalgales en base seca utilizable para biodiésel (< f_L), P_a es la velocidad de producción de biomasa microalgal y ρ_{CL} es la densidad de lípidos utilizables para su conversión a biodiésel (Zemke et al. 2008).

Teóricamente, la máxima velocidad de producción de lípidos potencialmente utilizables para su conversión a biodiésel reportado por Zemke et al. (2008) es de 43 L m^{-2} $año^{-1}$. Sin embargo, el potencial de las microalgas para la producción de biodiésel ha sido sobreestimado por diversas empresas que aseguran productividades iguales o

superiores al máximo teórico posible (Wijffels 2008, Waltz 2009). La eficiencia fotosintética se define como la fracción de la energía luminosa absorbida que es fijada como energía química en la biomasa durante el crecimiento fototrófico. La fijación fotosintética de un mol de CO_2 en biomasa microalgal con una composición representativa ($CH_{1.78}O_{0.35}N_{0.12}$) en sistemas con amonio como fuente de nitrógeno se considera que requiere de la absorción de 14 fotones, aunque de acuerdo a las estimaciones de autores diversos, esta magnitud puede variar de 6 a 16. La incorporación de 1 mol de carbono resulta así en la producción de 21.25 g de peso seco con un contenido energético (entalpía de combustión) de 547.8 kJ (Wijffels 2008). Cabe destacar que la totalidad de la radiación solar no es aprovechada en la fotosíntesis microalgal, sólo es útil el espectro comprendido entre los 400 y 700 nm de longitud de onda denominado "radiación fotosintéticamente activa" (PAR por sus siglas en inglés), región que sólo representa el 42.3% del total. La energía promedio de los fotones comprendidos en este rango es de 218 kJ. Los datos anteriores posibilitan determinar una eficiencia fotosintética máxima para las microalgas del 7.6% respecto a la radiación solar total, valor que a pesar de ser reportado por varios investigadores en un intervalo del 3 al 10%, es cercano o superior al máximo teórico precisado para plantas C_3 (2.4 - 4.3%) y C_4 (1.3 - 3.7%). El aprovechamiento parcial de la radiación fotosintéticamente activa es atribuido a fenómenos diversos, principalmente a la disipación de energía en los aparatos fotosintéticos en forma de calor o fluorescencia, con el fin de evitar el daño de éstos por radiación excesiva (fotoinhibición) (Pulz y Scheibenbogen, 1998, Janssen et al. 2003, Dismukes et al. 2008, Wijffels 2008).

COMENTARIOS FINALES

La energía solar es un recurso renovable abundante y de alta calidad en México, sobre todo en las zonas áridas del norte del país, ya que la radiación solar promedio es de 1,825 kWh m^{-2} $año^{-1}$ (Jiménez et al. 2007). Al considerar una eficiencia fotosintética del 7.6%, se determina que la máxima productividad teórica de biomasa seca microalgal con esa radiación sería de 194 tons ha^{-1} $año^{-1}$; asimismo, al suponer un contenido de triglicéridos del 30% ($g_{TAG}/g_{Biomasa}$), una eficiencia de transesterificación del 96% (Al-Zuhair 2007) y una densidad del biodiésel microalgal de 0.864 kg/L (Xu et al. 2006), se estima una producción hipotética del biocombustible (biodiésel) de 64,500 L ha^{-1} $año^{-1}$, magnitud que en la práctica no ha sido lograda hasta ahora. Acorde con Wijffels (2008), la tecnología actualmente disponible permitiría producir alrededor de 20,000 L ha^{-1} $año^{-1}$ de biodiésel. No obstante, este valor podría mejorarse, para ser cercano al máximo teórico, a través de avances en la selección de especies, las estrategias y sistemas de cultivo, los procedimientos de cosecha y extracción de aceites, aunados al mejoramiento del metabolismo de los microorganismos mediante recursos moleculares (Wijffels 2008, Waltz 2009). Además, debido a que las zonas desérticas no son útiles para cultivos agrícolas, el cultivo con microalgas no desplaza a los cultivos destinados para la alimentación. Estos datos indican claramente la superioridad del potencial de producción de biodiésel a partir de cultivos microalgales en contraste con el uso de aceites vegetales (tanto comestibles como no comestibles) como materia prima para la obtención de biodiésel.

LITERATURA CITADA

Acién, F., F. García, J. Sánchez, J. Fernández, E. Molina. 1998. Modelling of biomass productivity in tubular photobioreactors for microalgal cultures: effects of dilution rate, tube diameter and solar irradiance. Biotechnology and Bioengineering 58(6): 605–616.

Ackman, R., C. Tocher, J. McLachlan. 1968. Marine phytoplankter fatty acids. Journal of the Fisheries Research Board of Canada 25: 1603-1620.

Akkerman, I., M. Jansen, J. Rocha, R. H. Wijffels. 2002. Photobiological hydrogen production: photochemical efficiency and bioreactor design. International Journal of Hydrogen Energy 27: 1195-208.

Al-Zuhair, S. 2007. Production of biodiesel: possibilities and challenges. Biofuels, Bioproducts and Biorefining 1: 57-66.

Arredondo, B. O., R. Vázquez-Duhalt. 1991. Aplicaciones biotecnológicas en el cultivo de microalgas. Ciencia y Desarrollo 17: 99-111.

ASTM, 6751. -03a 2003 Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels. 1-6 pp

Ben-Amotz, A. 2008. Large scale open algae ponds. Workshop on Algal Oil for Jet Fuel Production. February 19-21, Colorado, E. E. U. U. [http://www.nrel.gov/biomass/algal_oil_workshop.html]

Banerjee, A., R. Sharma, Y. Chisti, U. C. Banerjee. 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Critical Reviews in Biotechnology 22: 245-279.

Benemann, J. R., P. Pursoff, W. J. Oswald. 1978. Engineering design and cost analysis of a large-scale microalgae biomass system, NTIS #H CP/T1605-01 UC-61. Washington DC: US Department of Energy.

Benemann, J. R., W.J. Oswald. 1996. Systems and economic analysis of microalgae ponds for conversion of CO_2 to biomass. Final report. US DOE-NETL. [http://www.osti.gov/bridge/servlets/purl/493389-FXQyZ2/webviewable/493389.pdf]

Campbell, M. N. 2008. Biodiesel: algae as a renewable source for liquid fuel. Guelph Engineering Journal 1: 2-7.

Carlozzi, P. 2000. Hydrodynamic aspetcs and *Arthrospira* growth in two outdoor tubular undulating row photobioreactors. Applied Microbiology and Biotechnology 54: 14-22.

Chen, G. Q., F. Chen. 2006. Growing phototrophic cells without light. Biotechnology Letters 28: 607-616.

Cheng, Y., W. Zhou, C. Gao, K. Lan, Y. Gao, Q. Wu. 2009. Biodiesel production from Jerusalem artichoke (*Helianthus tuberosus L.*) tuber by heterotrophic microalgae *Chlorella protothecoides.* Society of Chemical Industry 84: 777-781.

Chisti, Y. 2007. Biodiesel from microalgae. Biotechnology Advances 25:294-306.

Chiu, S. Y., C. Y. Kao, M. T. Tsai, S. C. Ong, C. H. Chen, C. S. Lin. 2009. Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. Bioresource Technology 100: 833-838.

Constantopolous, G., K. Bloch. 1967. Effect of light intensity on the lipid composition of *Euglena gracilis*. The Journal of Biological Chemistry 242: 3538-3542.

Converti, A., A. Lodi, C. Solisio, D. Soletto, M. del Borghi, J. Carvalho. 2006. *Spirulina platenisis* biomass as adsorbent for cooper removal. Ciencia y Tecnología alimentaria 5(2): 85-88.

de Swaaf, M., J. Pronk, L. Sijtsma. 2003a. Fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii.* Biotechnology and Bioengineering 61: 40-43.

de Swaaf, M., L. Sijtsma, J. Pronk. 2003b. High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii.* Biotechnology and Bioengineering 81: 666-672.

Dismukes, G., D. Carrieri, N. Bennette, G. Ananyev, M. Posewitz. 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology 19: 235-240.

Dorval, N., A. Parisien, B. Wang, C. Lan. 2009. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. Journal of Biotechnology 141(1-2): 31-41.

Doucha J, F. Straka, K. Lívanský. 2005. Utilization of flue gas for cultivation of microalgae (*Chlorella* sp.) in an outdoor open thin-layer photobioreactor. Journal of Applied Phycology 17: 403–412.

Dunahay, T. G., E. E. Jarvis, S. S. Dais, P. G. Roessler. 1996. Manipulation of microalgal lipid production using genetic engineering. Applied Biochemistry and Biotechnology 57-58: 223-231.

Freedman, B., Pryde, E. H., Mounts, T. L., 1984. Variables affecting the yield of fatty esters from transesterified vegetable oils. JAOCS 61: 1638-1643.

Freedman, B., Butterfield, R. O., Pryde, E. H., 1986. Transesterification kinetics of soybean oil. JAOCS 63: 1375-1380.

Eriksen, N. 2008. The technology of microalgal culturing. Biotechnology Letters 30(9): 1525-1536.

European Standard EN 5509. 2000. Animal and vegetable fats and oils-preparation of methyl esters of fatty acids. [http://esearch.cen.eu/CatWeb.aspx?id=2491009]

Fairless, D. 2007. The little shrub that could-maybe. Nature 449: 652-655.

Fedorov, A. S., S. Kosourov, M. L. Ghirardi, M. Seibert. 2005. Continuous H_2 photoproduction by *Chlamydomonas reinhardtii* using a novel two-stage, sulfate-limited chemostat system. Applied Biochemistry and Biotechnology 121-124: 403-412.

García-González, M., J. Moreno, J. Manzano, J. Florencio, M. Guerrero. 2005. Production of *Dunaliella salina* biomass rich in 9-*cis*-β-carotene and lutein in a closed tubular photobioreactor. Journal of Biotechnology 115(1): 81-90.

Garibay, A. 2010. Evaluación de condiciones de cultivo en la producción de lípidos con el alga *Neochloris oleoabundans* para su uso potencial como biodiésel. Tesis de Maestría en Ciencias Bioquímicas. Instituto de Biotecnología UNAM.

Garibay, A., R. Vázquez-Duhalt, M. Sánchez, L. Serrano-Carreón, A. Martínez. 2009. Biodiésel a partir de microalgas. Revista de la Sociedad Mexicana de Biotecnología y Bioingeniería 13 (3): 1-25. [http://www.smbb.com.mx/revista/Revista_2009_3/Biodiesel.pdf]

Gavrilescu, M., Y. Chisti. 2005. Biotechnology - a sustainable alternative for chemical industry. Biotechnology Advances 23: 471-499.

Ghirardi, M. L., J. P. Zhang, J. W. Lee, T. Flynn, M. Seibert, E. Greenbaum, A. Melis. 2000. Microalgae: a green source of renewable H_2. Trends in Biotechnology 18: 506-511.

Gouveia, L., A. Oliveira. 2009. Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology and Biotechnology 36: 269-274.

Gouveia, L., A. Marques, T. da Silva, A. Reis. 2009. *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. Journal of industrial Microbiology and Biotechnology 36: 821-826.

Grobbelaar, J. 2000. Physiological and technological considerations for optimizing mass algal cultures. Journal of Applied Phycology 12: 201-206.

Hu, Q., M. Sommerfield, E. Jarvis, M. Ghirardi, M. Poseewitz, M. Seibert, A. Darzins. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant Journal 54: 621-639.

Huntley, M., D. Redalje. 2007. CO_2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. Mitigation and Adaptation Strategies for Global Change 12(4): 573-608.

Indicadores Petroleros 2008. [http://www.ri.pemex.com/index.cfm?action=content&sectionID=16&catID=12155]

Janssen, M., J. Tramper, L. R. Mur, R. H. Wijffels. 2003. Enclosed outdoor photobioreactors: light regime, photosynthetic efficiency, scale up and future prospects. Biotechnology and Bioengineering 81: 193-210.

Jiménez, C., B. Cossio, D. Labella, F. Niell. 2003. The feasibility of industrial production of *Spirulina* (*Arthrospira*) in southern Spain. Aquaculture 217: 179–190.

Jiménez, A., A. Sánchez-Juárez, A. Fernández, X. Mathew, P. J. Sebastian. 2007. Converting solar radiation to electric power in Mexico. In: Towards a cleaner planet. Klapp, J., J. L. Cervantes-Cota, J. F. Chávez (eds.) Springer Berlin Heidelberg pp. 281-303.

Kapdan, I. K., F. Kargi. 2006. Bio-hydrogen production from waste materials. Enzyme and Microbial Technology 38: 569-582.

Khozin-Goldberg, I., Z. Cohel. 2006. The effect of phosphate starvation n the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. Phytochemistry 67: 696-701.

Laws, E., S. Taguchi, J. Hirata, L. Pang. 1986. High algal production rates achieved in a shallow outdoor flume. Biotechnology and Bioengineering 28: 191-197.

Lee, Y. K. 2001. Microalgal mass culture systems and methods: their limitation and potential. Journal of Applied Phycology 13(4): 307-315.

Li, X., H. Xu, Q. Wu. 2007. Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering 98(4): 764-771.

Li, Q., W. Du, D. Liu. 2008a. Perspectives of microbial oils for biodiesel production. Applied Microbiology and Biotechnology 80: 749-756.

Li, Y., M. Horsman, N. Wu, C. Lan, N. Dubois-Calero. 2008b. Biofuels from microalgae. Biotechnology Progress 24(4): 815-820.

Li, Y., M. Horsman, B. Wang, N. Wu, C. Lan. 2008c. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied Microbiology and Biotechnology 81(4): 629-636.

Liu, B., Z. Zhao. 2007. Biodiesel production by direct methanolysis of oleaginous microbial biomass. Journal of Chemical Technology and Biotechnology 82: 775-780.

Ma, F., Clements, L.D., Hanna, M.A., 1998. The effects of catalyst, free acids and water on transesterification of beef tallow. Trans ASAE 41: 1261-1264.

Martínez-Jiménez, A. 2008. Potencial de producción de biodiésel con algas oleaginosas. [http://www.dictus.uson.mx/bio3/bio1.html]

Martínez-Jiménez, A. y Gosset-Lagarda, G. (2007). Ingeniería metabólica de bacterias. En: Una ventana al quehacer científico. Instituto de Biotecnología de la UNAM. 373-384. [http://www.ibt.unam.mx/computo/pdfs/libro_25_aniv/capitulo_32.pdf]

Martínez Jiménez, A. 2009. Biocombustibles biotecnológicos. Ide@s CONCYTEG. Publicación del Consejo Nacional de Ciencia y Tecnología del Estado de Guanajuato. 4, 1198-1215. [http://octi.guanajuato.gob.mx/octigto/formularios/ideasConcyteg/Archivos/54042009_BIOCOMBUSTIBLES_BIOTECNOLOGICOS.pdf]

Mata, T. M., A. A. Martinis, N. S. Caetano. 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews 14: 217-232.

Mayfield, S. (2008). Algal model systems. Workshop on Algal Oil for Jet Fuel Production. February 19-21, Colorado, E. E. U. U. [http://www.nrel.gov/biomass/algal_oil_workshop.html]

Maxwell, E. L., A. G. Folger, S. E. Hogg. 1985. Resource evaluation and site selection for microalgae production systems. SERI/TR-215-2484. [http://www.osti.gov/bridge//searchresults.jsp?formname=searchform&Author=%22Maxwell,%20E.%22]

Meier, R. L. (1955). Biological cycles in the transformation of solar energy into useful fuels. In Solar Energy Research (Daniels, F., J. A. Duffie, eds). Madison, WI: University of Wisconsin Press. p. 179-183.

Melis, A. 2002. Green alga hydrogen production: Progress, challenges and prospects. International Journal of Hydrogen Energy 27: 1217-1228.

Meng, X., X. Yang, X. Xu, L. Zhang, Q. Nie, M. Xian. 2009. Biodiesel production from oleaginous microorganisms. Renewable Energy 34: 1-5.

Molina Grima, E., J. Fernández, F. Acién Fernández, Y. Chisti. 2001. Tubular photobioreactor design for algal cultures. Journal of Biotechnology 92: 113-131.

Moore, A. 2001. Blooming prospects? Humans have eaten seaweed for millennia; now microalgae are to be served up in a variety of novel health supplements, medicaments and preparations. European Molecular Biology Organization 2(6): 462-464.

Nichols, B. 1965. Light induced changes in the lipids of *Chlorella vulgaris*. Biochimica et Biophysica Acta 106: 274-279.

Oswald, W. J., C. Golueke. 1960. Biological transformation of solar energy. Advances in Applied Microbiology 2: 223-262

Otsuka, H. 1961. Changes of lipid and carbohydrate contents of *Chlorella* cells during the sulfur starvation, as studied by the technique of synchronous culture. Journal of General and Applied Microbiology 7: 72-77.

Patil, V., K. Q. Tran, H. R. Giselrod. 2008. Towards sustainable production of biofuels from microalgae. International Journal of Molecular Sciences 9: 1188-1195.

Pohl, P., H. Wagner. 1972. Control of fatty acid and lipid biosynthesis in *Euglena gracilis* by ammonia, light and DCMU. Zeitschrift für Naturforschung 27: 53-61.

Posten, C. 2009. Design principles of photo-bioreactors for cultivation of microalgae. Engineering in Life Sciences 9(3): 165-177.

Pulz, O., K. Scheibenbogen. 1998. Photobioreactors: design and performance with respect to light energy input. In: Advances in biochemical engineering/ biotechnology. Scheper T. (ed.) Springer-Verlag Berlin Heidelberg pp. 123-152.

Richmond, A. 2004. Handbook of microalgal culture: Biotechnology and applied phycology. Blackwell Science Ltd.

Robles-Medina, A., P. A. González-Moreno, L. Esteban-Cerdán, E. Molina-Grima. 2009. Biocatalysis: Towards ever greener biodiesel production. Biotechnology Advances 27: 398-408.

Rodolfi, L., G. Chini, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M. Tredici. 2008. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 102(1):100-112.

Roessler, P. G., L. M. Brown, T. G. Dunahay, D. A. Heacox, E. E. Jarvis, J. C. Schneider, S. G. Talbot, K. G. Zeiler. 1994. Genetic-engineering approaches for enhanced production of biodiesel fuel from microalgae. ACS Symposium Series 566: 255-270.

Rosenberg, A., J. Gouaux. 1967. Quantitative and compositional changes in monogalactosyl and digalactosyl diglycerides during light-induced formation of chloroplasts in *Euglena gracilis.* Journal of Lipid Research 8: 80-83.

Rosenberg, J., G. Oyler, L. Wilkinson, M. Betenbaugh. 2008. A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Current Opinion in Biotechnology 19: 430-436.

Sato, T., S. Usui, Y. Tsuchiya, Y. Kondo. 2006. Invention of outdoor closed type photobioreactor for microalgae. Energy Conversion Manage 47: 791-799.

Sawayama, S., S. Inoue, Y. Dote, S. Y. Yokoyama. 1995. CO_2 fixation and oil production through microalga. Energy Conversion and Management 36: 729-731.

Schenk, P., S. Thomas-Hall, E. Stephens, U. Marx, J. Mussgnug, C. Posten, O. Kruse, B. Hankamer. 2008. Second generation biofuels: High-efficiency microalgae for biodiesel production. Bioenergy Resource 1: 20-43.

Scragg, A., A. Illman, A. Carden, S. Shales. 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass and Bioenergy 23(1): 67-73.

Sharma, Y. C., B. Singh, S. N. Upadhyay. 2008. Advancements in development and characterization of biodiesel: a review. Fuel 87: 2355-2373.

Sheehan, J., T. Dunahay, J. Benemann, P. A. Roessler. 1998. A look back at the U. S. Departmet of Energy´s Aquatic Species Program - biodiesel from algae. National Renewable Energy Laboratory, Golden, CO. Report NREL/TP-580-24190. [http://www.nrel.gov/docs/legosti/fy98/24190.pdf]

Song. D., J. Fu, D. Shi. 2008. Exploitation of oil-bearing microalgae for biodiesel. Chinese Journal of Biotechnology 24: 341-348.

Spoehr, H., H. Milner. 1949. The chemical composition of *Chlorella*; affect of environmental conditions. Plant Physiology 24: 120-149.

Spolaore, P., C. Joannis-Cassan, E. Duran, A. Isambert. 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering 101: 8796.

Tanaka, Y., Okabe, A., Ando, S., 1981. Method for preparation of a lower alkyl ester of fatty acids. US Patent 4: 303-590.

Torrey, M. 2008. Algae in the tank. International News on Fats Oils and Related Materials 19(7): 432-437.

Torzillo, G., B. Pushparaj, F. Bocci, W. Balloni, R. Materassi, G. Florenzano. 1986. Production of *Spirulina* biomass in close photobioreactors. Biomass 11: 61-74.

Waltz, E. 2009. Algae trailblazer shuts. Nature Biotechnology 27(7): 586.

Watanabe, Y., J. Delanoue, D. Hall. 1995. Photosynthetic performance of a helical tubular photobioreactor incorporating the Cyanobacterium *Spirulina platensis.* Biotechnology and Bioengineering 47(2): 261-269.

Wen, Z., F. Chen. 2001. A perfusion-cell bleeding culture strategy for enhancing the productivity of eicosapentaenoic acid by *Nitzschia laevis*. Applied Microbiology and Biotechnology 57: 316-322.

Wen, Z., F. Chen. 2003. Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnology Advances 21(4): 273-294.

Werner, D. 1966. Die Kieselsaure im Stoffwechsel von *Cyclotella cryptica* reimann, Lewin and Guilard. Arch. Mikrobiol 55: 278-308.

Wijffels, R. H. 2008. Potential of sponges and microalgae for marine biotechnology. Trends in Biotechnology 26: 26-31.

World Bank, 2008. Focus B, Biofuel: Promise and Risks. [http://econ.worldbank.org/WBSITE/EXTERNAL/EXTDEC/EXTRESEARCH/EXTWDRS/

EXTWDR2008/0,,contentMDK:21410054~menuPK:3149676~pagePK:64167689~piPK:64167673~theSitePK:2795143,00.html]

Wu, Q., X. Miao. 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology 97: 841-846.

Xiong, W., X. Li, J. Xiang, Q. Wu. 2008. High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology 78: 29-36.

Xu, H., X. Miao, Q. Wu. 2006. High quality biodiesel production from microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126: 499-507.

Xu, L., P. J. Weathers, X. R. Xiong, C. Z. Liu. 2009. Microalgal bioreactors: Challenges and opportunities. Engineering in Life Sciences 9(3): 178-189.

Zemke, P., B. Wood, D. Dye. 2008. Technoeconomic analysis of algal photobioreactors for oil production. Workshop on Algal Oil for Jet Fuel Production. February 19-21, Colorado, E. E. U. U. [http://www.nrel.gov/biomass/algal_oil_workshop.html]

Zitelli, G., L. Rodolfi, N. Biondi, M. Tredici. 2006. Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns. Aquaculture 261: 932-943.

IV. JUSTIFICACIÓN

N. oleoabundans es una microalga oleaginosa que produce un 80% de triacilgliceroles (TAG) del total de sus lípidos. Muchos de los ácidos grasos son saturados que poseen 16-20 átomos de carbono [21], ideales para su uso como aceite comestible o para la producción de biodiesel [22].

Al inicio de esta tesis no se encontraban muchos trabajos relacionados con la producción de esta microalga para la obtención de metabolitos de reserva, y ninguno bajo condiciones heterotróficas de crecimiento. Sin embargo, en estudios preliminares (Comunicación Personal, Adriana Garibay Hernández) realizados en nuestro grupo de trabajo a nivel matraz, se observó que esta microalga era capaz de crecer bajo condiciones heterotróficas utilizando glucosa como fuente de carbono.

Reportes previos han mostrado que la relación C/N en cultivos con microalgas tiene efectos considerables en el metabolismo de reserva. Así, a una relación C/N alta se promueve la acumulación de lípidos [23]. En el caso de *N. oleoabundans* se ha observado que la estrategia de limitación del crecimiento por la fuente de nitrógeno (relación C/N alta) resulta efectiva para la acumulación preferencial de lípidos [15, 19, 22]. Un proceso de limitación de nitrógeno controlado podría ser llevado a cabo a través de cultivos exponencialmente alimentados controlando la velocidad específica de crecimiento. Así, además de promover la acumulación de metabolitos de reserva, también se podrían obtener altas densidades celulares en un solo proceso.

N. oleoabundans es una microalga que no tiene su genoma secuenciado. Este hecho limita el conocimiento a fondo de su metabolismo y por consiguiente, el desarrollo de herramientas de ingeniería genética que puedieran incrementar la capacidad de esta microalga para producir metabolitos de reserva a una velocidad de crecimiento comparable con bacterias o levaduras. Sin embargo, las herramientas 'omicas' como la transcriptómica, metabolómica o la proteómica

han sido utilizadas cuando existe esta limitante. Especificamente, la proteómica podría ayudar a entender el porqué son sintetizadas algunas proteínas claves en las vías metabólicas microalgales cuando se encuentran bajo ciertas condiciones de cultivo. Esto podría considerarse como el inicio del entendimiento del metabolismo de una especie de microalga no secuenciada.

V. HIPÓTESIS

La manipulación de la relación C/N y la velocidad de crecimiento en cultivos heterotróficos de *N. oleoabundans* conducirán al incremento en la concentración de la masa celular y a la acumulación de lípidos o carbohidratos, además el análisis del proteoma bajo estas diferentes estrategias de cultivo revelará puntos clave en la síntesis de proteínas implicadas en el metabolismo central y las vías de síntesis de metabolitos de reserva.

VI. OBJETIVOS

OBJETIVO GENERAL

Desarrollar cultivos de *N. oleoabundans* bajo condiciones estrictas de crecimiento heterotróficas con el fin de estudiar el efecto de la relación C/N y la velocidad de crecimiento sobre la composición macromolecular y el perfil proteómico de esta microalga.

OBJETIVOS ESPECIFICOS

1. Determinar fuentes de carbono que pueden ser metabolizadas bajo condiciones estrictamente heterotróficas por *N. oleoabundans.*

2. Estudiar el efecto de la relación C/N balanceada y alta sobre los parámetros cinéticos y estequiométricos, la acumulación de metabolitos de reserva y el perfil de ácidos grasos en *N. oleoabundans*.

3. Desarrollar cultivos alimentados y limitados por la fuente de nitrógeno bajo dos estrategias de alimentación: intermitente y exponencial; ambas

con nitrato de sodio para maximizar la producción de biomasa y metabolitos de reserva por *N. oleoabundans*.

4. Identificar y analizar las proteínas que se sintetizan de manera diferencial entre los cultivos de *N. oleoabundans* en condiciones de acumulación preferencial de lípidos y de carbohidratos a través de estudios de proteómica.

VII. PRESENTACIÓN DE MANUSCRITOS (Manuscripts presentation)

VII.1 Morales-Sánchez D., Tinoco R., Kyndt J. and Martínez A. (2013). **Heterotrophic growth of *Neochloris oleoabundans* using glucose as carbon source**. *Biotechnology for Biofuels* 6:100.

VII.2 Morales-Sánchez D., Tinoco R., Caro M. and Martínez A. (2014). **Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid and carbohydrate production (accumulation)**. *Submitted to 'Algal Research'.*

VII.3 Morales-Sánchez D., Kyndt J., Ogden K. and Martinez A. (2014). **Lipid accumulation triggered by pyruvate dehydrogenase accumulation and high pentose phosphate pathway activation was revealed through proteomic studies in heterotrophic N-limited cultures of *Neochloris oleoabundans***. *In preparation to be submitted to 'Applied and Environmental Microbiology'.*

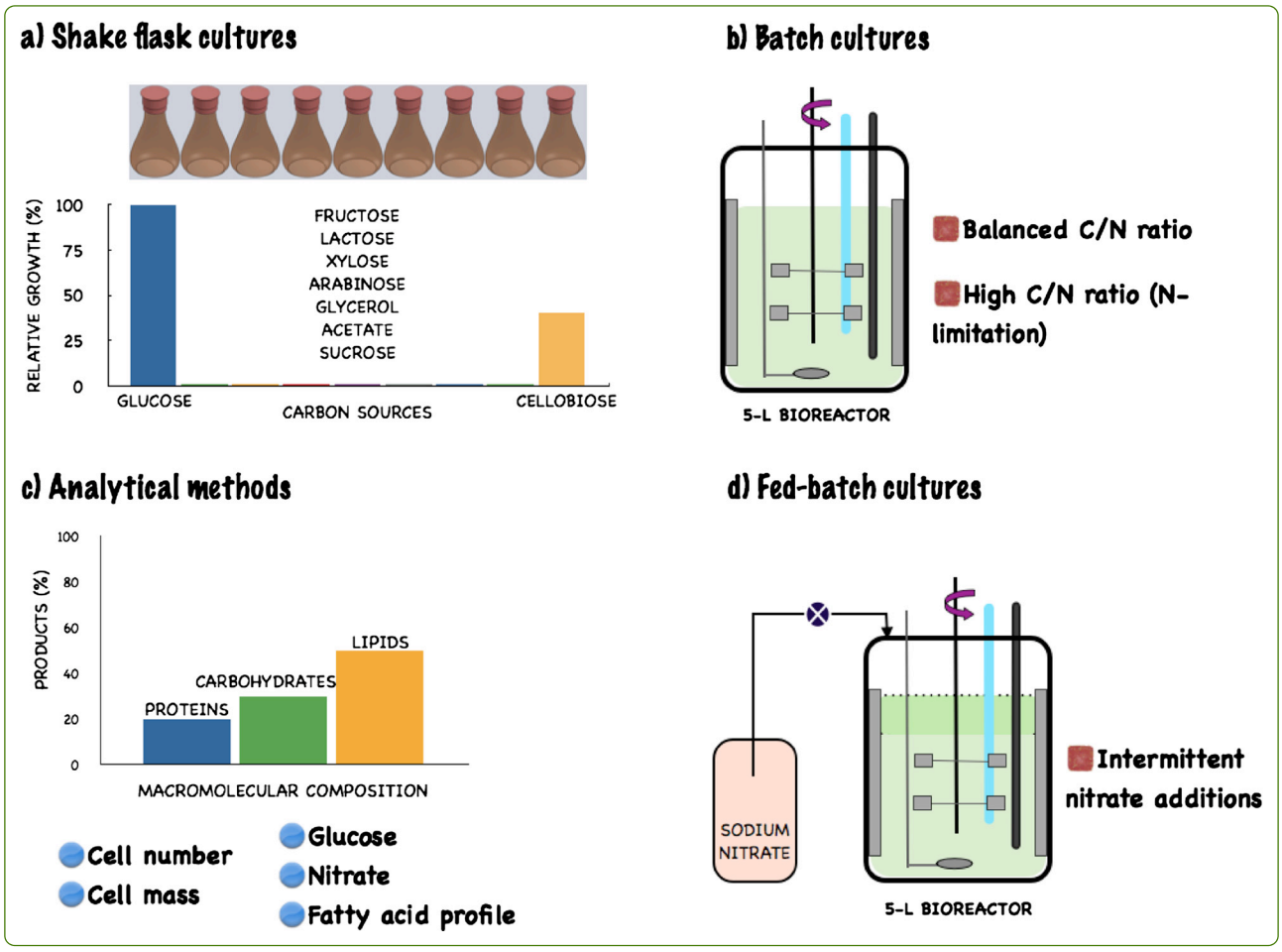
VII.1 Manuscrito:

Heterotrophic growth of *Neochloris oleoabundans* using glucose as carbon source

***Biotechnology for Biofuels* 2013, 6(100): 1-12.**

Morales-Sánchez D., Tinoco R., Kyndt J. and Martínez A.

Biotechnology for Biofuels



Heterotrophic growth of *Neochloris oleoabundans* using glucose as a carbon source

Morales-Sánchez *et al.*

BioMed Central

Morales-Sánchez *et al. Biotechnology for Biofuels* 2013, **6**:100
http://www.biotechnologyforbiofuels.com/content/6/1/100

Biotechnology for Biofuels

RESEARCH Open Access

Heterotrophic growth of *Neochloris oleoabundans* using glucose as a carbon source

Daniela Morales-Sánchez[1], Raunel Tinoco-Valencia[1], John Kyndt[2] and Alfredo Martinez[1*]

Abstract

Background: In comparison with phototrophic growth, heterotrophic conditions can significantly increase growth rates, final cell number and cell mass in microalgae cultures. *Neochloris oleoabundans* is a microalga of biotechnological interest that accumulates lipids under phototrophic and nitrogen-limited conditions. Heterotrophic flask culture experiments were conducted to identify carbon sources that can be metabolized by *N. oleoabundans*, and bioreactor batch and fed-batch (nitrate pulse additions) cultures supplemented with glucose were performed to study the cellular composition of the microalgae under balanced and high C/N ratios (glucose/nitrate).

Results: *N. oleoabundans* was able to grow using glucose and cellobiose as sole carbon sources under strict heterotrophic conditions. Under a balanced C/N ratio of 17 and using bioreactor batch cultures containing 3 g/L glucose, a maximal cell mass of 1.72 g/L was found, with protein being the major cell component (44% w/w). A maximal cell mass of 9.2 g/L was obtained using batch cultures at a C/N ratio of 278. Under these conditions, lipid accumulation was promoted (up to 52% w/w) through N-limitation, resulting in high lipid productivity (528.5 mg/L/day). Fed-batch cultures were performed at a C/N ratio of 278 and with nitrate pulse additions. This condition allowed a maximal cell mass of 14.2 g/L to be achieved and switched the metabolism to carbohydrate synthesis (up to 54% of dry weight), mainly in the form of starch. It was found that transmembrane transport under these conditions was dependent on a proton-motive force, indicating that glucose is transported by a symporter.

Conclusions: *N. oleoabundans* was able to grow under strict heterotrophic culture conditions with glucose or cellobiose as the only carbon source. The glucose used is transported by a symporter system. Batch cultures with a balanced C/N ratio accumulate proteins as the major cellular component; a high C/N ratio significantly increased the dry cell mass and resulted in a high lipid content, and a high cell density was achieved using fed-batch cultures promoting carbohydrate accumulation. These results suggest heterotrophic batch cultures of *N. oleoabundans* as an alternative for the production of proteins or lipids with simple culture strategies and minimal-mineral media supplemented with glucose.

Keywords: *Neochloris oleoabundans*, Heterotrophic growth, Glucose, Glucose transporter, Fed-batch, Nitrate, Lipids, Protein

Background

Recently, several photosynthetic microalgae have been identified as efficient biological systems for producing a wide variety of high-value chemicals and pharmaceuticals, such as phycobiliproteins, astaxanthin and polyunsaturated fatty acids (PUFAs) [1,2]. Consequently, several processes have been developed to obtain some of these compounds on a commercial scale; most of these developments are based on phototrophic growth using CO_2 as a carbon source [1,3].

Although most microalgae grow photoautotrophically, some are able to grow heterotrophically using organic substrates as sole carbon and energy sources [1]. The heterotrophic growth of microalga depends on the strain and culture conditions, and the consumption of the carbon source depends on the transport or diffusion of the carbon source across the membrane, and the enzymatic processes required for its incorporation into the central carbon metabolism [4]. Compared to photoautotrophic

* Correspondence: alfredo@ibt.unam.mx
[1]Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, A P 510-3, Cuernavaca, Morelos 62250, México
Full list of author information is available at the end of the article

growth, heterotrophic cultivation of microalgae eliminate light requirements, can significantly increase growth rates and cell mass, protein and lipid productivities [5-7]; bioreactor operation and maintenance is relatively simple and can be performed under strict axenic conditions; also cell masses obtained under heterotrophic conditions are higher because the energy density of the carbon source is higher in comparison with carbon dioxide [7] and cell densities can be increased using some culture strategies like fed-batch cultures, leading to a decrease in the costs of biomass harvesting [8,9]. However, heterotrophic cultures also have drawbacks: there is a limited number of microalgal species that can grow heterotrophically; energy expenses and costs by adding an organic substrate are higher; growth can be inhibited by an excess of organic substrate; and light-induced metabolites cannot be produced [7,8]. One of the most notable advantages of the phototrophic cultivation is that under such condition microalgae fixes carbon dioxide and produces oxygen, contributing to the reduction of carbon emissions to the atmosphere [10]; while heterotrophic cultures use an organic carbon source, consumes oxygen and generates some CO_2 during this kind of cultivations. Furthermore, phototrophic cultures permit the use of non-potable water and not arable land and do not displace food crops cultures [10]. Microalga can grow heterotrophically using the same media components used in phototrophic cultures, but with an organic carbon source instead of using a continuous flow of carbon dioxide and light. However, the cost of the organic carbon source -that is often high- and the production of CO_2 are major commercial and environmental concerns of heterotrophic cultures. The price of the glucose (obtained from starch that is produced from plants that are cultivated under phototrophic conditions, *e.g.* corn) is in the order of 0.6 US dollars per kg, while the use of carbon dioxide from flue gases can generate some bonus due to the reduction of emissions to the atmosphere [10]; although, additional cleanup steps of the flue gas are likely to be required.

A microalga suitable for heterotrophic culture should have the following physiological abilities: divide and metabolize without light, grow on easily sterilized culture media, adapt rapidly to environmental changes and withstand the hydrodynamic stresses generated in stirred tank bioreactors and peripheral equipment [1,4,8]. Several strains of algae, including *Chlorella protothecoides, Galdieria sulphuraria, Nitzschia laevis* and *Crypthecodinium cohnii* have been studied under heterotrophic growth conditions to achieve high quantities of dry cellular weight (DCW) and fatty acids, or high productivity of valuable chemicals [5,11-13].

The present study was carried out to investigate whether *Neochloris oleoabundans*, which is classified as an oleaginous microalga when cultured under phototropic conditions, is able to grow under heterotrophic conditions using various carbon sources. The following carbon sources were tested to determine whether *N. oleoabundans* can consume them under heterotrophic conditions: glucose, a sugar utilized by most microorganisms and microalgae [7]; cellobiose, a disaccharide consisting of $\beta(1 \rightarrow 4)$ linked D-glucose units, which is obtained from cellulose, an agroindustrial byproduct; xylose and arabinose, which are monosaccharides containing five carbon atoms, obtained from the hydrolysis of hemicellulose; sucrose, a disaccharide comprised of glucose and fructose, which is commonly obtained from sugar cane; fructose (fruit sugar), a simple monosaccharide found in many plants and the most water-soluble of all the sugars; lactose, a disaccharide consisting of galactose and glucose, which is found mostly in milk and dairy wastes and has previously been reported as a carbon source for *N. oleoabundans* under mixotrophic conditions [14]; glycerol, which is widely used for the cultivation of microalgae under heterotrophic conditions [7] (currently, glycerol has become very inexpensive because it is an abundant residual byproduct from the biodiesel industry [15,16]); and acetic acid, which is a common carbon source for many microbial species, including microalgae [7,17].

We characterized the performance of this microalga in batch and fed-batch heterotrophic cultures using glucose as the sole carbon and energy source (Figure 1). In addition, the type of the transmembrane transporter used by *N. oleoabundans* to transport glucose was determined.

Results and discussion

Determination of carbon sources metabolized under heterotrophic conditions by *N. oleoabundans*

Results from shake flask cultures indicate that *N. oleoabundans* does not use xylose, arabinose, sucrose, fructose, lactose, glycerol or acetate as carbon sources under strict heterotrophic growth, *i.e.*, dark conditions and mineral media; however, this organism is able to metabolize glucose and cellobiose to grow heterotrophically. Final dry cell weights of 2.3 g/L (dry cell mass productivity of 0.47 g/L/d) and 1.56 g/L (dry cell mass productivity of 0.3 g/L/d) were obtained at 5 days of cultivation of *N. oleoabundans* with 10 g/L of glucose or cellobiose, respectively (relative cell masses obtained in these cultures are reported in Figure 1a). The increase in dry cell weight or cell number and the consumption of xylose, arabinose, sucrose, fructose, lactose, glycerol or acetate was null in a period of 5 days; these experiments were repeated four times obtaining the same results. Hence it was concluded that under strict heterotrophic conditions and using

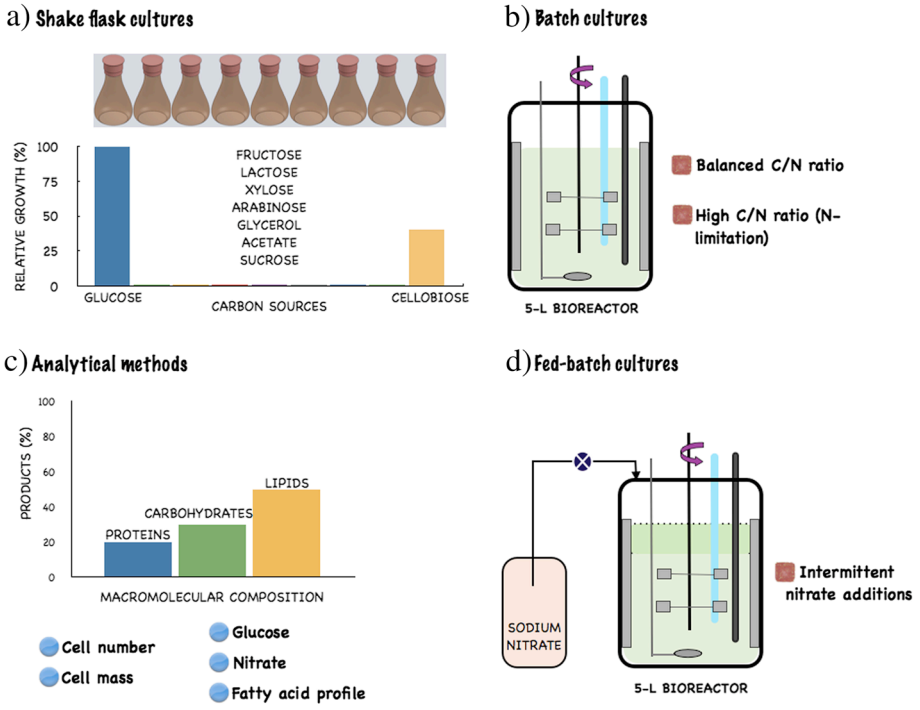


Figure 1 Schematic representation of the experimental strategy. (a) Cultures in shake flasks were used to determine the carbon sources that can be metabolized by *N. oleoabundans* under strict heterotrophic conditions and were also used, with glucose, to generate the inocula (Relative growth obtained after 5 days of cultivation). **(b)** Next, glucose was used for batch cultivations in 5-L bioreactors, two different C/N ratios were studied, a balanced ratio of 17 and a high ratio of 278 (nitrogen limited) **(c)** For the batch and fed-batch cultures the following parameters were determined: the cell number and cell mass, the glucose and nitrate consumption, the macromolecular composition of the microalgae (total protein, carbohydrates and lipids) and the fatty acid profile. **(d)** Fed-batch cultures, with intermittent nitrate additions, were performed in 5-L bioreactors.

mineral media such carbon sources cannot be metabolized by *N. oleoabundans*.

In a study done by Wu et al. [14] on the production of an extracellular biopolymer by *N. oleoabundans* using glucose or lactose (5 g/L) as a carbon source and a mineral medium, and the same components as the Bold's Basal Medium (BBM) used in our work, it was demonstrated that the microalga was able to grow on glucose; however, a three-fold lower biomass yield was obtained than that obtained in the present work. Unlike our results, Wu et al. [14] reported that *N. oleoabundans* is able to grow under mixotrophic conditions using lactose as a carbon source. To date, we do not have an explanation for this discrepancy, although we used strict heterotrophic conditions.

Glucose is the preferred carbon source for most living cells on earth. Additionally, glucose has been reported as a carbon and energy source in many heterotrophic cultures of microalgae [5,7,18,19]. Apart from glucose and cellobiose, *N. oleoabundans* apparently does not have the cellular metabolism to transport or catabolize the carbon sources tested. It has been shown with other microalgae that glucose allows higher rates of growth and respiration than do other carbon sources, such as other sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols [7,20]. Furthermore, in *Chlorella pyrenoidosa*, it was demonstrated that it is possible to generate more ATP under heterotrophic conditions using glucose as an energy source than under phototrophic conditions with light as an energy source [21].

Glucose promotes physiological changes in some microalgae species; for example, in *Chlorella vulgaris*, glucose strongly affects metabolic carbon assimilation pathways, the size of the cells, the quantity of storage materials, such as starch, lipids and protein, and the cellular contents of chlorophyll, RNA, and vitamins [7].

The pathways reported for glucose catabolism in algae are the Embden-Meyerhof and the Pentose Phosphate pathways [15]. The fact that *N. oleoabundans* can grow under dark conditions is noteworthy because some species of microalgae, such as *Dunaliella tertiolecta* and *Prymnesium parvum*, are unable to assimilate glucose in the dark despite the fact that they have the enzymatic activities needed to metabolize this sugar [15].

The observation that *N. oleoabundans* does not grow on the carbon sources tested in this work other than glucose

and cellobiose might be explained by: (1) a lack or low activity of certain enzymes, (2) the inability to simultaneously oxidize these compounds and supply the cell with reducing power for biosynthesis, (3) the lack of appropriate permeases, including those located in the plasmalemma and mitochondrial membrane [20], (4) and the putative presence of cellobiohydrolases in the enzymatic repertory of *N. oleoabundans*. Because the microalga *N. oleoabundans* is not adapted to elevated osmolarity, unlike marine microalgae that live in seawater and saline ponds [7,15], it can be speculated that *N. oleoabundans* could not grow using other carbon sources, such as glycerol.

Due to the higher cell density concentration obtained with glucose compared to cellobiose, we decided to characterize the growth, nutrient assimilation and macromolecular cell composition of *N. oleoabundans* under more controlled environmental conditions using batch cultures in 5-L bioreactors with glucose as a carbon and energy source (Figure 1).

Batch cultures with an equilibrated C/N ratio allows protein accumulation

A carbon and nitrogen balance calculation was performed to determine the number of moles of carbon and nitrogen required for the growth of *N. oleoabundans* without limiting one of these nutrients when cultured with BBM salts. Batch cultures were developed with 3 and 0.5 g/L of glucose and sodium nitrate, respectively, to equilibrate the atomic ratio of carbon and nitrogen at 17 (*i.e.*, a C/N ratio of 17); such value was estimated based in the elemental composition of *N. oleoabundans* cell reported by Pruvost et al. 2009 [22] and previous range values reported in the literature for other microalgal species [9,19,23]. To avoid any nutrient limitation other than those of glucose or sodium nitrate, all components of the BBM media were added at twice the normal concentration. As shown in Figure 2, a cell mass concentration of 1.72 g/L was attained, representing an increase by one order of magnitude compared to that present at the beginning of the culture period. The specific growth rate (μ), which was calculated based on the exponential part of the growth phase, was 0.05/h (Table 1); this value is equivalent to a duplication time (t_D) of 13 h. Exhaustion of the growth-limiting nutrients (glucose and nitrate) occurred by 5 days of culture, ending exponential growth.

Compared with a phototrophic culture of *N. oleoabundans* grown in the same culture media [22], heterotrophic cultivation decreased the duplication time by half and increased the cell density 3.5 times.

Throughout the cultivation time, the ratios between lipids, proteins and carbohydrates remained constant, with most of the weight representing protein (on average 42.5% (w/w), Table 2). Generally, the C/N ratio may influence cell composition by controlling the switch between protein, lipid and carbohydrate syntheses [9]. Due to this equilibrated C/N ratio, resulting from the carbon and nitrogen balance, the cell was not under any type of nutrient stress (until the fifth day) that could promote the accumulation of reserve metabolites; hence, the main macromolecule found in these cultures was protein, with an overall volumetric productivity of 150.3 mg/L/day (Table 1).

The lipid content was 23% at the sixth day of the culture, with octadecenoic acid (oleic acid, C18:1) as the main component (49.9%; Figure 3) of fatty acids, followed by hexadecanoic acid (palmitic acid, C16:0, 26.1%), octadecadienoic acid (linoleic acid, C18:2, 12.7%) and octadecanoic acid (stearic acid, C18:0, 8.0%). A similar fatty acid composition has been reported by Gouveia et al. [24] for *N. oleoabundans* grown under phototrophic conditions. Cerón et al. [25] stated that these types of lipids act mainly as storage in microalgae.

Batch cultures with high C/N ratios promotes lipid accumulation

To investigate the effect of high glucose concentration (50 g/L) and nitrogen limitation on growth and cell composition, batch cultures with a C/N ratio of 278 were analyzed. To avoid any nutrient limitation other than that of nitrate, all components of BBM were added at twice the normal concentration. The results presented in Figure 4 show that *N. oleoabundans* can grow at high glucose concentration (50 g/L) with a DCW yield of 0.62 g/g (Table 1). The growth rate obtained under these conditions was the same as that using 3 g/L glucose (0.05/h, see Table 1). When these cultures entered into N-limitation (after day 4), the cell density did not increase; but there was a 4-fold increase in the DCW (Figure 4).

During the first four days of cultivation, *i.e.*, during the 'no nitrogen limitation' phase where cell density exponentially increased, the ratio of lipids, proteins and carbohydrates was constant, being quite similar to the values for the batch culture with 3 g/L glucose. At day 9, *i.e.*, during nitrogen limitation, lipid accumulation was promoted, up to 50% of the DCW (Table 2). This increase in lipid content is indicative of a change in metabolism toward the synthesis of carbon and energy into reserve metabolites. Some green algae and diatoms can store carbon and energy in the form of lipids (*i.e.*, triacylglycerides, TAGs) under certain stress-inducing culture conditions [26,27], and this behavior has been extensively reported in *N. oleoabundans* when cultivated in phototrophic conditions under N-limitation [22,24,28,29]; however, to the best of our knowledge, this is the first time that such study has been performed for *N. oleoabundans* under nitrogen-limited heterotrophic conditions.

Previous reports have shown that a high C/N ratio promotes lipid accumulation for several other microalgae

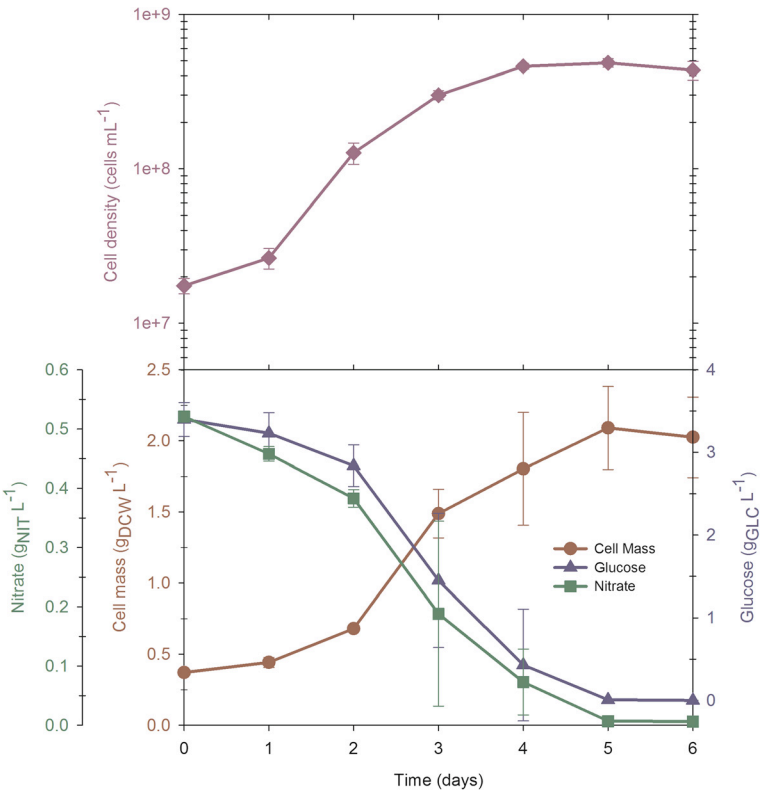


Figure 2 Batch cultivation of *N. oleoabundans* with a balanced C/N ratio (C/N = 17). The experiments were performed in triplicate and the results in the figure show the average and standard deviation.

under heterotrophic conditions [9]. Mainul et al. [23] found that when the C/N ratio in heterotrophic cultures of *Cryptococcus curvatus* was increased from 25 to 70, the oil content increased from 18% to 46%. At a high C/N ratio, the synthesis of nitrogen-containing compounds such as protein and nucleic acids is reduced, and cells enter into the stationary phase and begin to accumulate storage lipids [19]. Unlike other reserve metabolites such as carbohydrates, lipids are preferred as reserve metabolites under prolonged nutritional stress conditions. This has been ascribed to their higher reduction state, hydrophobic character and the fact that they can be efficiently packed into the cell and generate high amounts of energy upon oxidation, thus constituting the best reserve for rebuilding the cell after the nutritional stress has been alleviated [30-32]. The reported values for global lipid productivities in heterotrophic cultures range from 54 mg/L/day for *Chlorella vulgaris* to as high as 600 mg/L/day for *Schizochytrium limacinum* SR21 [33]. Our results indicate a global lipid productivity of 528.5 mg/L/day for nitrogen-limited cultures. This value is consistent with the values presented above for other microalgae and is approximately 4.9 times higher than that achieved in phototrophic cultures of *N. oleoabundans* [28].

Table 1 Kinetic and stoichiometric parameters of the cultures evaluated in this work

Culture	μ (day^{-1})	X_{MAX} (g_{DCW} L^{-1})	$Y_{X/GLC}$ ($g_{DCW}g_{GLC}^{-1}$)	$Y_{X/NIT}$ ($g_{DCW}g_{NIT}^{-1}$)	Q_X ($mg_{DCW}L^{-1}$ day^{-1})	Q_{PROT} ($mg_{PROT}L^{-1}$ day^{-1})	Q_{LIP} ($mg_{LIP}L^{-1}$ day^{-1})	Q_{CARB} ($mg_{CARB}L^{-1}$ day^{-1})
Batch C/N = 17	0.05 ± 0.001	1.72 ± 0.30	0.57 ± 0.10	3.3 ± 0.56	344.0 ± 8.0	150.3 ± 1.7	82.6 ± 4.8	106.3 ± 1.0
Batch C/N = 278	0.05 ± 0.004	9.2 ± 0.15	0.62 ± 0.08	4.8 ± 0.43	1,022.2 ± 44.4	147.2 ± 5.1	528.5 ± 7.2	340.9 ± 4.6
Fed-batch	0.02 ± 0.002	14.2 ± 0.46	0.3 ± 0.14	3.1 ± 0.67	1,420.0 ± 50.0	164.7 ± 4.26	478.5 ± 7.1	770.1 ± 6.7

μ: Specific growth rate.
X_{MAX}: Maximum cell mass produced.
$Y_{X/GLC}$: Yield of biomass on glucose when the culture was in nutrient sufficient and during the feed in the case of fed-batch culture.
$Y_{X/NIT}$: Yield of biomass on nitrate when the culture was in nutrient sufficient and during the feed in the case of fed-batch culture.
Q_X: Biomass productivity at maximum total cell mass produced.
Q_{PROT}, Q_{LIP}, Q_{CARB}: Protein, lipid or carbohydrate productivity at maximum cell mass produced.

Table 2 Macromolecular composition of *N. oleoabundans* cells from batch and fed-batch cultures

Culture	Proteins ($g_{PROT}\ g_{DCW}^{-1}*100$)		Carbohydrates ($g_{CARB}\ g_{DCW}^{-1}*100$)		Lipids ($g_{LIP}\ g_{DCW}^{-1}*100$)	
	S	M	S	M	S	M
Batch C/N = 17	41.2 ± 0.4	43.7 ± 1.9	31.7 ± 1.3	30.9 ± 3.5	24.8 ± 0.3	24.0 ± 0.9
Batch C/N = 278	42.9 ± 1.7	14.4 ± 1.2	32.1 ± 0.4	33.3 ± 0.5	23.3 ± 1.2	51.7 ± 1.7
Fed-batch	40.7 ± 1.4	11.6 ± 0.9	27.5 ± 2.0	54.2 ± 0.1	27.5 ± 1.9	33.7 ± 0.6

S: at the start of the culture.
M: at the time of maximum cell mass produced.

As seen in Figure 3, the fatty acid profile of *N. oleoabundans* at a C/N ratio of 278 is very similar to that obtained at a C/N ratio of 17, but the relative content of stearic acid (C18:0) is higher and the relative content of oleic acid (C18:1) is lower for cells cultured at a C/N of 278, but C18:1 still is the principal fatty acid component. Furthermore, C20 fatty acids (arachidic acid, C20:0 and gadoleic acid, C20:1) constituted less than 1% (of the fatty acid content) under nitrogen limiting conditions.

Fed-batch cultures with nitrate pulse additions switches metabolism to carbohydrate accumulation

Fed-batch cultures with 'nitrate pulse additions' were performed to obtain a high cell density culture and determine the macromolecular composition of *N. oleoabundans* under such conditions. As described in the Methods section, this culture started with a working volume of 3.5 L (50 g/L glucose) in the bioreactor and consisted of three stages (Figure 5). First, a batch culture stage of three days was developed. When the sodium nitrate concentration decreased to a level of 0.05 g/L, the feed stage was initiated; the nitrate feed was manually controlled to maintain a residual nitrate concentration between 0.10 and 0.6 g/L. This profile was developed to maintain a specific rate of nitrate consumption during the fed-batch culture, close to its maximum value. To prevent nutrient limitation other than nitrate, all BBM medium salts (at half of the usual concentration) were also added each time nitrate was supplemented. When a DCW concentration of 14.2 g/L was reached, the final stage was initiated by ending the supply of nitrate (and BBM salts) with the purpose of developing a stationary, nitrogen-limited phase. The last nitrate addition was performed on the sixth day of cultivation, when 21.6 g/L of glucose remained in the medium; nitrate was depleted on the seventh day, and the residual glucose concentration was 11.7 g/L at this time. The final stage was completed when the residual glucose was depleted.

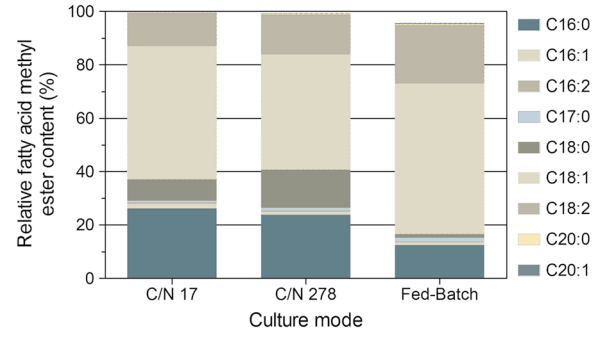


Figure 3 Fatty acid methyl ester profile in batch cultures with a C/N = 17 and C/N = 278 and fed-batch culture with an initial C/N = 278. The determinations were performed in triplicate and the results in the figure show the average.

A maximum biomass concentration of 14.2 g/L dry weight was reached at 10 days of cultivation, when glucose was depleted (Figure 5). During the batch and fed-batch stages, cell density increased exponentially, and the estimated specific growth rates were 0.05 and 0.02/h, respectively (Table 1). When nitrate was depleted, cell density no longer increased, but the DCW continued to increase (Figure 5); this finding might suggest the formation and accumulation of a reserve metabolite from metabolism of the consumed glucose. As expected, this culture reached a higher cell mass, approximately eight and two times higher than batch cultures with C/N ratios of 17 (3 g/L glucose) and 278 (50 g/L glucose), respectively.

Table 2 shows the cellular composition of *N. oleoabundans* in the fed-batch culture. While the cells were under nutrient-sufficient conditions, the major cellular fraction was protein. During the third stage (at 7 days), while the cells were under nitrogen limitation but still had sufficient glucose, the cells accumulated carbohydrates with an overall volumetric productivity of 770.1 mg/L/day (Table 1). The carbohydrates were hydrolyzed with α-amylase and gluco-amylase, and the following monosaccharides were found: glucose (with a relative content of 90%, w/w), xylose (6% w/w) and arabinose (~1% w/w). Because the carbohydrates were hydrolyzed with enzymes that digest starch, it is suggested that the main content of these carbohydrates is starch. These results indicate that stages of low and relatively high levels of nitrate over short periods

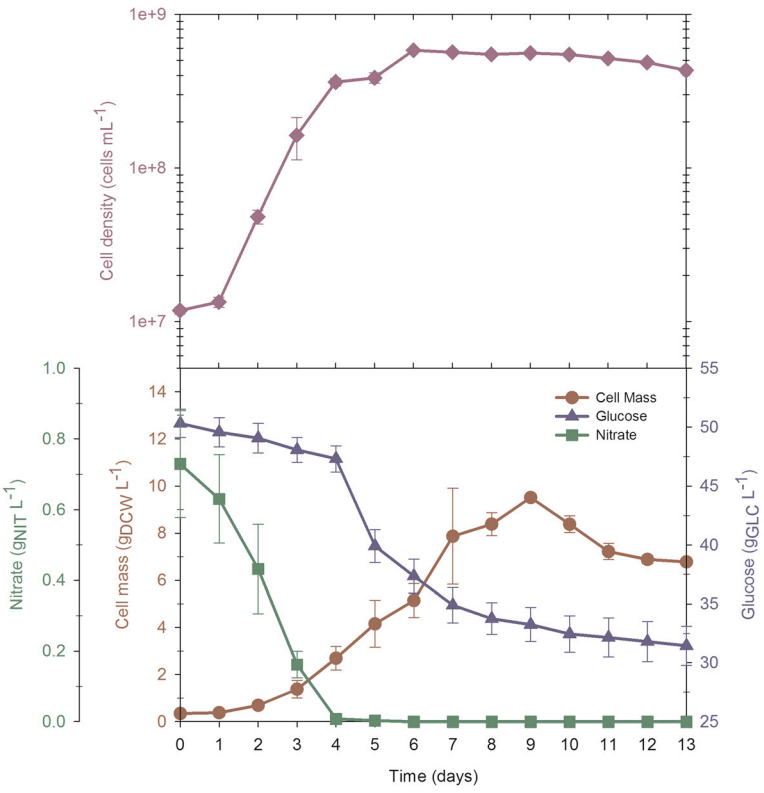


Figure 4 Batch cultivation of *N. oleoabundans* with a high C/N ratio (C/N = 278). The experiments were performed in duplicate and the results in the figure show the average and standard error.

promoted carbohydrate rather than lipid accumulation; carbohydrates are most likely preferred as reserve metabolites under short-term nutritional stress conditions, similar to the situation in plants. In *Arabidopsis*, as in most vascular plants, starch plays an important role in the day-to-day carbohydrate metabolism of the leaf. Glucose produced during photosynthesis is stored mainly in the form of starch granules in the chloroplast, this storage material is used to change the availability of photosynthates obtained during the diurnal cycle of light and dark. Starch that has accumulated during the day through light reactions is degraded during the subsequent night through Calvin cycle or dark reactions, providing a constant supply of carbohydrate in the absence of photosynthesis. Therefore, the plant starch can be seen as a short-term carbohydrate reservoir and its frequency termed transitory starch [34].

As seen in Figure 3, the fatty acid profile of *N. oleoabundans* in fed-batch cultures was different than that in the batch cultures. The saturated fatty acid content was reduced (palmitic acid, C16:0 and stearic acid, C18:0), but longer-chain and unsaturated fatty acids (oleic acid, C18:1 and linoleic acid, C18:2) were synthesized in higher proportions; oleic acid (C18:1) was again the principal fatty acid component. In addition, C:20 fatty acids (arachidic acid, C20:0 and gadoleic acid, C20:1) again represented less than 1% of total fatty acids in the fed-batch culture.

Transmembrane glucose transport and chlorophyll content

Transmembrane glucose transport was investigated in cells growing in batch cultures containing 3 g/L glucose and [^{14}C]-glucose tracer; the ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was incorporated as a proton gradient decoupler. It is known that CCCP causes an uncoupling of the proton gradient, which is established during the normal activity of electron transport in the cell wall; if a carbon source is transported through a symporter system then CCCP will uncouple its entrance into the cell. As shown in Figure 6, when added to *N. oleoabundans* cells CCCP inhibits the glucose transport across the membrane, indicating that the cells can transport glucose only in the presence of a proton gradient; hence suggesting that this microalga uses a glucose symporter to transport this sugar into the cell. Calculations based on the rates of consumption and uptake suggest that glucose consumption depends on the transport capacity of the cell because the glucose

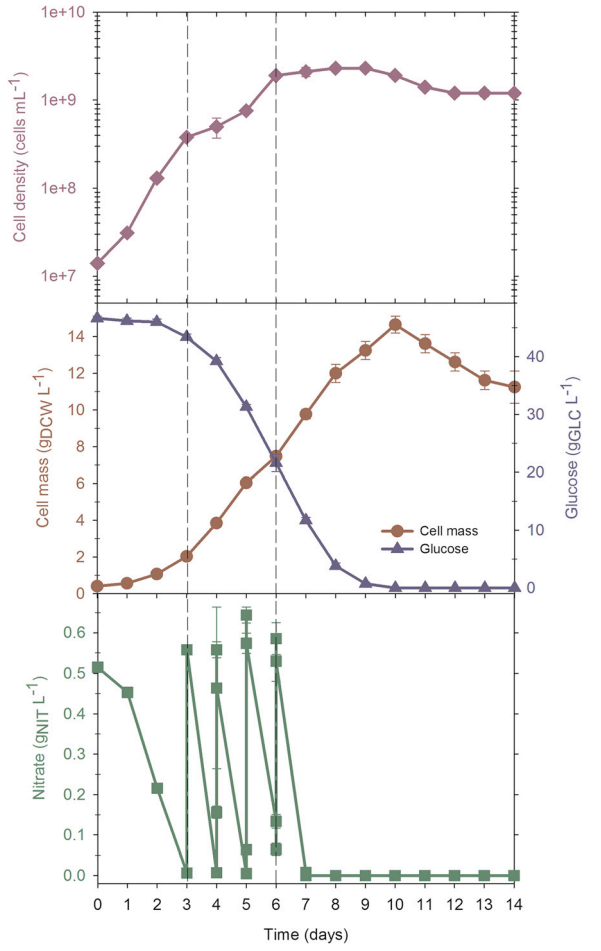


Figure 5 Fed-batch cultivation of *N. oleoabundans* with an initial C/N = 278 and nitrate pulse additions. Vertical dashed lines indicate the start and end of the feeding stage. The experiments were performed in duplicate and the results in the figure show the average and standard error.

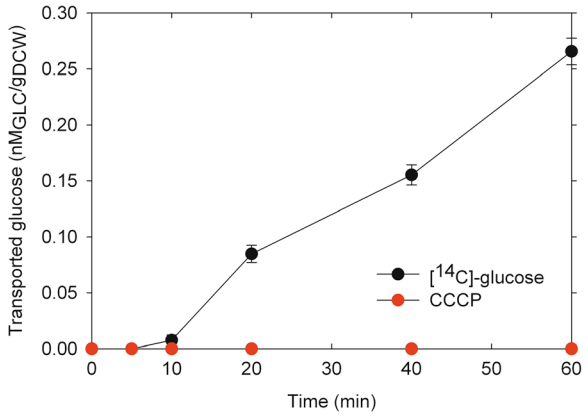


Figure 6 Transport kinetic of [^{14}C]-glucose with the ionophore carbonyl cyanide m-chlorophenyl hydrazone (red circle) and with [^{14}C]-glucose alone (black circle). The determinations were performed in triplicate and the results in the figure show the average and standard deviation.

consumption and transport rates are very similar (8 μmol/g/min and 5 μmol/g/min, respectively).

As in *Chlorella* cells, transmembrane transport in *N. oleoabundans* is proton-motive force-dependent, indicating that the transporter system is a glucose symporter. It has been demonstrated that *Chlorella* cells possess an inducible active hexose/H^+ symport system responsible for the uptake of glucose from the medium [13,35-37]. This system transports sugars and protons with 1:1 stoichiometry, and the cell consumes one molecule of ATP per molecule of sugar transported [13,37]. With glucose as the inducer, the minimum time necessary to induce the synthesis of the hexose/H^+ symport system proteins in *Chorella* is 15–18 min [13,37,38].

The total chlorophyll content in cells was determined at the fifth day of batch cultures using the spectrometric method reported by Pruvost et al. [22]. Although cultures were performed under strict dark conditions, the total chlorophyll content ranged from 1.0–1.5% (μg chlorophyll/μg DCW). Such values are 2.3–3.5-fold lower than values found under phototropic conditions [22]. However, the quantities of chlorophyll found under heterotrophic conditions indicate a constitutive expression of the photosynthetic system.

Conclusions

N. oleoabundans was able to grow under strict heterotrophic cultures conditions with glucose or cellobiose as the only carbon source; however, no growth was obtained using xylose, arabinose, fructose, sucrose, lactose, glycerol or acetic acid. Transport studies indicate that glucose is transported by a symporter system, although further study is needed to determine the identity of this transporter. Using different cultivation strategies with variable C/N ratios under controlled conditions (pH, temperature and aeration rate) in a 5-L bioreactor allows metabolism switching. Batch cultures with balanced C/N ratios (17) accumulate proteins (up to 44% w/w) as the major cell component; a high C/N ratio (nitrogen limitation during the stationary phase) significantly increases the dry cell mass and yields a high lipid content (up to 52% w/w). High cell density and carbohydrate accumulation (up to 54% (w/w), mainly starch) were achieved using fed-batch cultures (50 g/L initial glucose and the addition of nitrate pulses). These results suggest heterotrophic batch cultures of *N. oleoabundans* as an alternative for the production of proteins or lipids with simple culture strategies and mineral media supplemented with glucose, which could potentially be obtained by hydrolyzing the glucan fraction of lignocellulose.

Methods

Algal strain, media and inocula development

The microalgae *Neochloris oleoabundans* UTEX 1185 was obtained from the Algae Culture Collection of the University of Texas (Austin, TX, USA). Stock cultures were maintained by sub-cultivation in 250-mL shake flasks containing 100 mL of mineral medium (Bold's Basal Medium; BBM) supplemented with 10 g/L glucose in the dark. Cultures were incubated in an orbital shaker (300 rpm, 25°C; Barnstead MaxQ 4000, USA). BBM [28] comprises the following salts (in mM): $NaNO_3$ 2.94, $MgSO_4 \cdot 7H_2O$ 0.3, K_2HPO_4 1.29, KH_2PO_4 0.43, $CaCl_2$ 0.17, NaCl 0.43, Na-EDTA 0.171, KOH 0.553, and H_3BO_3 0.185, and the following trace elements (in μM): $FeSO_4 \cdot 7H_2O$ 18, $ZnSO_4 \cdot 7H_2O$ 30.7, $MnCl_2 \cdot 4H_2O$ 7.3, $CuSO_4 \cdot 5H_2O$ 6.3, MoO_3 4.9 and $CoNO_3 \cdot 6H_2O$ 1.7 dissolved in H_2SO_4 10.2 μM. All medium components were heat-sterilized (121°C, 20 min). To prepare the inocula, *N. oleoabundans* was cultivated in 250-mL Erlenmeyer flasks with 100 mL of BBM at an initial pH of 7 and at 25°C and 300 rpm. All cultures in flasks and bioreactor were initiated with a 10% (v/v) inoculum, roughly equivalent to an initial cell number of 1×10^7–2×10^7 cells/mL or 0.4 g_{DCW}/L.

Analysis of carbon sources that support heterotrophic growth of *N. oleoabundans*

Cultures in 250-mL Erlenmeyer flasks were used to test common carbon sources that are used for the heterotrophic growth of microalgae or bacteria. The carbon sources studied were glucose, xylose, arabinose, fructose, sucrose, lactose, cellobiose, glycerol and sodium acetate. Culture conditions were similar to those used for inocula development and included 10 g/L of the carbon source (except for sodium acetate, which was used at 5 g/L), and the cultures were incubated for 5 days.

Cultivation in bioreactors

A 5-L bioreactor (LSL Biolafitte, USA) was used for batch and fed-batch cultivation of heterotrophic *N. oleoabundans*. Initial working volumes for the batch and fed-batch cultures were 4 and 3.5 L, respectively. The culture conditions for both operation modes were as follows: 25°C, pH7.0 (adjusted by the automatic addition of 1 N H_3PO_4 and 1 N KOH) and 10% (v/v) of the initial inoculum. To determine if the *N. oleoabundans* cells are sensitive to the stirring and/or aeration rates, a preliminary set of experiments were performed using 1 L of air/L of medium/min and three different stirring speeds: 150, 300 and 600 rpm. The growth of the cells, measured as cell density (cells/mL) and dry cell mass, was the same for the three set of experiments, indicating that no mechanical stress was developed at such conditions. Hence batch cultures were developed at 300 rpm (using three Rushton Turbines with 6 blades), and an aeration rate of 1 L of air/L of medium/min; under these conditions, the dissolved oxygen was always greater than 80% of the air saturation value. An initial sodium nitrate concentration of 0.5 g/L was used for the batch cultures, and two sets of experiments were performed with initial glucose concentrations of 3 and 50 g/L, *i.e.*, equivalent to C/N ratios of 17 and 278, respectively.

Kinetic and stoichiometric parameters (specific growth rate; maximum cell mass produced; yield of biomass on glucose; yield of biomass on nitrate; and volumetric productivities for biomass, proteins, lipids or carbohydrates) of the 17 C/N ratio batch culture were determined at mid-log phase. In the 278 C/N ratio batch cultures, these parameters were calculated during the first four days of culture, *i.e.,* when the cultures were not limited by the carbon and/or nitrogen sources.

The initial medium (3.5 L) for the fed-batch cultures also contained 0.5 g/L sodium nitrate and 50 g/L glucose. The consumption of nitrate was previously determined from batch cultures; the times and quantity of sodium nitrate added were based on these values. The specific rate of consumption of sodium nitrate was determined, and the fed-batch cultures were fed based on this rate with intermittent sodium nitrate additions to maintain the concentration between 10 and 600 mg/L. In these cultures, the initial values of agitation and aeration were 300 rpm (using three Rushton Turbines with 6 blades) and 1 L of air/L of medium/min, but the dissolved oxygen was maintained above 20% air saturation by stepwise increments in the agitation speed and airflow rate. For the fedbatch cultures, a mass balance, considering the volume of liquids added (nitrate solution, acid and base used for pH control, and nutrient additions) and removed (sampling and evaporation) was performed to correct the actual values for the cell density and cell mass. Kinetic and stoichiometric parameters of the fed-batch cultures were determined during the feeding phase (pseudo-stationary feeding state) of the culture.

To detect any interference in glucose consumption samples from all cultures (flasks and bioreactors) were tested every day for the presence of contaminants, spreading several dilutions of the broths into solid-rich-media (1.5% agar, 1% tryptone, 0.5% yeast extract and 1% sodium chloride); cultures were discarded if contaminants were detected in solid media after 1–3 days of incubation at 37°C.

Analytical methods

The cell density (cells/mL) was determined by direct counting using a Neubauer chamber and a light microscope equipped with a 40× objective. The algal dry cell weight (DCW) was evaluated gravimetrically by filtration

through a pre-dried and pre-weighed 0.45-μm pore size nitrocellulose membrane filter (Millipore).

Lipids were extracted using solvents and gravimetrically quantified as reported by Band et al. [39]. Briefly, samples with culture media were centrifuged (14,000 × g, 15 min, 4°C), and the cell pellet was resuspended in methanol/dichloromethane (2:1; v/v) containing 0.5 mg of butylated hydroxytoluene and stored at 4°C for at least 12 h. The cell debris was separated by centrifugation (14,000 × g, 15 min, 4°C). After centrifugation, the lipid-containing supernatant was transferred to another tube, and the residue was extracted twice with 4 mL of methanol/dichloromethane (1:1; v/v). Methanol from the solution containing the extracted lipids was separated by adding deionized water. To remove residual water, the resulting organic phase (containing lipids, dichloromethane and residual amounts of water) was filtered through anhydrous sodium sulfate. Finally, solvents were removed using an evaporator (Waterbath B-480 Büchi, Switzerland) at 40°C and atmospheric pressure, and the lipids were gravimetrically quantified.

Carbohydrate content was determined using the phenol-sulfuric acid method after acid hydrolysis of the sample [40], and the protein content was determined using the Lowry method after alkaline hydrolysis of the sample [41].

Glucose was determined in the culture supernatants using a biochemical analyzer (YSI 2700 Select, Yellow Spring Instruments, Ohio, USA). Nitrate concentration was quantified using an ion-selective probe calibrated with sodium nitrate standards (10, 100, and 1,000 mg/L; Ion Concentration Controller Microprocessor Based IC 7685, B&C Electronics Srl, Lombardy, Italy; ISE: Nitrate NO31502, Van London-Phoenix Co, Texas, USA).

After lipid extraction, the total carbohydrates found in *N. oleoabundans* cells from fed-batch cultures were hydrolyzed using α-amylase (Liquozyme SC DS, Novozymes, Franklinton, NC, USA) and gluco-amylase (Spirizyme fuel, Novozymes, Franklinton, NC, USA); the carbohydrate composition (glucose, xylose and arabinose) was determined by HPLC analysis (Waters U6 K, Millipore Co., Milford, MA, USA) using an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA), and a 5 mM H_2SO_4 solution was used as the mobile phase (0.5 mL/min) at 60°C. A refractive index detector was used to identify the sugars in the hydrolysate (Model 2410, Waters, Millipore Co).

To determine the fatty acid composition, oil samples were chemically derivatized using the boron trifluoride method [10]. Briefly, 10 mg of a sample of total lipids were processed with 2 mL of NaOH (0.3 N-methanol 90% v/v) at 75°C during 2 hours. The non saponifiable lipids (upper layer) were extracted 5 times with 2 mL of hexane and centrifugation (5,000 × g, 3 min, 4°C). The lower phase was acidified with 0.3 mL of HCl 6 N, and the free fatty acids were extracted with 2 mL of hexane by centrifugation (5,000 × g, 3 min, 4°C); to remove residual water, this phase (containing free fatty acids, hexane and residual amounts of water) was filtered through anhydrous sodium sulfate. The solvent was removed using an evaporator (Waterbath B-480 Büchi, Zwitzerland) at 60°C and 500 mmHg vacuum; and the free fatty acids were gravimetrically quantified. Derivatization was performed using a boron trifluoride solution, 0.5 mL (14% in methanol) were added to the fatty acid sample and the mixture was heated in sealed vials and a water bath at 60°C for 20 min, and then rapidly cooled to room temperature. Hexane (3 mL) and a saturated solution of sodium chloride (1 mL) were added, and after centrifugation (5,000 × g, 3 min, 4°C), the upper phase, containing the methyl esters was collected and filtered through anhydrous sodium sulfate to remove residual water. Finally, samples containing the methyl esters and hexane were placed into chromatographic vials. The obtained organic phase was analyzed in a GC-MS system (GC: 6890 N, Agilent Technologies, Wilmington, DE, USA; Mass selective detector: 5973 N, Agilent Technologies, Wilmington, DE, USA). Samples (1 μL) were injected in split mode (30:1) into a fused silica capillary column packed with phenyl methyl siloxane (30 m × 0.250 mm, 0.25 μm, HP-5MS Agilent Technologies, Wilmington, DE, USA), using helium (99.999% purity) as the carrier gas at an on-column flow of 1 mL/min. The temperature of the oven was set at 50°C for 7 minutes, then raised at a rate of 5°C/min to 250°C and held for 15 minutes. The mass detector was operated as follows: the filament was turned on at minute 6, the transfer line from the GC to the MS was maintained at 280°C, the Quadrupole was maintained at 150°C and the ionization source was maintained at 230°C. Electron ionization was operated at 70 eV. The analysis was performed in the full scan mode over the m/z range of 20 to 700.

Initial glucose transport rates were determined according to Utrilla et al. [42]. Cells were harvested from cultures in the mid log phase (14,000 × g for 10 min at 4°C), cooled on ice, washed in 1× BBM salts, and dissolved in the same medium without a carbon source at a cell density of 1×10^8 cells/mL. Because the cells were kept on ice (5 minutes) before the transport experiments, metabolic activity was reduced; nonetheless, it is assumed that the amount of sugar transport protein present is the same as that present during growth under culture conditions. For the [^{14}C]-glucose uptake assays, 540 μL of cell suspension were incubated for 15 min at 25°C, and the kinetics experiment was then started by adding 60 μL of [^{14}C]-glucose (0.5 mM, 5 mCi/mmol). The suspension was maintained at 25°C with shaking,

and 50 μL samples were taken at 0, 5, 10, 20, 40 and 60 min intervals. These samples were then filtered immediately through membrane filters (pore size 0.45 μm) and washed three times with BBM salts. The filters were dried and placed in vials with 5 mL of Ecolite scintillation cocktail (ICN Biomedicals, Costa Mesa, CA, USA). Radioactivity was measured using a scintillation counter (Beckman LS6000IC, Fullerton, CA, USA). The [^{14}C]-glucose uptake rates were calculated from the initial linear data based on a plot of intracellular [^{14}C]-glucose versus time.

Abbreviations

BBM: Bold's basal medium; CCCP: Carbonyl cyanide m-chlorophenyl hydrazine; C/N: Carbon to nitrogen ratio; CO_2: Carbon dioxide; DCW: Dry cell weight; Q_X: Biomass productivity at maximum cell mass produced; Q_{PROT}, Q_{LIP}, Q_{CARB}: Protein, Lipid or carbohydrate productivity at maximum cell mass produced; TAGs: Triacylglycerides; t_D: Duplication time; v/v: Volume per volume; w/w: Weight per weight; X_{MAX}: Maximum cell mass produced; $Y_{X/GLC}$: Yield of biomass on glucose; $Y_{X/NIT}$: Yield of biomass on nitrate; μ: Specific growth rate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DM-S carried out the experiments, acquired the data and drafted the manuscript. RT-V performed the fatty acid profile experiments and participated in the results analysis. JK participated in the results interpretation and analysis and critically revised the manuscript. AM conceived the study, participated in the design of the study, performed results interpretation and analysis, and critically revised the manuscript. All authors read and approved the final manuscript.

Authors' information

DM-S is currently a PhD student at the Biotechnology Institute-UNAM. She is working with heterotrophic cultures of *Neochloris oleoabundans* to study biomass and lipid production with sugars from lignocellulose hydrolysates. RT-V is an Academic Technician at the Pilot Plant of the Biotechnology Institute-UNAM. He is working with microalgae cultures and laccase production by *Pleurotus ostreatus*. JK is currently Director of Professional Sciences and Assistant Professor at Bellevue University (Nebraska, US). His research background is in the area of developing algae for enhanced biofuel production, as well as photoactive proteins and optogenetics. AM is currently Professor at the Biotechnology Institute-UNAM, his main research interest is in the metabolic engineering and bioprocess development with *Escherichia coli* for biofuels and biopolymer precursors production, as well as physiological studies with oleaginous microalgae for biofuel and feedstock applications.

Acknowledgements

We thank Jose Utrilla for his technical support with glucose transport measurements and Mario Caro and Leobardo Serrano for technical support in the Fermentation Pilot Plant of the Biotechnology Institute-UNAM. This work was supported by the Universidad Nacional Autónoma de México (UNAM), grant DGAPA/PAPIIT/UNAM IT200312. DMS held a scholarship from The Mexican Council of Science and Technology CONACyT.

Author details

[1]Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, A P 510-3, Cuernavaca, Morelos 62250, México. [2]College of Science and Technology, Bellevue University, 1000 Galvin Road South, Bellevue, NE 68005, USA.

Received: 19 December 2012 Accepted: 8 July 2013
Published: 13 July 2013

References

1. Chen GQ, Chen F: **Growing phototrophic cells without light.** *Biotechnol Lett* 2006, **28:**607–616.
2. Pereira H, Barreira L, Mozes A, Florindo C, Polo C, Duarte CV, Custódio L, Varela J: **Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae.** *Biotechnol Biofuels* 2011, **4:**61.
3. Olaizola M: **Commercial development of microalgal biotechnology from the test tube to the marketplace.** *Biomol Eng* 2003, **20:**459–466.
4. Mojtaba A, Mohd S, Rosfarizan M, Raha A, Arbakariya B: **Improvement of medium composition for heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, using response surface methodology.** *Biochem Eng J* 2011, **53:**187–195.
5. Cheng Y, Zhou W, Gao C, Lan K, Gao Y, Wu Q: **Biodiesel production from Jerusalem artichoke (*Helianthus tuberosus L.*) tuber by heterotrophic microalgae *Chlorella protothecoides*.** *Soc Chem Ind* 2009, **84:**777–781.
6. O'Grady J, Morgan J: **Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol.** *Bioprocess Biosyst Eng* 2010, **34:**121–125. Short communication 1.
7. Perez-Garcia O, Escalante FME, de-Bashan LE, Bashan Y: **Heterotrophic cultures of microalgae: metabolism and potential products.** *Water Res* 2011, **45**(1):11–36.
8. Chen F: **High cell density culture of microalgae in heterotrophic growth.** *Trends Biotechnol* 1996, **14:**412–426.
9. Eriksen N: **The technology of microalgal culturing.** *Biotechnol Lett* 2008, **30**(9):1525–1536.
10. Gouveia L, Oliveira C: **Microalgae as a raw material for biofuels production.** *J Ind Microbiol Biotechnol* 2009, **36:**269–274.
11. Graverholt O, Eriksen N: **Heterotrophic high cell-density fed-batch and continuous flow cultures of *Galdieria sulphuraria* and production of phycocyanin.** *Appl Microbiol Biotechnol* 2007, **77:**69–75.
12. Wen ZY, Chen F: **A perfusion-cell bleeding culture strategy for enhancing the productivity of eicosapentaenoic acid by *Nitzschia laevis*.** *Appl Microbiol Biotechnol* 2001, **57:**316–322.
13. de Swaaf M, Sijtsma L, Pronk J: **High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*.** *Biotechnol Bioeng* 2003, **81:**666–672.
14. Wu N, Li Y, Lan C: **Production and rheological studies of microalgal extracellular biopolymer from lactose using the green alga *Neochloris oleoabundans*.** *J Polym Environ* 2011, **19:**935–942.
15. Neilson AH, Lewin RA: **The uptake and utilization of organic carbon by algae: an essay in comparative biochemistry.** *Phycologia* 1974, **13:**227–264.
16. Cintolesi A, Clomburg JM, Rigou V, Zygourakis K, Gonzalez R: **Quantitative analysis of the fermentative metabolism of glycerol in *Escherichia coli*.** *Biotechnol Bioeng* 2012, **109**(1):187–198.
17. Droop M: **Heterotrophy of carbon.** In *Algal physiology and biochemistry.* Edited by Stewart WDP. Oxford: Blackwell Scientific; 1974:530–559.
18. Vazhappilly R, Chen F: **Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth.** *J Am Oil Chem Soc* 1998, **75:**393–397.
19. Jiang Y, Chen F: **Effects of temperature and temperature shift on docosahexaenoic acid production by the marine microalgae *Crypthecodinium cohnii*.** *J Am Oil Chem Soc* 2000, **77:**613–617.
20. Griffiths DJ, Thresler CL, Street HE: **The heterotrophic nutrition of *Chlorella vulgaris*.** *Ann Bot* 1960, **2:**1–11.
21. Yang C, Hua Q, Shimizu K: **Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions.** *Biochem Eng J* 2000, **6:**87–102.
22. Pruvost J, Van Vooren G, Cogne G, Legrand J: **Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor.** *Bioresour Technol* 2009, **100**(23):5988–5995.
23. Mainul H, Phillipe J, Louis M, Alain P: **Influence of nitrogen and iron limitations on lipid production by *Cryptococcus curvatus* grown in batch and fed-batch culture.** *Process Biochem* 1996, **31**(4):355–361.
24. Gouveia L, Marques A, de Silva T, Reis A: ***Neochloris oleoabundans* UTEX #1185: a suitable renewable lipid source for biofuel production.** *J Ind Microbiol Biotechnol* 2009, **36:**821–826.
25. Cerón García M, Fernández Sevilla J, Acién Fernández F, Molina Grima E, García Camacho F: **Mixotrophic growth of *Phaeodactylum tricornutum* on glycerol: growth rate and fatty acid profile.** *J Appl Phycol* 2000, **12:**239–248.

26. Valenzuela J, Mazurie A, Carlson RP, Gerlach R, Cooksey KE, Peyton BM, Fields MW: **Potential role of multiple carbon fixation pathways during lipid accumulation in *Phaeodactylum tricornutum*.** *Biotechnol Biofuels* 2012, **5:**40.
27. Xia C, Zhang J, Zhang W, Hu B: **A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by *Mucor circinelloides*.** *Biotechnol Biofuels* 2011, **4:**15.
28. Tornabene T, Holzer G, Lien S, Burris N: **Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*.** *Enzyme Microb Technol* 1983, **5:**435–440.
29. Li YQ, Horsman M, Wang B, Wu N, Lan C: **Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*.** *Appl Microbiol Biotechnol* 2008, **81**(4):629–636.
30. Thompson GA: **Lipids and membrane function in green algae.** *Biochim Biophys Acta* 1996, **1302:**17–45.
31. Rodolfi L, Chini G, Bassi N, Padovani G, Biondi N, Bonini G, Tredici M: **Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor.** *Biotechnol Bioeng* 2008, **102**(1):100–112.
32. Courchesne NM, Parisien A, Wang B, Lan CQ: **Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches.** *J Biotechnol* 2009, **141:**31–41.
33. Yokochi T, Honda D, Higashihara T, Nakahara T: **Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21.** *Appl Microbiol Biotechnol* 1998, **49:**72–76.
34. Streb S, Zeeman SC: **Starch metabolism in Arabidopsis.** *The Arabidopsis book* 2012, **10:**e0160. doi:10.1199/tab.0160.
35. Tanner W: **Light-driven active uptake of 3-O-methylglucose via an inducible hexose uptake system of *Chlorella*.** *Biochem Biophys Res Commun* 1969, **36:**278–283.
36. Komor E: **Proton-coupled hexose transport in *Chlorella vulgaris*.** *FEBS Lett* 1973, **38:**16–18.
37. Komor E, Tanner W: **The hexose-proton symport system of *Chlorella vulgaris*: specificity, stoichiometry and energetics of sugar-induced proton uptake.** *Eur J Biochem* 1974, **44:**219–223.
38. Komor E, Haass D, Komor B, Tanner W: **The active hexose-uptake system of *Chlorella vulgaris*. Km-values for 6-deoxyglucose influx and efflux and their contribution to sugar accumulation.** *Eur J Biochem* 1973, **39:**193–200.
39. Band CJ, Arredondo-Vega BO, Vazquez-Duhalt R, Greppin H: **Effect of salt-osmotic upshock on the edaphic microalga *Neochloris oleoabundans*.** *Plant Cell Environ* 1992, **15:**129–133.
40. Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F: **A colorimetric method for the determination of sugars.** *Nature* 1951, **168:**167–168.
41. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: **Protein measurement with the folin phenol reagent.** *J Biol Chem* 1951, **193**(1):265–275.
42. Utrilla J, Gosset G, Martinez A: **ATP limitation in a pyruvate formate lyase mutant of *Escherichia coli* mg1655 increases glycolytic flux to D-lactate.** *J Ind Microbiol Biotechnol* 2009, **36:**1057–1062.

doi:10.1186/1754-6834-6-100
Cite this article as: Morales-Sánchez *et al.*: Heterotrophic growth of *Neochloris oleoabundans* using glucose as a carbon source. *Biotechnology for Biofuels* 2013 **6**:100.

VII.2 Manuscrito Sometido:

Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid and carbohydrate production (accumulation).

***Algal Research* 2014**

(Sometido: Diciembre de 2013)

Morales-Sánchez D., Tinoco R., Caro M. and Martínez A.

Elsevier Editorial System(tm) for Algal Research
Manuscript Draft

Manuscript Number:

Title: Nitrogen-limited heterotrophic fedbatch cultures of the microalgae Neochloris oleoabundans to enhance lipid and carbohydrate accumulation

Article Type: Full Length Article

Section/Category: Algal Cultivation

Keywords: Neochloris oleoabundans; heterotrophic growth; exponentially fed-batch; specific growth rate; glucose; lipid accumulation, carbohydrate accumulation

Corresponding Author: Dr. Alfredo Martinez, PhD

Corresponding Author's Institution: Universidad Nacional Autonoma de Mexico - Instituto de Biotecnologia

First Author: Daniela Morales-Sánchez, Ph.D.

Order of Authors: Daniela Morales-Sánchez, Ph.D.; Raunel Tinoco-Valencia, M.Sc.; Mario A Caro-Bermúdez , B.Sc.; Alfredo Martinez, PhD

Abstract: The effect of the specific growth rate (μ) on the accumulation of reserve metabolites of N. oleoabundans, during heterotrophic-fedbatch cultivation with excess glucose and limiting nitrate in a 40-L bioreactor, was studied in a system consisting of three stages. During the first stage, the batch cultivation without carbon or nitrogen limitation, the cells grew at a μ of 0.045 h-1 and the major cellular component, on a dry cell weight (DCW) basis, were proteins (44%, w/w). In the second stage, two different exponential nitrate-feeding profiles were performed at μ of 0.042 and 0.035 h-1. The results show that a small reduction in the μ (only 20%) has a drastic effect in the biochemical composition of the microalga, promoting a high accumulation of lipids and carbohydrates. During the third stage the cells were in a nitrogen-starving condition, glucose excess, and at 0.042 h-1, the lipids constituted 53.8% of the cell mass, reaching a cell mass of 20.9 gDCW/L, and productivities of biomass, lipids and carbohydrates of 1,900, 1,020 and 500 mg L-1 day-1, respectively; so far these are the highest productivity values reported for N. oleoabundans cultures either under phototrophic, mixotrophic or heterotrophic growth.

Suggested Reviewers: Jeremy Pruvost Ph.D.
University of Nantes, France
jeremy.pruvost@univ-nantes.fr
Expert in the culture of microalgae

Rene Wijffels Ph.D.
Wageningen University, The Netherlands
rene.wijffels@wur.nl
Expert in microalgal cultivations

Opposed Reviewers: Choul-Gyun Lee

Conflict of interest

Instituto de Biotecnología
UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

December 12, 2013

Dear Professors J. Olivares, R.T. Sayre
Editors-in-Chief:
Dear Professor Rene Wijffels
Editor
Algal Research

We are submitting the manuscript "**Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid and carbohydrate accumulation"** by Morales-Sánchez *et al*., in order to consider it for publication in the **Algal Research Journal**.

In this work, an exponential fed-batch strategy (EFBC), under nitrogen limitation and glucose excess conditions, was used to obtain high cell mass production of the microalga *N. oleoabundans*, to study its physiological behavior at two different growth rates, focused in the type of reserve metabolite accumulated. The EFBC performed at 0.042 h^{-1} behaves as a batch culture. During the fedbatch stage in the EFBC performed at 0.035 h^{-1} the yields of lipid and carbohydrate on nitrate and the yields of lipid and carbohydrate on glucose were 1.7 to 4-fold higher than in the EFBC 0.042. These results demonstrate that when the microalgae growth rate is reduced, in this instance reducing the specific nitrate consumption rate, carbon is preferably directed towards the synthesis of reserve metabolites with a higher efficiency than protein synthesis accordingly with the nitrate stress condition

During the post fedbatch stage, when cells were under nitrogen starving conditions, the remnant glucose was used to increase the cell mass, augmenting the lipid content in a greater extent in the culture preceded by the EFBC 0.042 and reducing the carbohydrate content in a greater extend in the culture preceded by the EFBC 0.035.

The exponential fed proved to be a successful strategy to obtain a high cell mass productivity and a high global lipid and carbohydrate productivity, which are the

TELS. (52) 56 22 76 00 TELS. (7) 329 16 00 FAX. 317 23 88 APDO. 510-3, CUERNAVACA, MORELOS 62250, MÉXICO

highest values reported so far for *N. oleoabundans* cultures either under phototrophic, mixotrophic or heterotrophic growth.

We state that all the authors (Daniela Morales-Sánchez, Raunel Tinoco-Valencia, Mario A. Caro-Bermúdez and Alfredo Martinez) mutually agree that this research work should be submitted to Algal Research; it is an original work of the above mentioned authors; and that the article has not been published or submitted in any other peer-reviewed journal.

We would be very pleased if you find this manuscript suitable for publication in Algal Research.

With my best Regards,

Dr. Alfredo Martinez
(Corresponding Autor)
Departamento de Ingeniería Celular y Biocatálisis
Instituto de Biotecnología. Universidad Nacional Autónoma de México
A.P. 510-3, Cuernavaca, Morelos 62250, MEXICO
Phone: (52+777) 329 16 01 Fax: (52+777) 317 2388
e-mail: alfredo@ibt.unam.mx

Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid and carbohydrate accumulation

Daniela Morales-Sánchez, Raunel Tinoco-Valencia, Mario A. Caro-Bermúdez and Alfredo Martinez*

Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Apdo. Postal 510-3, Cuernavaca, Morelos 62250, México.

*Corresponding Author:
Alfredo Martinez
Tel: (52+777) 329 1601
Fax: (52+777) 317 2388
E-mail: alfredo@ibt.unam.mx

E-mail addresses:
DMS: dmorales@ibt.unam.mx
RT: raunel@ibt.unam.mx
MC: mcaro@ibt.unam.mx
AM: alfredo@ibt.unam.mx

Abstract

The effect of the specific growth rate (μ) on the accumulation of reserve metabolites of *N. oleoabundans*, during heterotrophic-fedbatch cultivation with excess glucose and limiting nitrate in a 40-L bioreactor, was studied in a system consisting of three stages. During the first stage, the batch cultivation without carbon or nitrogen limitation, the cells grew at a μ of 0.045 h^{-1} and the major cellular component, on a dry cell weight (DCW) basis, were proteins (44%, w/w). In the second stage, two different exponential nitrate-feeding profiles were performed at μ of 0.042 and 0.035 h^{-1}. The results show that a small reduction in the μ (only 20%) has a drastic effect in the biochemical composition of the microalga, promoting a high accumulation of lipids and carbohydrates. During the third stage the cells were in a nitrogen-starving condition, glucose excess, and at 0.042 h^{-1}, the lipids constituted 53.8% of the cell mass, reaching a cell mass of 20.9 g_{DCW}/L, and productivities of biomass, lipids and carbohydrates of 1,900, 1,020 and 500 mg L^{-1} day^{-1}, respectively; so far these are the highest productivity values reported for *N. oleoabundans* cultures either under phototrophic, mixotrophic or heterotrophic growth.

Keywords: *Neochloris oleoabundans*; heterotrophic growth; exponentially fed-batch; specific growth rate; glucose; lipid accumulation, carbohydrate accumulation.

1. Introduction

Microalgae, being at the base of the food chain, are critical components of many habitats on the planet and are the major oxygen producers. Moreover, they are of commercial importance in the food, feed, aquaculture and pharmaceutical industry as natural sources of high-value products such as proteins, carbohydrates, carotenoids, fatty acids, steroids and algal toxins [1-4]. Oleaginous microalgae produce substantial amounts of neutral lipids, primarily comprised of triacylglycerides (TAGs) of favorable fatty acid chain length, making them an ideal feedstock for conversion to renewable diesel and jet fuel [5, 6].

Microalgae are commonly grown in phototrophic conditions using CO_2 as the carbon source. However, a low biomass concentration and low lipid production have been identified as the major inconvenients in this cultivation mode. Although certain species are obligate photoautotrophs, numerous microorganisms currently classified as microalgae are in fact obligate heterotrophs and others are capable of both photoautotrophic and heterotrophic metabolism, either sequentially or simultaneously [7]. Heterotrophic cultivation without light and with the controlled addition of an organic source of carbon and energy is similar to procedures established for aseptic cultivation of bacteria, streptomyces, fungi or yeast, in multipurpose stirred closed tanks that are sterilized by heat [7]. This cultivation mode for microalgae can be performed under specific controlled conditions and provides the possibility to achieve fast growth and high yield of products on a large scale [8-10].

66 *Neochloris oleoabundans* is a microalga that can grow photoautotrophically or
67 heterotrophically under different culture conditions [10-16]. Heterotrophic growth of *N.*
68 *oleoabundans* supplied with glucose as carbon source in batch cultures under nitrogen
69 limiting conditions resulted in high biomass and high content of lipid in cells [10]. It is
70 also well known that this microalga accumulates lipids under phototropic conditions
71 when nitrogen becomes limited [13, 15-17]. In a previous work [10] a maximal cell mass
72 of 9.2 g_{DCW} L^{-1} was obtained in 9 days using batch heterotrophic cultures at a C/N
73 (glucose/sodium nitrate) ratio of 278. Under these conditions the accumulation of lipids
74 was promoted (up to 52% w/w) through a long period of N-starvation (5 days) resulting
75 in high lipid productivity (528.5 mg_{LIP} L^{-1} day^{-1}). Also, fed-batch cultures were performed
76 at a C/N ratio of 278 and nitrate intermittent additions [10]; this short period of N-
77 limitation condition allowed to reach a maximal cell mass of 14.2 g_{DCW} L^{-1} and promoted
78 the accumulation of carbohydrate, mainly in the form of starch, as the larger reservoir
79 metabolite (up to 54% of the dry cell weight) and lipids (33.7%). Such study
80 demonstrated the need to limit the microalgae growth with the nitrogen source for a long
81 period in order to promote the metabolism to lipid synthesis. This objective could be
82 achieved using exponentially fedbatch cultures (EFBC), *i.e.* controlling the specific
83 growth rate (μ), maintaining a limiting nitrate concentration for a longer time in the
84 culture and promoting a higher cell density.
85 A fedbatch culture is a modified batch culture supplied with nutrient medium
86 continuously or intermittently during cultivation [8]. The μ can be controlled at or below
87 its maximum value by controlling substrate additions in an exponential-continuous
88 manner. This strategy allows having a limiting substrate concentration in the medium

during the feed. Based on this theory it is possible to design an exponentially fedbatch strategy, feeding the nitrogen source, allowing to maintain a maximum or lower μ to have a prolonged nitrogen-limiting conditions, but at the same time allowing the duplication of cells.

In the present work, a basic strategy of substrate feeding and process control was set up, using exponentially fedbatch cultures of *N. oleoabundans*, to sustain a higher cell mass concentration than the one obtained in batch cultures and to study the effect of two controlled μ, during the exponential growth phase, on the reserve metabolite synthesis.

2. Material and Methods

2.1 Algal strain, media and inocula development

The microalgae *Neochloris oleoabundans* UTEX 1185 was maintained by sub-cultivation in 250-mL shake flasks containing 100 mL of mineral medium (Bold's Basal Medium; BBM) supplemented with 10 g L^{-1} glucose, under dark conditions. Cultures were incubated in an orbital shaker (300 rpm, 25ºC; Barnstead MaxQ 4000, USA). The inoculum size in all cultivations was 10% (v/v). The components of BBM [11] are as follows (in mM): $NaNO_3$ 2.94, $MgSO_4 \cdot 7H_2O$ 0.3, K_2HPO_4 1.29, KH_2PO_4 0.43, $CaCl_2$ 0.17, NaCl 0.43, EDTA 0.171, KOH 0.553, H_3BO_3 0.185 and the following trace elements (in μM) $FeSO_4 \cdot 7H_2O$ 18, $ZnSO_4 \cdot 7H_2O$ 30.7, $MnCl_2 \cdot 4H_2O$ 7.3, $CuSO_4 \cdot 5H_2O$ 6.3, MoO_3 4.9, $CoNO_3 \cdot 6H_2O$ 1.7, H_2SO_4 10.2. All medium components were heat-sterilized (121 ºC, 20 min). For the inocula preparation, *N. oleoabundans* was cultivated

in 5-L bioreactor (LSL Biolafitte, USA) with 3 L of BBM at pH of 7 (adjusted by the automatic addition of 1 N H_3PO_4 and 1 N KOH), 25 ºC, 300 rpm (using three Rushton Turbines with 6 blades) and an aeration rate of 1 L of air/L of medium/min (the dissolved oxygen was always greater than 80% of the air saturation value).

2.2 Exponentially fedbatch cultures (EFBCs)

A 40-L nominal volume fermenter (B. Braun Biotech International, Melsungen, Germany) operated at a constant pH of 7.0, 25ºC, and at an initial volume of 20 L, 300 rpm and 20 L of air min^{-1} was used. The dissolved oxygen concentration was maintained at a value above 20% of air saturation, controlled by manual increases of the impeller speed. After an initial batch phase of 4 days, the fedbatch operation was initiated. The fedbatch scheme was implemented using a programmable logic controller (PLC) with a predetermined exponentially increasing feeding profile. In an EFBC upon reaching a pseudosteady state, the nitrate concentration can remain constant and close to zero, hence the specific growth rate (μ) can be controlled at a constant and predetermined value. The nitrate flow rate (F) profiles were established according to the following equation [18]:

$$F = \frac{X_0 V_0 \exp(\ \ t)}{Y_{X/S} S_i}$$

where S_i is the substrate concentration in the inlet stream, V_0 and X_0 are the volume and biomass at the initiation of the fedbatch phase, t is the elapsed time of the fedbatch phase, and $Y_{X/S}$ is the cell yield on nitrate. This equation shows that the EFBC can be employed for controlling μ at a predetermined value by selecting the limiting substrate

135 flow rate, knowing the cell yield and the initial volume and cell concentration. According
136 to the measured V_0 and X_0, and the pre-determined μ, a S_i was chosen based on the
137 minimum resolution flow rate of the feeding pump used (Masterflex, head Easy-Load,
138 model 7518-60, Cole-Parmer, Barrington, IL); thus, 0.035 and 0.042 h^{-1} values for μ
139 were programed. In both cultures, S_i was 30 g/L of sodium nitrate, V_0 was 20 L and $Y_{X/S}$
140 was taken as a constant value of 8 x 10^{11} cells/g_{NIT}; this value was estimated from data
141 reported previously [10] and is equivalent to 3.3 g_{DCW}/g_{NIT} under non nitrogen limiting
142 conditions. An estimation of the maximum μ (approximately 0.045 h^{-1}) was obtained
143 from previous batch cultures without any nutrient limitation [10]. To contend with the
144 culture evaporation, the air flow in the inlet was saturated with water. To prevent a
145 nutrient limitation, other than the sodium nitrate, all BBM medium salts (at a final
146 concentration of 0.5X) were added separately to the concentrated fed of sodium nitrate.
147 The cultures were designed to add 10 L of a concentrated sodium nitrate solution (30
148 g_{NIT} L^{-1}).
149 The EFBC used in this work were designed to ensure a limiting nitrate condition during
150 the feeding period, *i.e*. the nitrate consumption matched the supply of the nitrogen fed
151 and therefore the nitrate concentration detected in the culture broth was zero during this
152 period and was run to allow between 20-30-fold increases in the cell number. This
153 strategy enables the imposition of a nutritional metabolic stress through nutrient
154 limitation. During the EFBC the volume changed in an exponential way with the elapsed
155 time. Although the change in volume over time is different from zero and no steady state
156 in biomass concentration is reached during the exponential feeding [18], this approach
157 permits the increase in one or two logarithmic scales the concentration of cell mass and

158 biomass productivity of some species of microalgae like *Chlorella protothecoides* [19].

159

160 *2.3 Analytical methods and kinetic and stoichiometric parameters calculations*

161 The cell number (cells mL^{-1}) was determined by direct counting using the Neubauer
162 chamber and a light microscope with the 40X objective. The algal dry cell weight (DCW)
163 was evaluated gravimetrically by filtration through a 0.45 μm pore size pre-dried and
164 pre-weighed nitrocellulose membrane filter (Millipore). Lipids were extracted with
165 solvents and gravimetrically quantified. More details of this method were previously
166 described [10]. The carbohydrate content was determined by the phenol-sulfuric acid
167 method, after acid hydrolysis of the sample [20], and the protein content by the Lowry
168 method, after alkaline hydrolysis of the sample [21]. Glucose in culture supernatants
169 was determined using a biochemical analyzer (YSI 2700 Select, Yellow Spring
170 Instruments, Ohio, USA). Nitrate concentration was quantified using an ion selective
171 probe calibrated with sodium nitrate standards (10, 100, 1000 mg L^{-1}; Ion Concentration
172 Controller Microprocessor Based IC 7685, B&C Electronics Srl, Lombardía, Italy; and
173 nitrate probe ISE: Nitrate NO31502, Van London-Phoenix Co, Texas, USA). To
174 determine the fatty acid composition, oil samples (7 mg) were chemically derivatized
175 using the borum trifluoride method [22]. The obtained organic phase was analyzed in a
176 GC-MS system (GC: 6890N, Agilent Technologies, Wilmington, DE, USA; Mass
177 selective detector: 5973N, Agilent Technologies, Wilmington, DE, USA). The details of
178 the method are described elsewhere [10].

179

The cell mass, substrates and metabolite concentration were determined as the increase or decrease of their concentration during the batch, fed-batch, post fed-batch phase and during the total elapsed time of the culture. The specific growth rates (μ) were determined by fitting the cell number data versus time to exponential regressions for each stage. The cell mass yield on glucose and nitrate ($Y_{X/GLC-NIT}$), yield of lipids, proteins and carbohydrates on glucose ($Y_{LIP-PROT-CARB/GLC}$), yield of lipids, proteins and carbohydrates on nitrate ($Y_{LIP-PROT-CARB/NIT}$) were estimated as the coefficient of the linear regression of the respective metabolite concentration versus the concentration of glucose or nitrate consumed during each stage, in $g_{DCW}/g_{GLC-NIT}$ and $g_{LIP-PROT-CARB}$ / $g_{GLC-NIT}$. The specific glucose or nitrate consumption rate ($q_{GLC-NIT}$) was determined as the ratio of μ to $Y_{X/GLC-NIT}$. These parameters were calculated taking into account the values during the elapsed time for each stage. The volumetric productivities of cell mass and metabolites were determined as the increase of the concentration of the cell mass and metabolites in each stage or in the total cultivation period of the stage or the total cultivation time, respectively. A carbon balance was performed: the percentage of C-mol in the reserve materials from glucose was calculated taking into account the carbon content of glucose (40%), carbohydrates (40%) and lipids (76.6%) and the total amount of glucose consumed and carbohydrates and lipids accumulated for each stage.

3. Results and discussion

To evaluate the effect of the growth rate on the reserve metabolite synthesis and relevant kinetic and stoichiometric parameters, EFBC's of *N. oleoabundans* were

operated at two specific growth rates: 0.042 and 0.035 h^{-1}, which will be identified as EFBC 0.042 and EFBC 0.035. These μ correspond to an approximate value of the maximum growth rate obtained during the exponential growth of this microalga in batch cultures (0.045) and to a value below this maximum rate, respectively. As the volume of the reactor changed significantly a mass balance, considering the volume of liquids added (nitrate solution, acid and base used for pH control, and nutrient additions) and removed (sampling), was performed in order to correct the actual values for the cell density, cell mass, glucose and nitrate concentration

Figure 1 and table 1 show the complete overview of both EFBC's and results for each phase will be described separately in the following sections.

3.1 Batch phase culture evaluation

In both cultures (EFBC 0.042 and EFBC 0.035), the first stage was a batch culture of 4 days with an initial C/N ratio of 278. In this stage the microalga grew without nitrogen or glucose limitation. To avoid any nutrient limitation other than those of glucose or sodium nitrate, all components of BBM media were added at twice the normal concentration. As shown in table 1 and figure 1b, a cell mass concentration of 1.4-1.51 g_{DCW} L^{-1} was attained at the end of this first stage in both cultures, compared to the cell mass concentration inoculated, these values correspond to a 9-fold increase. The μ, based on the increase of cells was 0.045 h^{-1}, this value was similar to the one obtained in previously reported batch cultures with a balanced C/N ratio [10]. At these conditions the major cellular components were proteins (44% in both EFBC's; figure 2), these results were previously observed in heterotrophic batch cultures without any nutrient

limitation [10]. As seen in figure 1 and 2 both cultures behave similar during the first stage, hence the data reported in these figures was pooled to calculate the kinetic and stoichiometric parameters shown in tables 1 and 2 for this phase. It is worth mentioning that the values reported in tables 1 and 2 are different to previously documented data of *N. oleoabundans* grown under heterotrophic conditions, owing to the following differences between both studies: a 10-fold change in the bioreactor scale, different salts concentration in the media, differences in bioreactor configuration and different oxygen transfer rates, among others.

3.2 Fedbatch phase culture evaluation: lower μ *promote lipid and carbohydrate accumulation*

Figure 1 shows the results of the EFBC's operated at a μ of 0.042 and 0.035 h^{-1}. The vertical dashed lines, black and gray, denote the time of initiation and termination of the feeding phase in the EFBC 0.042 and the black vertical dashed line and the gray dotted line correspond to the fedbatch phase in the EFBC 0.035. For both EFBC's, a pseudo-steady state was rapidly reached after initiation of the fedbatch phase as observed from a controlled exponential increase in biomass concentration (figures 1a and b) and a nitrate concentration of zero detected by the nitrate selective probe (figure 1d). This indicates that a constant specific nitrate consumption rate was obtained during the fedbatch phase, having a higher nitrate uptake rate for the EFBC 0.042 (1.2-fold higher than the value obtained for the EFBC 0.035; table 1) as a consequence of the differences in the μ. During the feed phase with nitrogen limitation, cells continued duplicating (figure 1a). The fedbatch phase was designed to allow 20 cell duplications

for EFBC 0.042 and 30 for EFBC 0.035. The initial nitrate flow rates were 0.3 and 0.15 mL min^{-1} for EFBC 0.042 and EFBC 0.035, respectively. The initial concentration of glucose in the fedbatch stage was about 45 g_{GLC} L^{-1} for both EFBC's.

During the fedbatch phase of the EFBC 0.042 a cell mass of 11.73 g_{DCW} L^{-1} was produced with a glucose uptake rate of 0.085 g_{GLC} g_{DCW} h^{-1} (table 1), which resulted in a cell mass yield on glucose of 0.45 g_{DCW} g_{GLC}. As shown in figure 2, the protein and carbohydrate concentrations from the batch to fedbatch phase didn't change significantly, however the lipid concentration increased 5.5%. The volumetric concentration for these components was 5.66 g_{PROT} L^{-1}, 2.85 g_{LIP} L^{-1} and 4.24 g_{CARB} L^{-1}.

The increase on cell mass during the EFBC 0.035 was very similar to the value obtained for the EFBC 0.042; but, since the nitrate supply rate was lower, the specific rates for glucose and nitrate consumption were 2.5 and 1.2-times lower, hence the volumetric cell mass productivity decreased 2.04-fold when compared the value obtained for the EFBC 0.042. However, the yield of cell mass on glucose was 28% higher in the culture than in the EFBC 0.042 (table 1), but the cell mass yield on nitrate was the same for both cultures; suggesting a greater incorporation of reserve materials into the cells during the fed phase of EFBC 0.035. During this phase the content of lipids and carbohydrates increased 8.3 and 19.3%, in comparison with the batch phase; but the proteins decreased 27.5%; accordingly the volumetric concentration of these compounds were 1.88 g_{PROT} L^{-1}, 5.78 g_{LIP} L^{-1} and 6.84 g_{CARB} L^{-1}, respectively.

Table 2 shows the stoichiometric parameters obtained during the fed-batch stage of both EFBC. The actual yield of protein on glucose ($Y_{PROT/GLC}$), *i.e.* the consumed glucose converted to proteins during such period of time, was similar in both EFBC's,

this result indicates that glucose was transformed into proteins with the same efficiency regardless of the μ. The protein content was low in comparison with lipids and carbohydrates, meaning that the synthesis of reserve metabolites was higher.

Although the lipid content was higher for the EFBC 0.035, the cell masses at the end of the feeding period were similar (figure 1), but the time frame to reach these values was 2 days longer for this culture, hence the lipid productivities (Q_{LIP}) were similar in both EFBC's (table 1). The lipid concentration in the EFBC 0.035 was 2-fold higher than the EFBC 0.042. The effect of the feeding time was observed in the protein productivity (Q_P) as well, this value was 3-times lower in the EFBC 0.025 due to the longer feeding time and the higher lipid and carbohydrate accumulation, which partitioned the carbon flow in a greater proportion towards the protein synthesis but also towards the lipid and carbohydrate synthesis when compared to the EFBC 0.042. As seen in figure 2, the highest protein content for this culture was reached during this stage The protein yield on nitrate ($Y_{PROT}/_{NIT}$) was 3-fold higher in the EFBC 0.042 indicating that the nitrate was used to build proteins instead of promoting the formation of reserve metabolites and suggesting that a strict nitrogen limitation wasn't obtained under this condition because cells were growing at a μ close to the maximum.

In the EFBC 0.035 the lipid and carbohydrate yields on nitrate ($Y_{LIP\text{-}CARB}/_{NIT}$) and the lipid and carbohydrate yields on glucose ($Y_{LIP\text{-}CARB/GLC}$) were 1.7 and 4-times higher, respectively, than in the EFBC 0.05. This means that under a lower growth rate the carbon and nitrogen flow are preferably directed toward the synthesis of reserve metabolites with a higher efficiency accordingly with the nitrate stress condition.

The carbohydrate concentration and productivity (Q_C) were 3 and 1.7-fold higher in the

295 EFBC 0.035, respectively, than in the EFBC 0.042, indicating a high carbohydrate
296 synthesis in response to a nitrogen limiting conditions.
297 The fed was stopped according to the predetermined profile, thereby the next stage
298 proceeded without nitrate, but there was a remnant glucose concentration of 19.3 and
299 29.8 g_{GLC} L^{-1} for the EFBC 0.042 and EFBC 0.035, respectively.
300 Several studies have demonstrated that alteration in nutrient concentrations in short
301 periods can modify the growth and secondary metabolism of microalgae and some
302 microalgae species like *Chlorella vulgaris* are prone to reserve carbohydrates for energy
303 [23]. Carbohydrates are most likely preferred as reserve metabolites under short-term
304 nutritional stress conditions, as observed in plants [24]. The obtained cell mass in our
305 previous report, using an intermittent fed of nitrate (Table 4, [10]), was 6.7 g_{DCW} L^{-1}
306 lower than both EFBC reported here, showing that the exponential strategy was more
307 effective than the intermittent strategy to obtain higher cell masses.
308
309 *3.3 Post fedbatch phase culture evaluation: nitrogen starvation promote lipid*
310 *accumulation*
311 After the nitrate feeding was stopped the third-stage proceeded with 19.3 and 29.8 g L^{-1}
312 of residual glucose (figures 1), the cultures were in a nitrogen-starving condition,
313 accordingly the number of cells did not increase, but there was an 1.6 and 2-fold cell
314 mass increase in the EFBC 0.042 and EFBC 0.035, respectively. During this stage the
315 EFBC 0.035 showed a specific glucose uptake rate that increased 2-fold and a cell
316 mass productivity that was 1.4-fold higher in comparison with the EFBC 0.042.
317 Moreover, the physiological response of *N. oleoabundans* at these two μs was quite

different. At a higher μ (EFBC 0.042) the lipid accumulation was 53.8% of the dry cell weight; being the oleic (55.3%), linoleic (22.7%) and palmitic (13.3%) acids the main components determined at the maximum produced cell mass (day 11). At this condition a higher amount of carbon was directed to lipid biosynthesis and the yield of lipid in glucose and the lipid productivity, during this stage, were about 1.5 and 1.3-fold higher, respectively, than the EFBC 0.035 (table 2). The final content of proteins, carbohydrates and lipids were of 17.5, 26.9 and 53.8% for the EFBC 0.042 and 17.4, 38.1 and 44.6% for EFBC 0.035. These results suggest the use of metabolic engineering to eliminate the synthesis of starch, *i.e*. deleting the gene(s) encoding for glucose 1-phosphate adenylyltransferase [25] could increase the lipid contend in this microalgae.

Feeding the nitrogen source at a lower μ (EFBC 0.035) led to a decrease in the lipid accumulation (44.6%) but the carbohydrate content was 1.5-fold higher (38.1%) than the EFBC 0.042 (26.9%). The yield of carbohydrate in glucose ($Y_{CARB/GLC}$) was 3-fold higher indicating that a higher total carbon flow was directed towards the carbohydrate synthesis with a productivity 3-times higher than the EFBC 0.042. The protein content was 17.4% and similar in both EFCB's. The lipid profile in this culture determined at the maximum produced cell mass (day 13) was different from the EFBC 0.042, but similar to the profile found in phototrophic cultures of *N. oleoabundans* [17], the oleic acid decreased 7.4%, the palmitic acid was the second major component in the profile (27.6%) and the third major component was the linoleic acid (14.3%), indicating that the saturated fatty acid content increased when compared with the EFBC 0.042. Some changes on the relative content of different fatty acids were reported among different culture strategies with *Chlorella protothecoides* [19], indicating a complicated regulation

of the lipid metabolism under different physiological conditions. The lipid, protein and carbohydrate productivity were higher in the EFBC 0.035 due to the higher cell mass obtained (table 2). These reserve materials have a high nutritional content making the biomass of *N. oleoabundans* a suitable source of food supplements for marine and terrestrial organisms [26].

In general, the lipid accumulation in microorganisms is stimulated by an excess of a carbon source and a limitation in one (or more) of the other nutrients, especially nitrogen. Microbial lipid accumulation is often a biphasic process. In the first phase, exponential cell division occurs, in the second phase a small decrease in the μ slightly increases the accumulation of lipids [27]. This behavior was clearly observed in the EFBC 0.042 sugesting that this strategy is suitable to obtain a high cell mass concentration and a high global lipid productivity (table 3), which is the highest value reported so far for *N. oleoabundans* cultures. On the other hand, the EFBC 0.035 started producing carbohydrates from the fed-batch stage and probably that was the reason to accumulate fewer lipids than the EFBC 0.042. Nevertheless the global lipid productivity was 10% higher in the EFBC 0.035 because this culture contains a higher cell mass concentration.

3.4 Comparing the energy content in microalgae containing reserve materials with ethanol production by yeast.

The energy content in the microalgal biomass obtained at the best production conditions attained in this work (EFBC 0.035) was estimated using the considerations stated in table 4 and the next lines. The total carbon supply was 1,500 g of glucose or 600 g of C.

364 Based on the elemental composition of *N. oleoabundans* [17], an amount of 407.5 g of
365 C was incorporated into the total microalgal biomass with a productivity of 1.04 g_C L^{-1}
366 day^{-1} ($Y_{X/GLC}$ = 52%; table 3). The theoretical calculations were based on protein,
367 carbohydrate and lipid caloric contents of 15.5, 13.0 and 38.3 MJ kg^{-1}, respectively
368 [16,28], as well as on the corresponding productivity values attained at EFBC 0.035
369 (table 3).
370 Assuming that the total amount of carbohydrates and lipids are extracted and can be
371 converted to ethanol and biodiesel and using the factors indicated in table 5, a total
372 amount of 483.6 MJ m^{-3} can be reached. From these values, the specific energy content
373 of the microalga cultured under heterotrophic conditions was estimated as 25 MJ kg_{DCW}^{-1}
374 . This value is within the previously reported range for several microalgae: 14.2 – 29.0
375 MJ kg^{-1} [28,29], including the same microalga cultured under phototrophic – nitrogen
376 limiting conditions: 20.8 MJ kg^{-1} [16].
377 As a comparison the conversion of the 1,500 g of glucose to ethanol (maximum
378 theoretical yield of 0.51 g_{EtOH}/g_{GLC} and a fermentation efficiency of 95%) [30] 649.57 MJ
379 m^{-3} yield can be obtained. Furthermore, assuming that the microalga can accumulate up
380 to 80% (w/w) of lipid content (no carbohydrate accumulation), it is hypothetically
381 possible to obtain 616.02 MJ m^{-3}. Using the total biomass (lipids, proteins and
382 carbohydrates, 795.9 g of DCW) with the energy content obtained before, it could be
383 feasible to obtain 662.5 MJ m^{-3}, which is similar to the energy values obtained from
384 yeast. From this analysis is clear that the conversion of glucose or other types of sugars
385 to microalgal cell mass containing a high amount of reserve materials (including only
386 lipids) is not favorable in terms of energy content in comparison with the conversion of

such sugars to ethanol. Therefore, from the perspective of biofuel production it is better to produce low cell density cultures of yeast with ethanol as an end-product than high cell density cultures with microalgae with lipids and/or carbohydrates as end-products.

4. Conclusions

The exponentially fed-batch strategy, under nitrogen limitation and glucose excess conditions, enabled us to obtain high cell mass production of *N. oleoabundans*. The physiological behavior of the microalgae at the two different growth rates was reflected in the type of reserve metabolite accumulated. The EFBC performed at 0.042 h^{-1} behaves as a batch culture, *i.e*. the protein, carbohydrate and lipid contend was similar to the values obtained in batch cultures without nutrient limitations; this growth rate is similar to the values obtained during the batch stage (0.045 h^{-1}), because the nitrate was practically fed at the same specific consumption rate to maintain maximum growth rate, hence there wasn't a strict nitrogen stress at this μ. During the fedbatch stage in the EFBC 0.035 the yields of lipid and carbohydrate on nitrate and the yields of lipid and carbohydrate on glucose were 1.7 to 4-fold higher than in the EFBC 0.042. These results demonstrate that when the microalgae growth rate is reduced, in this instance reducing the specific nitrate consumption rate, carbon is preferably directed towards the synthesis of reserve metabolites with a higher efficiency than protein synthesis accordingly with the nitrate stress condition

During the post fedbatch stage, when cells were under nitrogen starving conditions, the remnant glucose was used to increase the cell mass, augmenting the lipid content in a

greater extent in the culture preceded by the EFBC 0.042 and reducing the carbohydrate content in a greater extend in the culture preceded by the EFBC 0.035.

The final contents of carbohydrates and lipids were 38.1 and 44.6% and 26.9 and 53.8%, for EFBC 0.035 and EFBC 0.042, respectively, corroborating that cultures with nitrogen starving conditions, under glucose excess, accumulate reserve materials and that small variations in the nitrate-limiting conditions and μ switch the degree of reserve materials between lipids and carbohydrates.

The exponential fed proved to be a successful strategy to obtain a high cell mass productivity (1,900 mg_{DCW} $L^{-1}day^{-1}$) and a high global lipid productivity (1,020 $mg_{LIP}L^{-1}day^{-1}$), which are the highest values reported so far for *N. oleoabundans* cultures either under phototrophic, mixotrophic or heterotrophic growth.

Acknowledgements

We thank Leobardo Serrano for technical support in the fermentation Pilot Plant of the Biotechnology Institute-UNAM. This work was supported by the Universidad Nacional Autónoma de México (UNAM), grant DGAPA/PAPIIT/UNAM IT200312 and the Consejo Nacional de Ciencia y Tecnología (CONACyT) – México, grant 213566. DMS held a scholarship from CONACyT.

References

[1] A. Jamers, R. Blust, W. de Coen, Omics in algae: paving the way for a systems biological understanding of algal stress phenomena?, Aquat. Toxicol. 92 (2009) 114-121.

[2] J.L. Guil-Guerrero, R. Navarro-Juarez, J.C Lopez-Martinez, P. Campra-Madrid, M.M. Rebolloso-Fuentes, Functional properties of the biomass of three microalgal species, J. Food Eng. 65 (2004) 511-517.

[3] X. Meng, J. Yang, X. Xu, L. Zhang, Q. Nie, M. Xian, Biodiesel production from oleoaginous microorganism, Renew. Energ. 34 (2009) 1-5.

[4] C. Barrow, F. Shahidi, Marine nutraceuticals and functional foods. CRC Press, Taylor & Francis Group (2008).

[5] M.T. Guarnieri, A. Nag, S.L. Smolinski, A. Darzins, M. Seibert, P.T. Pienkos, Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga, PLoS ONE 6(10) (2011) e25851.

[6] J. Traller, M. Hildebrand, High throughput imaging to the diatom *Cyclotella cryptica* demonstrates substantial cell-to-cell variability in the rate and extent of triacylglycerol accumulation, Algal Research 2 (2013) 244-252.

[7] F. Bumbak, S. Cook, V. Zachleder, S. Hauser, K. Kovar, Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations, Appl. Microbiol. Biotechnol. 91 (2011) 31-46.

[8] G.Q. Chen, F. Chen, Growing phototrophics cells without light, Biotechnol. Lett. 28 (2006) 607-616.

[9] D. Mitra, J. Leeuwen, B. Lamsal, Heterotrophic/mixotrophic cultivation of oleaginous *Chlorella vulgaris* on industrial co-products, Algal Research 1 (2012) 40-48.

[10] D. Morales-Sánchez, R. Tinoco, J. Kyndt, A. Martínez, Heterotrophic growth of *Neochloris oleoabun*dans using glucose as a carbon source, Biotechnol. Biofuels 6 (2013) 100.

[11] T.G. Tornabene, G. Holzer, S. Lien, N. Burris, Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*, Enzyme Microb. Technol. 5 (1983) 435-440.

[12] Y. Li, M. Horsman, B. Wang, N. Wu, C.Q. Lan, Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*, Appl. Microbiol. Biotechnol. 81 (2008) 629-636.

[13] J. Pruvost, G. Van Vooren, G. Cogne, J. Legrand, Investigation of biomass and lipids production with Neochloris oleabundans in photobioreactor, Bioresour. Technol. 100(23) (2009) 5988-5995.

[14] C. Sousa, A. Compadre, M.H. Vermuë, R.H. Wijffel, Effect of oxygen at low and high light intensities on the growth of *Neochloris oleoabundans*, Algal Research 2 (2013) 122-126.

[15] A.M. Santos, P.P. Lamers, Janssen M, R.H. Wijffels, Biomass and lipid productivity of *Neochloris oleoabundans* under alkaline-saline conditions, Algal Research 2 (2013) 204-2011.

[16] A. Garibay-Hernández, R. Vazquez-Duhalt, L. Serrano-Carreón, A. Martinez, Nitrogen limitation in *Neochloris oleoabundans*: A reassessment of its effect on cell growth and biochemical composition. Appl. Microbiol. Biotechnol. In press (2013).

http://dx.doi.org/10.1007/s12010-013-0454-1.

[17] L. Gouveia, A. Marques, T. da Silva, A. Reis, *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production, J. Ind. Microbiol. Biotechnol. 36 (2009) 821-826.

[18] A. Martínez, O.T. Ramírez, F. Valle, Effect of growth rate on the production of ß-galactosidase from Escherichia coli in Bacillus subtilis using glucose-limited exponentially fed-batch cultures, Enzyme Microb. Technol. 22 (1998) 520-526.

[19] W. Xiong, X. Li, J. Xiang, Q. Wu, High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production, Appl. Microbiol. Biotechnol. 78 (2008) 29-36.

[20] M. Dubois, K. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, A colorimetric method for the determination of sugars, Nature 168 (1951) 167-168.

[21] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193(1) (1951) 265-275.

[22] L. Gouveia, C. Oliveira, Microalgae as a raw material for biofuels production, J. Ind. Microbiol. Biotechnol. 36 (2009) 269-274.

[23] G. Dragone, B.D. Fernandes, A.P. Abreu, A.A. Vicente, J.A. Teixeira, Nutrient limitation as a strategy for increasing starch accumulation in microalgae, Appl. Energy 88 (2011) 3331-3335.

[24] S. Streb, S.C. Zeeman, Starch metabolism in Arabidopsis, The Arabidopsis book (2012) 10:e0160.doi: 10.1199/tab.0160.

[25] R. Radakovits, R.E. Jinkerson, A. Darzins, M.C. Posewitz, Genetic engineering of algae for enhanced biofuel production. Eukaryotic. Cell. 9(4) (2010) 486-501.

500 [26] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic
501 cultures of microalgae: metabolism and potential products. Water Res. 45(1) (2011)
502 11-36.
503 [27] M. de Swaaf, L. Sijtsma, J. Pronk, High-cell-density fed-batch cultivation of the
504 docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*, Biotechnol.
505 Bioeng. 81 (2003) 666-672.
506 [28] J. Ma, O. Hemmers, Thermo-economic analysis of microalgae co-firing process for
507 fossil fuel-fired power plants. ASME Proceedings 4th International Conference on
508 Energy Sustainability (2010) Phoenix, AZ. ISBN: 978-0-7918-4394-9 l.
509 [29] A.M. lllman, A.H. Scragg, S.W. Shales, Increase in Chlorella strains calorific values
510 when grown in low nitrogen medium, Enzyme Microb. Tech. 27 (2000) 631-635.
511 [30] Garay-Arroyo A., A.A. Covarrubias, I. Clark, I. Niño, G. Gosset, A. Martinez.
512 Response to different environmental stress conditions of industrial and laboratory
513 *Saccharomyces cerevisiae* strains. Appl. Microbiol. Biotechnol. 63 (2004) 734-741.

Table 1. Kinetic and stoichiometric parameters for *Neochloris oleoabundans* cells grown in EFBC 0.042 and EFBC 0.035. The parameters were calculated for each phase considering initial and final values for each period.

Parameter	Batch stage	Fed-Batch stage		Post fed-batch stage	
		EFBC 0.042	EFBC 0.035	EFBC 0.042	EFBC 0.035
μ (h^{-1})	0.045 ±0.001	0.042 ±0.003	0.035 ±0.001	NA	NA
X (g_{DCW} L^{-1})	1.45 ±0.15	11.733 ±0.247	11.513 ±0.095	7.67 ±2.19	13.62 ±0.072
$Y_{X/GLC}$ (g_{DCW} g_{GLC}^{-1})	0.32 ±0.07	0.5 ±0.05	0.64 ±0.01	0.45 ±0.061	0.46 ±0.009
$Y_{X/NIT}$ (g_{DCW} g_{NIT}^{-1})	2.9 ±0.14	2.8 ±0.001	2.8 ±0.002	NA	NA
q_{GLC} (g_{GLC} g_{DCW}^{-1} h^{-1})	0.07 ±0.02	0.085 ±0.01	0.034 ±0.002	0.007 ±0.005	0.015 ±0.0004
q_{NIT} (g_{NIT} g_{DCW}^{-1} h^{-1})	0.016 ±0.0007	0.015 ±0.001	0.013 ±0.001	NA	NA
Q_X (g_{DCW} L^{-1} day^{-1})	0.365 ±0.035	5.87 ±0.12	2.88 ±0.02	1.9 ±0.3	2.72 ±0.01

μ: Specific growth rate
X: Cell mass produced in each stage
$Y_{X/GLC}$: Yield of biomass on glucose
$Y_{X/NIT}$: Yield of biomass on nitrate
q_{GLC}: Glucose uptake rate
q_{NIT}: Nitrate uptake rate
Q_X: Biomass productivity at maximum cell mass produced in each stage
NA: Not Applicable

Table 2. Kinetic and stoichiometric parameters for the biochemical composition of *Neochloris oleoabundans* grown in EFBC 0.042 and EFBC 0.035. The parameters were calculated for each stage considering initial and final values for each period.

Parameter	Batch stage	Fed-Batch stage		Postfed-batch stage	
		EFBC 0.042	EFBC 0.035	EFBC 0.042	EFBC 0.035
$Y_{LIP/GLC}$ (g_{LIP} g_{GLC}^{-1})	0.083 ±0.015	0.11 ±0.01	0.484 ±0.004	0.38 ±0.02	0.25 ±0.03
$Y_{PROT/GLC}$ (g_{PROT} g_{GLC}^{-1})	0.197 ±0.002	0.18 ±0.009	0.16 ±0.03	0.17 ±0.09	0.09 ±0.01
$Y_{CARB/GLC}$ (g_{CARB} g_{GLC}^{-1})	0.16 ±0.017	0.14 ±0.02	0.57 ±0.01	0.03 ±0.004	0.09 ±0.01
$Y_{LIP/NIT}$ (g_{LIP} g_{NIT}^{-1})	0.66 ±0.09	0.11 ±0.008	0.19 ±0.009	NA	NA
$Y_{PROT/NIT}$ (g_{PROT} g_{NIT}^{-1})	1.56 ±0.28	0.188 ±0.0008	0.062 ±0.007	NA	NA
$Y_{CARB/NIT}$ (g_{CARB} g_{NIT}^{-1})	1.25 ±0.33	0.14 ±0.009	0.23 ±0.007	NA	NA
Q_{LIP} (g_{LIP} L^{-1} day^{-1})	0.055 ±0.006	1.14 ±0.08	1.15 ±0.05	1.9 ±0.42	1.52 ±0.05
Q_{PROT} (g_{PROT} L^{-1} day^{-1})	0.13±0.008	1.89 ±0.008	0.62 ±0.07	0.67 ±0.21	0.55 ±0.12
Q_{CARB} (g_{CARB} L^{-1} day^{-1})	0.11 ±0.004	1.41 ±0.09	2.27 ±0.06	0.21 ±0.10	0.6 ±0.05

$Y_{LIP/GLC}$: Yield of lipids on glucose
$Y_{PROT/GLC}$: Yield of proteins on glucose
$Y_{CARB/GLC}$: Yield of carbohydrates on glucose
$Y_{LIP/NIT}$: Yield of lipids on nitrate
$Y_{PROT/NIT}$: Yield of proteins on nitrate
$Y_{CARB/NIT}$: Yield of carbohydrates on nitrate
Q_{LIP}: Lipid productivity
Q_{PROT}: Protein productivity
Q_{CARB}: Carbohydrate productivity
NA: Not Applicable

Table 3. Global kinetic and stoichiometric parameters of EFBC 0.042 and EFBC 0.035.

Parameters	EFBC 0.042	EFBC 0.035
X_{MAX} (g_{DCW} L^{-1})	20.9 ±2.43	26.53 ±0.4
$Y_{X/GLC}$ (g_{DCW} g_{GLC}^{-1})	0.45 ±0.03	0.52 ±0.009
$Y_{X/NIT}$ (g_{DCW} g_{NIT}^{-1})	0.72 ±0.07	0.9 ±0.01
$Y_{LIP/GLC}$ (g_{LIP} g_{GLC}^{-1})	0.24 ±0.01	0.24 ±0.01
$Y_{PROT/GLC}$ (g_{PROT} g_{GLC}^{-1})	0.08 ±0.01	0.09 ±0.01
$Y_{CARB/GLC}$ (g_{CARB} g_{GLC}^{-1})	0.11 ±0.003	0.19 ±0.01
$Y_{LIP/NIT}$ (g_{LIP} g_{NIT}^{-1})	0.39 ±0.03	0.4 ±0.01
$Y_{PROT/NIT}$ (g_{PROT} g_{NIT}^{-1})	0.12 ±0.03	0.15 ±0.01
$Y_{CARB/NIT}$ (g_{CARB} g_{NIT}^{-1})	0.175 ±0.01	0.32 ±0.02
Q_X (g_{DCW} L^{-1} day^{-1})	1.9 ±0.22	2.04 ±0.03
Q_{LIP} (g_{LIP} L^{-1} day^{-1})	1.02 ±0.17	0.93 ±0.04
Q_{PROT} (g_{PROT} L^{-1} day^{-1})	0.32 ±0.07	0.35 ±0.05
Q_{CARB} (g_{CARB} L^{-1} day^{-1})	0.50 ±0.08	0.74 ±0.04

X_{MAX}: Maximum cell mass produced
$Y_{X/GLC}$: Yield of biomass on glucose
$Y_{X/NIT}$: Yield of biomass on nitrate
$Y_{LIP/GLC}$: Yield of lipid on glucose; $Y_{PROT/GLC}$: Yield of protein on glucose; $Y_{CARB/GLC}$: Yield of carbohydrate on glucose
$Y_{LIP/NIT}$: Yield of lipid on nitrate; $Y_{PROT/NIT}$: Yield of protein on nitrate; $Y_{CARB/NIT}$: Yield of carbohydrate on nitrate
Q_X: Biomass productivity at maximum cell mass produced
Q_{LIP}: Lipid productivity at maximum cell mass produced
Q_{PROT}: Protein productivity at maximum cell mass produced
Q_{CARB}: Carbohydrate productivity at maximum cell mass produced

Table 4.Energy content of biofuels that can be obtained from 1,500 g of glucose, when this raw material is converted to ethanol by yeast or to lipids, carbohydrates and cell mass by *N. oleoabundans* grown at a μ of 0.035 h^{-1}.

Biofuels	Energy content (MJ m^{-3})
$EC_{Net\ Energy}$	662.5
$EC_{Bioethanol}$ [a]	128.2
$EC_{Biodiesel}$ [b]	353.24
$EC_{Total\ Liquid\ Fuel}$	483.6
$EC_{80\%Lipids}$ [c]	616.02
$EC_{Bioethanol\ Yeast}$ [d]	649.6

Net energy and liquid fuel Energy Contend are displayed (EC_j, where j denotes net energy, biodiesel, bioethanol or total liquid fuel energy, which is the sum of the biodiesel and bioethanol fuel energy values).

[a] According to data obtained from Morales-Sánchez et al. [10], it was considered that starch comprised 90% of the produced carbohydrates. Bioethanol production was calculated under the following considerations: 1.10 $g_{GLC}\ g_{Starch}^{-1}$; 98% efficiency for enzymatic starch hydrolysis; and glucose to ethanol yield of 0.51 $g_{Ethanol}\ g_{GLC}^{-1}$ [16]. A bioethanol density (at 25ºC) of 0.785 kg L^{-1} and a heating value of 79.8 MJ Gal^{-1} were used [16].

[b] It was assumed that TAGs (tryacylglycerides) accounted for the 78% of the total fatty acids comprised in the lipid fraction, according to Morales-Sánchez et al. [10]. For biodiesel production, an alkaline transesterification efficiency of 98% of the available TAGs was considered [16]. A biodiesel heating value of 38 MJ kg^{-1} was considered [16].

[c] Calculations were made assuming an 80% (w/w) of lipids of the total DCW and the considerations of b.

[d] As a comparison the conversion of 1,500 g of glucose to ethanol, considering a maximum theoretical yield of 0.51 g_{EtOH}/g_{GLC} and a fermentation efficiency of 95% [30], is included.

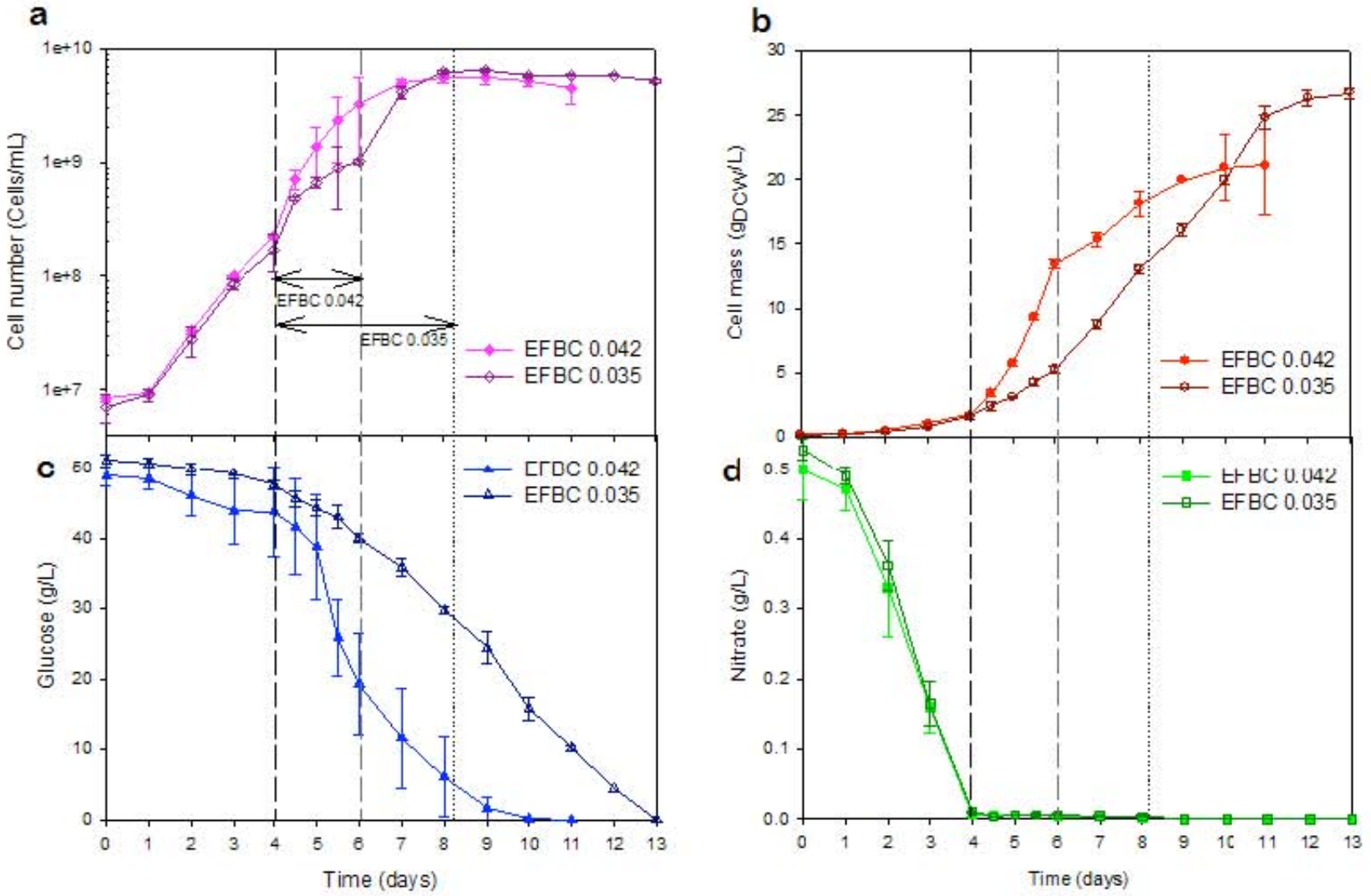
Figure legends

Figure 1. Exponentially fed-batch cultivation of *N. oleoabundans* in the EFBC 0.042 and the EFBC 0.035 The initial glucose concentration was about 50 g/L and nitrate was added in an exponentially way. The vertical dashed lines, black and gray, denote the time of initiation and termination of the feeding phase in the EFBC 0.042 and the black vertical dashed line and the gray dotted line correspond to the fedbatch phase in the EFBC 0.035. The experiments were performed in duplicate and the results in the figure show the average and standard error.

Figure 2. Reserve metabolite production in the three stages of the EFBC 0.042 and the EFBC 0.035. The determinations were performed in triplicate and the results in the figure show the average and standard deviation.

Figure 3. Fatty acid methyl ester profile in the EFBC 0.042 and the EFBC 0.035. The determinations were performed in triplicate and the results in the figure show the average and standard deviation.

590 Fig. 1

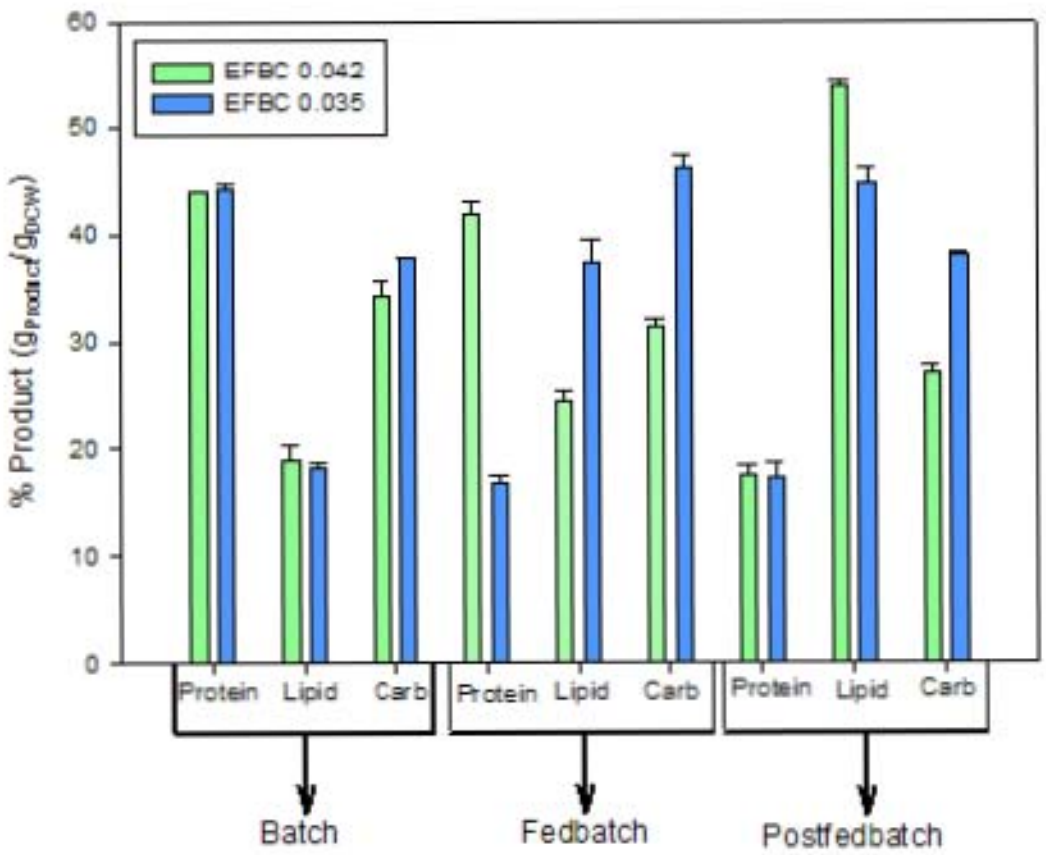


591

592

593 Fig. 2

594

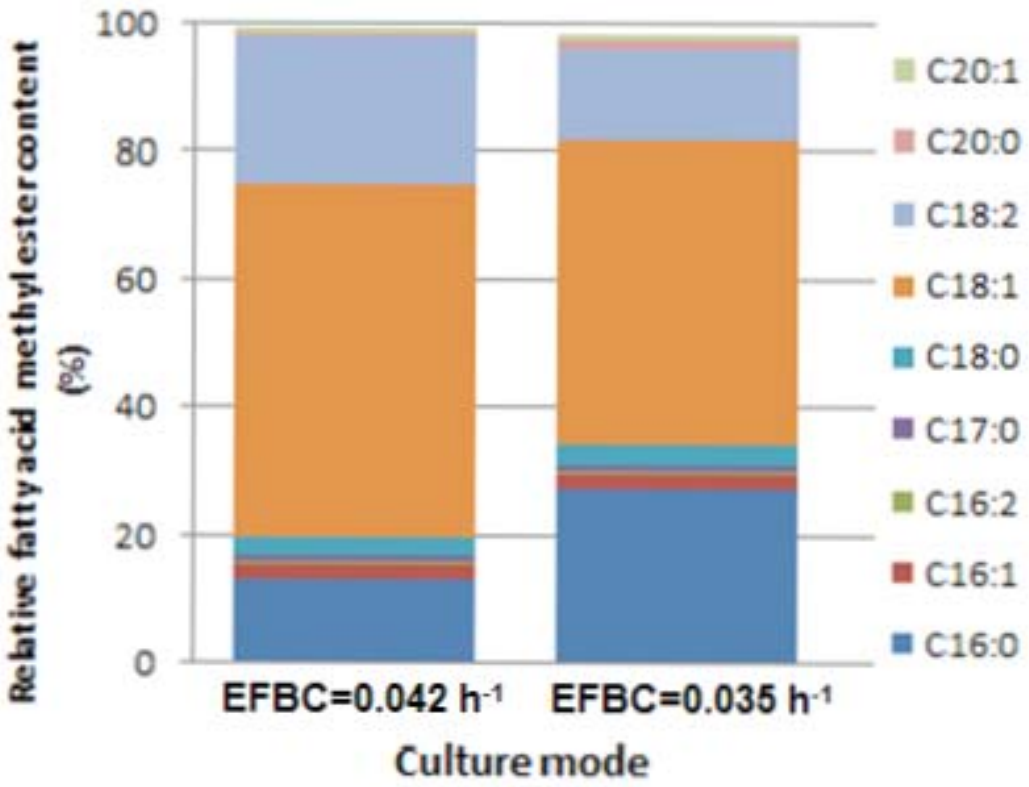


595

596

597 Fig 3

598



599

600

Acknowledgements

We thank Leobardo Serrano for technical support in the fermentation Pilot Plant of the Biotechnology Institute-UNAM. This work was supported by the Universidad Nacional Autónoma de México (UNAM), grant DGAPA/PAPIIT/UNAM IT200312 and the Consejo Nacional de Ciencia y Tecnología (CONACyT) – México, grant 213566. DMS held a scholarship from CONACyT.

HIGHLIGHTS

1. *Neochloris oleoabundans* was cultivated at 2 growth rates (μ) in fedbatch cultures
2. The exponential feeding strategy allowed to obtain high cell density mass
3. Post-fedbatch at a μ close to the maximum, N-limited, promotes lipid accumulation
4. A 20% reduction in the μ increases the accumulation of lipids and carbohydrates
5. Cultures at the lower μ cause an increase in the saturated fatty acids

VII.3 Manuscrito por Someter:

Lipid accumulation triggered by pyruvate dehydrogenase accumulation and high pentose phosphate pathway activation was revealed through proteomic studies in heterotrophic N-limited cultures of *Neochloris oleoabundans*

.

***Applied and Environmental* Microbiology 2014**

Manuscrito por someterse en febrero de 2014

Morales-Sánchez D., Tinoco R., Kyndt J., Ogden K. and Martínez A.

Lipid accumulation triggered by pyruvate dehydrogenase pool accumulation and high pentose phosphate pathway activation was revealed through proteomic studies in heterotrophic N-limited cultures of *Neochloris oleoabundans*

Daniela Morales-Sánchez[1], John Kyndt[2], Kimberly Ogden[3] and Alfredo Martínez[1]*

[1]Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Apdo. Postal 510-3, Cuernavaca, Morelos 62250, México.

[2]College of Science and Technology, Bellevue University. 1000 Galvin Road South, Bellevue, Nebraska 68005, United States of America.

[3]Department of Chemical and Environmental Engineering, College of Engineering, University of Arizona. 1133 E. James E. Rogers Way, Harshbarger 108, Tucson, Arizona 85721-0011, United States of America.

*Corresponding Author:
Alfredo Martinez
Tel: (52+777) 329 1601
Fax: (52+777) 317 2388
E-mail: alfredo@ibt.unam.mx

E-mail addresses:
DMS: dmorales@ibt.unam.mx
JK: John.Kyndt@bellevue.edu

KO: ogden@email.arizona.edu

Abstract

Neochloris oleoabundans is an oleaginous microalgae of biotechnological and commercial interest. Our previous work under strict heterotrophic cultivation indicated that this microalgae can accumulate lipids under nitrogen starvation in batch cultures, but is also able to accumulate carbohydrates under fedbatch cultures with nitrate pulse additions. Proteomic studies were carried out to compare the protein expression under two different scenarios, preferential lipid accumulation and preferential carbohydrate accumulation conditions.

Under long periods of nitrogen starvation, the lipid synthesis pathway was found highly activated. To supply precursors for lipid production, the pyruvate dehydrogenase protein was overexpressed. Under these conditions, the central metabolism pathways resulted highly activated to redirect the carbon flow towards the pyruvate dehydrogenase and ATP synthesis. Furthermore, the pentose phosphate pathway, specifically the enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were overexpressed to supply reducing power in the form of NADPH for lipid synthesis and inorganic nitrogen assimilation.

Carbohydrate synthesis related enzymes that channel carbon to starch and sugar synthesis such as UDP-glucose pyrophosphorilase and starch synthase were overexpressed when short durations of nitrogen limitation were encountered, as in the case of plants. However, the ADP-glucose pyrophosphorilase was overexpressed under preferential lipid accumulation conditions, indicating that under prolonged nitrogen

starvation conditions, this enzyme acts hydrolyzing the starch chains to channel the carbon flow to the lipid synthesis, a dual function that is already known in this protein.

These results have started to shed light on how coordinated metabolic processes redirect the flow of fixed carbon toward biosynthesis and storage of lipid and carbohydrate in response to the time of nitrogen starving conditions.

Keywords: *Neochloris oleoabundans*, heterotrophic growth, glucose, nitrogen-limitation, proteomics, lipid anabolism, carbohydrate anabolism.

1. Introduction

Microalgae are aquatic photosynthetic microorganism able to transform the carbon dioxide into biochemicals that can subsequently be processed into pharmaceuticals, cosmetics, nutraceuticals, functional food, biofuels and value bioactive compounds, such as: pigments, antioxidants, β-carotenes, polysaccharides, triglycerides, vitamins, ω-3 and ω-6 fatty acids, hydrocolloid alginates and, carrageenan (Guil-Guerrero et al. 2004; Meng et al. 2009; Barrow and Shahidi 2008; Pereira et al., 2011). Important advantages of oleaginous microalgae cultivations over oleaginous plants, such as Jatropha, soybeans and palm oil, include the use of photosynthetic process to convert sun energy into chemical energy, completing an entire growth cycle every few days (Patil et al. 2008; Mata et al 2010). Microalgae can be cultivated on non-arable land and, depending on the species, using fresh, saline or waste-water, also microalgae can

be propagated using auxotrophic, mixotrophic and heterotrophic cultures (Gouveia and Oliveira 2009; Perez-Garcia et al, 2009). However, despite the great potential of algae-based products, our understanding of algal metabolism is still largely inadequate (Guarnieri et al. 2011). The most promising oleaginous algal strains, thus far identified by growth experiments and lipid content screening, don't have sequenced or fully annotated genomes (Rismani-Yazdi et al. 2012). The lack of available genome sequence information limits the development of basic biological understanding required for strain improvement (Guarnieri et al. 2011). Rapidly developing post-genomic, system biology approaches such as transcriptomics, proteomics, and metabolomics have become essential for understanding how microorganisms respond and adapt to changes in their physical environment (Guarnieri et al. 2011). With microalgae, the proteomic studies had been mainly focused on model organisms that are not oleaginous but have sequenced genomes (Moellering and Benning 2010; Nguyen et al. 2011). There are a great number of oleaginous microalgae, with technological and commercial interest, from which the knowledge of their metabolisms is often scarce and are unsequenced. This is the case for the oleaginous microalgae *Neochloris oleoabundans*, that was studied some time ago (Tornabene et al. 1983; Kawata et al. 1998; Li et al. 2008; Gouveia and Oliveira 2009; Gouveia et al. 2009; Levine et al. 2010; Wan and Lan 2011; Wu et al. 2011; Murray et al. 2012; Sousa et al. 2013; Santos et al. 2013; Giovanardi et al. 2012) and recently reassessed under phototrophic-nitrogen limited conditions (Pruvost et al. 2009; Garibay-Hernández et al. 2013), including a proteomic study (Rismani-Yazdi et al. 2012) and also using heterotrophic conditions with glucose as carbon source (Morales-Sánchez et al. 2013). We had shown that *N. oleoabundans*

is able to grow under strict heterotrophic conditions using glucose as the only carbon source and by modifying the cultivation mode it was possible to change the biochemical composition of this microalgae: by using a high carbon/nitrogen ratio (glucose/nitrate; C/N=278) in batch cultures it was possible to significantly increase the specific lipid content (up to 52% of the dry weight); fedbatch cultures, with a high glucose concentration (50 g/L) and intermittent nitrate feeding, promoted the specific carbohydrate accumulation (up to 54% of the dry weight), mainly as starch; and balanced batch cultures with a C/N ratio of 17 led to a high percentage of proteins (up to 44% of the dry weight) (**figure 1**; Morales-Sánchez et al. 2013). Those cultivation studies let to the intriguing questions: what is the reason of the metabolism switch between the conditions that promote lipid or carbohydrate accumulation? A proteomic study can provide a broad overview of the important metabolic processes and could start to shed light on how coordinated metabolic processes redirect the flow to fixed carbon toward biosynthesis and storage of lipids and carbohydrates under different times of starvation.

Essentially, proteomics is a large scale comparative technique that reveals protein differential expression between two (or more) set of samples that differ in some variable (Simpson 2003; Rodriguez-Moya and Gonzalez 2010); this methodology with a proper analysis enables to obtain an integrated overview of cellular processes including metabolic pathways (Yu et al. 2012).

In the present study, we performed a study to compare the proteomes under lipid and carbohydrate accumulation conditions from heterotrophic cultures of the unsequenced microalga *N. oleoabundans* to elucidate the key proteins involved in the metabolic

pathway interactions and regulatory mechanisms involved in these two different conditions.

2. Materials and Methods

2.1 Algal strain and culture conditions

The details of culture conditions are described elsewhere (Morales-Sánchez et al. 2013); briefly, the edaphic eukaryotic green microalgae *Neochloris oleoabundans* strain UTEX 1185 was cultivated in 250-mL shake flasks (initial pH 7, 300 rpm, 25ºC), containing 100 mL of Bold's Basal mineral medium (BBM) supplemented with 10 g/L glucose, under dark conditions, for inocula preparation. The batch and fed-batch cultures, performed in bioreactors, were initiated with a 10% (v/v) inoculum, roughly equivalent to an initial cell number of 1 - 2 x 10^7 cells/mL or 0.4 g_{DCW}/L, and controlled at pH 7, 25 °C and an initial flow rates of 1 L of air / L of medium / min and a stirrer speed of 300 rpm; the dissolved oxygen was maintained above 20 % of air saturation by stepwise increments in the agitation speed and airflow rate as required. The analytical methods used for cell density, dry cell weight and glucose determination, as well as the methods for lipid, protein and carbohydrate extraction and determination were previously described (Morales-Sánchez et a. 2013).

2.2 Isolation of soluble protein fraction

From batch cultures (initial glucose 50 g/L and a C/N=278), that promoted the accumulation of lipids (as percentage of dry cell weight: lipids 51.7%, carbohydrates

33.3% and proteins 14.4%) and fedbatch cultures (initial glucose 50 g/L and intermittent nitrate feeding) that promoted the specific accumulation of carbohydrates (as percentage of dry cell weight: lipids 33.7%, carbohydrates 54.2% and proteins 11.6%) (**figure 1**), a total volume of 500-mL were harvested at the time of maximum cell mass produced, by centrifugation (14,000 x g, 5 min, 4 ºC). Cells pellets were resuspended in 50 mL of 50 mM Tris - 20 mM NaCl buffer (pH 7.5), homogenized to break up cell aggregates and frozen at -20 ºC overnight. To prevent protein degradation during the freezing, protease inhibitors were added before the freezing (Calbiochem cocktail V used in a 100x final concentration). Cell disruption was achieved using an automated French press (Sorvall-Ribi RF-1, Refrigerated Sorvall, Inc., Norwalk, Conn, USA) at a pressure of 20,000 psi, cell suspension was passed through it for twice. Cell debris were removed by ultracentrifugation (100,000 x g, 1 hour, 4 ºC) and the supernatant was collected at 4 ºC. Protein concentration was estimated using the Bradford Protein Assay (Bradford, 1976). The samples were then dialyzed against an 85% sucrose solution for 2 hours. In order to extract the proteins, HPLC grade acetone was added to the cell extracts in a ratio of 4 parts of acetone to one part of soluble protein solution, this mixture was stored for 24 hours at -20 ºC. The precipitate was collected by centrifugation (3,000 x g, 10 minutes, 4 ºC) and the pellet was washed three times with acetone. The protein pellet was air dried and resuspended in a buffer solution containing 7 M urea, 2 M thiourea, 4% CHAPS, and 30mM Tris at pH 9.1. A sample was run on a 1D SDS PAGE gel. Twenty µL of the extracted proteins were added to 20 µL of loading buffer (2x Laemmli buffer; Laemmli 1970): 1mL bromophenyl blue and 87 µL of the reducing agent ß-mercaptoethanol. The samples were then boiled for 10 minutes

and loaded into a precast 12% agarose gel (Bio-Rad Laboratories, Hercules, CA, USA). The protein pellet resuspended in the buffer solution was sent to the Proteomics facility at the University of Arizona for a LC-MS/MS analysis.

2.3 Proteome analysis

2.3.1 Clean-up

The algae protein samples were resuspended in a solution of 7M urea, 2M thiourea, 4% CHAPS, and 30 mM Tris of pH 9.1 after which ice cold acetone was added to 8x the volume, precipitating the protein. The precipitant was collected by centrifugation at 3,000 x g for 20 minutes at 4°C and the acetone was removed. The pellet was then air-dried. 500 µL of buffer 50mM Tris pH 9.7 was added and each sample was transferred to an eppendorf tube. To each tube 1 mL of phenol/chloroform/isoamyl alcohol solution was added. Each tube was vortexed for about 1 minute, and then centrifuged for 10 minutes at 15,000 x g. The solution was then splited into three layers, a chloroform layer, a precipitated protein layer, and a phenol layer. The chloroform and phenol layers were removed, pooled and centrifuged again, resulting in more precipitated protein product. The protein products were pooled and resuspended in urea buffer, and again precipitated in 8x volume of ice cold acetone. The protein pellet was again washed in acetone, and air dried. After this, the pellet was further cleaned using a 2D Clean-up kit following the manufacturers protocol (GE Heathcare).

2.3.2 LC-MS/MS

Figure 2 shows the overview of the proteomic LC-MS/MS experimental setup. The LC

run was used to separate peptides prior to MS/MS analysis on the LTQ Orbitrap Velos mass spectrometer.

Due to the lipids presents in the sample with lipid accumulation a delipidation of the sample was performed before the LC-MS/MS analysis. Lipids were extracted with chloroform-methanol as previously described (Zheleznova et al. 2012). Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) analysis was performed with 500 nanograms of trypsin-digested total protein, using a LTQ Orbitrap Velos mass spectrometer (Thermo fisher Scientific, San Jose, CA, USA), equipped with an Advion nanomate ESI source (Advion, Ithaca NY, USA) following ZipTip (Millipore, Billerica, MA, USA) C18 sample clean-up according to the manufacturer's instructions. Peptides were eluted from a C18 precolumn (100-µm id x 2 cm, Thermo Fisher Scientific) onto an analytical column (75-µm ID x 10 cm, C18, Thermo Fisher Scientific) using a 5% hold of solvent B (acetonitrile, 0.1% formic acid) for 5 minutes, followed by a 5-7% gradient of solvent B during 5 minutes, 7-15% gradient of solvent B over 45 minutes, 15-35% gradient of solvent B over 60 minutes, 35-40% gradient of solvent B over 28 minutes, 40-85% gradient of solvent B over 5 minutes, 85% hold of solvent B for 10 minutes and finally a return to 5% in 1 minute and another 10 minute hold of 5% solvent B. All flow rates were at 400 nL/min. Solvent A consisted of water and 0.1% formic acid. Data dependent scanning was performed by the Xcalibur v 2.1.0 software using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning *m/z* 400-1600, followed by collision-induced dissociation (DIC) tandem mass spectrometry (MS/MS) of the fourteen most intense ions in the linear ion trap analyzer. Precursor ions were selected by the monoisotopic precursor selection (MIPS) setting with selection or

rejection of ions held to a +/- 10 ppm window. Dynamic exclusion was set to place any selected *m/z* on an exclusion list for 45 seconds after a single MS/MS. All MS/MS spectra were searched against the Chlorophyta protein database downloaded October 26, 2012 from Uniprot (http://www.uniprot.org/taxonomy/3041) using Thermo Proteome Discoverer 1.3 (Thermo Fisher Scientific). At the time of the search, this Chlorophyta protein database contained 93,288 entries. Proteins were identified at 99% confidence with XCorr score cut-offs as determined by a reversed database search. The protein and peptide identification results were also visualized with Scaffold v 3.6.1 (Proteome Software Inc., Portland, OR), a program which uses Bayesian statistics to reliably identify more spectra. Proteins were accepted that passed a minimum of two peptides identified at 95% peptide confidence and 99.9% protein confidence by the Peptide and Protein Profit algorithms, respectively, within Scaffold.

The quantitative analyses were done with the spectrum counting method, which is the total number of MS/MS spectra matching peptides from a protein. The normalization was made using the normalized spectral abundance factor (NSAF) (Zybailov et al. 2006). The NSAF for a protein *k* is the number of spectral counts (SpC, the total number of MS/MS spectra) identifying a protein *k*, divided by the protein's length (L, in amino acids), divided by the sum of SpC/L for all N proteins in the experiment (Zybailov et al. 2006).

After analysis of each independent biological replicate (three replicate), a final dataset was generated that contained only proteins that were present in all three datasets. From the proteomic analysis it was obtained the abundance (percentage) of every protein according with the normalization mentioned before. We compared the

protein abundance loading equal amount of total protein for every sample (cpLa and cpCa) so that we can relatively say something of the abundance of any one common protein between samples. The fold change between cpLa and cpCa was obtained from the protein abundance of cpLa that was divided by the protein abundance of cpCa, so the value of the cpCa is always 1 and the expression level change up or down in cpLa. It is shown only the fold change that had significant difference between cpLa and cpCa ($p<0.5$).

3. Results

A total of 398 proteins were identified as differentially expressed between the cells from cultures that promotes Lipid accumulation (cpLa) and cultures that promotes Carbohydrate accumulation (cpCa). From this set of proteins, 34% were identified using the UniProt database against the MS/MS theoretical spectra of proteins reported from green microalgae (*Chlorphyta*) (**figure 3a**). The peptide sequences obtained from the MS/MS in this work that couldn't be identified in the *Chlorphyta* database were aligned to the peptide sequences from other algae, cyanobacteria and plants through a BLAST (Basic Local Alignment Search Tool, NCBI; **figure 3a**), 61% of proteins homologs were identified using this method. The remaining 5% were identified as uncharacterized proteins (**figure 1c**). The majority of the peptide sequences showed significant matches to other closely related green microalgae species (**figure 3b**) including *Chlorella variabilis* (62% of all peptide and spectra sequences), *Chlamydomonas reinhardtii* (16%), and *Volvox carteri* (4%).

In the following sections the major differences found in the relative protein abundances between cpLa and cpCa are compared for pathways related to: the lipid and nitrogen metabolism; carbohydrate metabolism; glycolysis, tricarboxylic acid cycle and pentose phosphate; photosynthesis; and other core pathways.

3.1 Lipid metabolism

Figure 4 shows the proteins involved in the fatty acid pathway that were identified in the UniProt and NCBI databases. The relative abundance (fold change) is indicated as a ratio with red numbers for cpLa and blue for cpCa. It can be seen that only five proteins involved in the biosynthesis of lipids are overexpressed in cpLa than in cpCa.

In cpLa the overexpression of the following proteins was found: the acyl carrier protein (ACP; 15-fold higher); the biotin carboxylase (BC: 6-fold higher), which is a component of the acetyl-CoA carboxylase complex that activates CO_2 by attaching it to nitrogen in a biotin ring in an ATP-dependent reaction (**figure 4a**), and three enzymes that are part of the multienzymatic complex of the fatty acid synthase (**figure 4b**), the enoyl-ACP reductase (ENR; 3-fold higher), which reduces the double bond of *trans-Δ^2-butenoyl-ACP* yielding butyril-ACP; the malonyl-CoA:ACP transacylase (MAT; 3-fold higher), which transfers the malonyl group from CoA to ACP; and the β-ketoacyl-ACP synthase (KAS; 2-fold higher), which acts in the first reaction in the synthesis of a fatty acid chain condensing the acyl and malonyl groups to yield acetoacetyl-ACP, an acetoacetyl group attached to ACP through the –SH group of the phosphopantetheine. But, the Acetyl-CoA carboxylase (ACC), a protein that previously was postulated as the

rate limiting step in the pathway, which is a heteromeric protein that catalyzes the first step in fatty acid biosynthesis transforming the acetyl-CoA into malonyl-CoA by addition of carbon dioxide, had a lower relative abundance (3.3-fold lower). The overexpression of BC, ACP, MAT, ENR and KAS, with the concomitant overexpression of several proteins from the glycolysis, the pentose phosphate pathway, the glyoxylate cycle and very high relative level of the pyruvate dehydrogenase (see below), suggest that the flux of glucose to acetyl-CoA and the priming of the initial fatty acid steps, catalyzed by the fatty acid synthase complex, controls the carbon flux towards the FA formation.

3.2 Nitrogen metabolism

Next the abundance of enzymes involved in nitrogen metabolism was compared. Proteins related to the nitrogen metabolism were overexpressed in cpLa compared to cpCa. The differentially expressed enzymes were the nitrate reductase (NR; 2-fold), which catalyzes NADPH reduction of nitrate to nitrite and serves as a central point for integration of metabolism by governing flux of reduced nitrogen by several regulatory mechanisms; glutamine synthase (GS; 5-fold), which catalyzes the formation of glutamine through the condensation of glutamate and NH_4^+ having as an intermediary γ-glutamil phosphate attached to the enzyme; glutamate synthase (3-fold), which catalyzes the formation of glutamate through the reductive amination of the α-ketoglutarate using glutamine as the nitrogen donor; glutamate dehydrogenase (3-fold), which converts α-ketoglutarate to glutamate and vice versa; and the nitrogen regulatory protein (8-fold), which plays a part in at least two independent responses to changes in the nitrogen status of the cell (**figure 5**): (1) the regulation of the activity of glutamine

synthetase (GS) by adenylylation/deadenylylation of the enzyme and (2) the regulation of expression from nitrogen-regulated (*nrt)* promoters by the phosphorylation/dephosphorylation of the transcriptional activator protein NtrC (Arcondéguy et al. 2001).

3.2 Carbohydrate metabolism

Under carbohydrate accumulation conditions (cpCa) the proteins UDP-glucose pyrophosphorylase (UGPase) and the starch synthase which are involved in the carbohydrate biosynthesis, were overexpressed compared to the cpLa condition (**figure 6**) but it is interesting to note that one of the most important proteins in the pathway, the glucose 1-phosphate adenylyltransferase (also called glucose pyrophosphorylase or AGPase) who catalyzes the addition of a phosphate group from ATP to form ADP-glucose, the glucosyl donor used by starch synthases, was overexpressed in cpLa than in cpCa. That also was observed for the proteins involved in the branching of the starch, such as the branching enzyme and the glucan branching isoamylase.

Some proteins involved in the breakdown of carbohydrates such as glucan 1,4-α-glucosidase, glycogen debranching protein and glucan 1,6-α-glucosidase (details of the pathway are not shown) were at least two-fold overexpressed in cpLa than in cpCa indicating that it was possible to direct this carbon source –possibly destined to the starch synthesis– to the fatty acid synthesis.

3.3 Central metabolism

Figure 7 shows an overview of the central metabolism and the expression level of proteins found in both conditions, cpLa and cpCa.

In cpLa we found an overexpression of proteins involved in the glucose catabolism through glycolysis, the pentose phosphate pathway, the glyoxylate cycle and the tricarboxylic acid cycle. The proteins overexpressed in the glycolysis were glucose 6-phosphate isomerase (2.5-fold), fructose 1,6-bisphosphate aldolase (2.3-fold), triose phosphate isomerase (3-fold), glyceraldehyde 3-phosphaste dehydrogenase (2.4-fold), which has a NADH generation reaction coupled, phosphoglycerate kinase (6-fold), which has an ATP generation reaction coupled, phosphoglycerate mutase (6-fold), enolase (12-fold) and, pyruvate kinase (2-fold), which has an ATP generation reaction coupled. In the case of the pentose phosphate pathway the proteins glucose 6-phosphate dehydrogenase (6-fold) and 6-phosphogluconate dehydrogenase (5-fold), both having a NADPH reaction coupled, transketolase (3-fold) and, ribose 5-phosphate isomerase (2-fold) were overexpressed in cpLa. The most remarkable difference between these two conditions was the significantly higher expression (22-fold) of the protein pyruvate dehydrogenase in cpLa, this protein is the first component enzyme of pyruvate dehydrogenase complex (PDHC) which transform the pyruvate into acetyl-CoA by a process called pyruvate decarboxylation. The glyoxylate cycle was apparently highly activated in cpLa in which an overexpression of proteins involved in this cycle was observed. Such proteins are aconitase (3-fold), succinyl-CoA synthase (3-fold), succinate dehydrogenase (2-fold), fumarase (5-fold), malate synthase (3-fold) and, isocitrate lyase (3-fold). Accordingly, isocitrate dehydrogenase and a-ketoglutarate dehydrogenase in the tricarboxylic acid cycle were underexpressed 0.59 and 0.14-fold, respectively.

3.4. Amino acid biosynthesis

Besides of being building blocks for proteins, amino acids are also key metabolic intermediates in living cells. Proteins that are involved in the biosynthesis of amino acids showed an overexpression in cpLa: Ornithine aminotransferase (3-fold), cysteine synthase (5-fold), phosphoglicerate dehydrogenase (2-fold), serine glioxylate aminotransferase (4-fold), diaminopimelate epimerase (4-fold), phosphoserine aminotransferase (3-fold), Δ-1-pymoline-5-carboxylate dehydrogenase (3-fold), ornithine carbamoyltrasnferease (4-fold), methylmalonate-semialdehyde dehydrogenase (3-fold), 4-hydroxyphenylpiruvate dioxygenase (2-fold), isopropylmalate synthase (2-fold). Only two proteins involved in amino acid biosynthesis were overexpressed in cpCa: threonine synthase (2-fold) and adenosyl homocysteine (2-fold). The amino acids that are synthesized by these proteins are Pro, Thr, Cys, Met, Ser, Gly, Lys, Glu, Arg, Phe, Tyr, Trp, Val, Leu, Ile, Asp and Arg.

3.5 Photosynthesis

Proteins involved in the photosynthesis process were found OVER-EXPRESED in both conditions. However, cpLa showed an overexpression of these proteins compared with cpCa. These proteins include: ferredoxin-NAD+ reductase (1.5-fold) which is an homologue of ferredoxin $NADP^+$ oxidoreductase, both are electronic transporters which transfer electrons from reduced ferredoxin to NAD^+ (Lehninger et al. 2013); photosystem II proteins (2-fold), which act in the reaction center P680 to drive electrons through cytochrome b_6f with concomitant movement of protons across the thylakoid membrane (Lehninger et al. 2013); photosystem I proteins (1.5-fold), which act

in the reactor center P700 to transport electrons to the Fe-S protein ferredoxin, then to $NADP^+$ yielding NADPH (Lehninger et al. 2013); thioredoxin reductase (1.5-fold), which is critical for redox regulation of protein function and signaling via thiol redox control and in the regulation of chloroplast photosynthetic enzymes (Arnér and Holmgren 2000); chlorophyll a/b binding protein (4-fold), which acts as light-harvesting complexes (Lehninger et al. 2013); ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) (3-fold), which catalyzes the covalent condensation of CO_2 and ribulose 1,5-bisphosphate and the rupture of the six carbon unstable intermediate, forming two molecules of 3-phosphoglicerate, one of which carriers the new carbon as CO_2 introduced in the carboxyl group (Lehninger et al. 2013); phosphoribulokinase (11-fold), which acts in the last step of the Calvin cycle regenerating ribulose 1,5-bisphosphate from ribulose 5-phosphate in an very exergonic reaction, as the phosphate anhydride bond in ATP is swapped for a phosphate ester in ribulose 1,5-bisphosphate (Lehninger et al. 2013); transketolase (2-fold), which is present in the regeneration of ribulose 1,5-bisphosphate, this protein contains thiamine pyrophosphate as its prosthetic group and requires Mg^{2+} and catalyzes the reversible transfer of a 2-carbon ketol group (CH_2OH-CO-) from a ketose phosphate donor (Lehninger et al. 2013); fructose 6-phosphate, to an aldose phosphate acceptor, glyceraldehyde 3-phosphate, forming the pentose xylulose 5-phosphate and the tetrose erythrose 4-phosphate (Lehninger et al. 2013); ribose 5-phosphate isomerase (2-fold), which catalyzes the isomerization of the ribose 5-phosphate to ribulose 5-phosphate in the third stage of the Calvin cycle (Lehninger et al. 2013); sedoheptulose 1, 7-bisphosphatase (3-fold), which converts the bisphosphate to sedoheptulose 7-phosphate (Lehninger et al. 2013).

3.6 Other pathways

In cpLa we found overexpressed GDP-mannose pyrophosphorylase (GMPP; 3-fold) who catalyses the synthesis of GDP-mannose, which is the precursor for mannose residues in galactomannan, glycoprotein and glycosylphosphatidylinositol (GPI) anchor. These GPI proteins are involved in morphogenesis and cell wall organization (de Jesus et al. 1989).

In cpCa were overexpressed proteins involved in the cell cycle such as elongation factor proteins (4-fold), cell cycle proteins (5-fold) and ribosomal proteins (9-fold). Elongation factors are a set of proteins that are used in protein synthesis in the cell. In the ribosome, they facilitate translational elongation together with the ribosomal proteins, from the formation of the first peptide bond to the formation of the last one (Berg et al. 2002).

In cpLa was an overexpression of ATP synthase (13.5-fold). ATP is the energy required for the metabolic activity and for biosynthesis of reserve metabolite. Fatty acid biosynthesis requires higher energy (7 ATP) than carbohydrate biosynthesis (4 ATP).

under both conditions (cpLa and cpCa), Expression differences in proteins that are related to the cell protection under stress were found in both conditions.. The heat shock protein Hsp was one of the most abundant proteins identified in this work, but the fold change in both cpLa and cpCa was not significantly different in between. This protein is involved in cellular protection under thermal stress and other kind of stress (Schorda et al. 2000). However, superoxide dismutase and catalase were overexpressed in cpLa in 9 and 6-fold, respectively.

Also in cpLa, it was an overexpression of proteins that are involved in the synthesis of antioxidants such as tocopherol cyclase (3-fold), pyridoxal biosynthesis lyase (10-fold) and L-ascorbate oxidase (2-fold).

4. Discussion

Lipid biosynthesis, catabolism and pathways that modify the length and saturation of fatty acids, have not been thoroughly investigated for algae as for terrestrial plants (Radakovits et al. 2010).

In this work we investigated the proteomic profile of *N. oleoabundans* cells obtained from two different culture strategies: lipid and carbohydrate accumulation conditions. In the lipid biosynthesis pathway it was found that the acyl carrier protein (ACP) and the protein biotin carboxylase were higher expressed in cpLa. The ACP is an essential cofactor in the synthesis of fatty acids and is part of the fatty acid synthase complex in plants (Radakovits et al. 2010). The biotin carboxylase protein is a component of the acetyl-CoA carboxylase complex (ACCase), which catalyzes the carboxylation of ACP and then transfer that carboxyl group through transcarboxylases to form malonyl-CoA (Radakovits et al. 2010). This protein is defined as the first enzyme in the actual fatty acid synthesis pathway and generally thought as the gateway to channel acetyl CoA into the fatty acid synthesis pathway.

On the other hand, the ACCase protein showed a higher expression in cpCa compared with cpLa. This result may seem contradictory since it has been reported that one of the first limiting steps in the fatty acid synthesis is the conversion of acetyl CoA to malonyl CoA, a reaction catalyzed by ACCase (Radakovits et al. 2010). However, it has

been demonstrated that a single protein is unable to activate or deactivate an entire metabolic pathway, the action of other components of the pathway is required, in this case the activation of other proteins involved in the fatty acid biosynthesis and the flow control in metabolic pathways is often shared by the activity of several enzymes (Radakovits et al. 2010).

Furthermore, there have been many efforts to increase the lipid content in several biological systems overexpressing the gene encoding the ACCase, but apparently the flux of this pathway is not simply controlled by this enzyme because it was not able to increase the content of lipids through this strategy (Radakovits et al. 2010). Dunahay and coworkers (1995) overexpressed the gene encoding to the ACCase in cells of the diatom *Cyclotella cryptica*. Although they were able to increase the activity of the ACCase, the lipid production was not increased (Sheehan et al. 2010).

Our results are consistent with the recent finds by Rismani-Yazdi and coworkers (2012). They performed a transcriptomic study from *N. oleoabundans* cells cultured phototrophically and under nitrogen limiting conditions; they found that while the gene encoding ACCase was repressed under this condition, the biotin containing subunit of ACCase, biotin carboxylase (BC) was significantly up-regulated in response to nitrogen starvation. In photosynthetic organisms, two different forms of ACCase have been identified, one located in the plastid and the other located in the cytosol. The plastidal ACCase is a heteromeric multi-subunit enzyme that contains BC, whereas the cytosolic ACCase is a homomeric multifunctional protein that does not contain BC (Rismani-Yazdi et al. 2012). In our proteome analysis we identified only one ACCase protein but we could not determine to which class of ACCase this protein belong. In the plastid –the

primary site of lipid biosynthesis in microalgae– we have observed a high relative abundance of the biotin carboxylase, the subunit heteromeric isoform that catalyzes the very first step of carboxylation. Possibly the overexpression of the ACCase observed in cpCa was due to the presence of cytosolic ACCase, where no lipid synthesis occurs regularly while the BC subunit observed in the LRS sample belongs to the plastid ACCase.

Some of the proteins with high expression in cpLa were the nitrate reductase (NR), an enzyme which catalyzes the reduction of nitrate to nitrite (Perez-Garcia et al. 2011), the Nitrogen regulatory protein II (NRP) and the glutamine synthetase (GS), which are interrelated to each other in the nitrogen metabolism. In cyanobacteria, the concentration and activity of glutamine synthetase is controlled in response to the availability of the nitrogen source (**figure 5**). PII is a tetrameric signal protein encoded by the *glnB* gene and is a component of the adenylation cascade involved in the regulation of the activity of glutamine synthetase. Under nitrogen limiting conditions, when the ratio of 2-ketoglutarate to glutamine decreases, the PII is phosphorylated at a serine residue to form PII-UMP. PII-UMP allows the deadenylation of glutamine synthetase and therefore its activation (Sibold et al. 1991). Since the isolation of the first *glnB* mutant, there have been indications that PII has a function beyond regulating ATase activity. Subsequent genetics studies with *E. coli*, *Salmonella enterica* serovar Typhimurium, *K. aerogenes,* and *Klebsiella pneumonia* identified a two-component nitrogen regulatory (*ntr*) system encoded by the *ntrBC* genes that was responsible for the global transcriptional control of enzymes of nitrogen assimilation and catabolism. The link between NtrBC and PII was demonstrated by further genetic studies in *E. coli*

that led to the proposal that PII modulated the activity of the histidine protein kinase sensor protein NtrB. This established PII as the key link between changes in the intracellular nitrogen status and the activity of the transcriptional activator protein NtrC (**figure 5**; Arcondéguy et al. 2001). Under physiological conditions, the uridylylation state of PII appears to be regulated mainly by the glutamine concentration, suggesting that the UTase/UR enzyme serves as an intracellular nitrogen sensor. This hypothesis is consistent with the constitutive expression of *glnD*, so that UTase/UR is always present in the cell and its activity is modulated in response o nitrogen availability, with the result that the uridylylation level of PII reflects the intracellular nitrogen status of the cell. The PII protein in *Arabidopsis thaliana* is encoded by the *GLB1* gene. The transcription of *GLB1* mRNA is induced by light or sucrose and is repressed by the amino acids asparagine, glutamine, and glutamate (Arcondéguy et al. 2001). One hypothesis that could explain the behavior of lipid-rich *N. oleoabundans* is that the microalgae was under nitrogen limiting conditions for a long time in cpLa (batch culture with C/N 278 ratio) and this caused the cell to redirection nearly all carbon source towards the synthesis of metabolites of reserve with higher energy like lipids and the nitrogen metabolism allowed a high catabolic activity in this pathway with the purpose of scavenge internal nitrogen reserves when the cell was in nitrogen starvation (Guarnieri et al. 2011).

These results suggest a high catabolic activity in response to a nitrogen limited condition, probably to take advantage of the nitrogen available in the environment and to scavenging of the internal nitrogen reserves (Guarnieri et al. 2011).

The carbohydrate metabolism is an essential part of this work because the fed-batch cultures obtained a high amount of carbohydrates (up to 50% DCW). The relevant behavior of this culture was that the addition of sodium nitrate pulses as a feed directed the carbon flow towards the synthesis of carbohydrates. Nitrate feeding was carried out when its concentration in the media decreased to approximate values of 100 mg/L, this way the culture was under nitrogen limiting conditions for a short time (Morales-Sánchez et al. 2013). It is believed that carbohydrates are probably preferred as reserve metabolites for a short term under nutritional stress conditions, as in the case of plants. In Arabidopsis, as in many vascular plants, starch plays an important role in the metabolism of carbohydrates in the day to day in leaves. The glucose produced during photosynthesis is stored mainly in the form of starch granules in the chloroplast and one of its functions is to change the availability of photosynthates obtained from the diurnal cycle of light and darkness. Starch accumulated during the day via the light reactions is degraded during the subsequent night in the Calvin cycle dark reactions providing a constant supply of carbon in the absence of photosynthesis. Therefore, the starch in plants can be seen as a pool of carbohydrate of short-term and is often known as a 'transitory starch' (Streb and Zeman 2012).

In the proteome analysis almost all the proteins involved in the carbohydrate synthesis had a higher relative abundance in cpCa (**figure 6**).

Although the pyrophosphorolytic reactions leading to the production of gluconeogenic intermediates such as ADP-glucose (ADPG, the universal starch precursor) and UDP-glucose (UDPG) are readily reversible, they mainly proceed toward the direction of nucleotide sugar synthesis (Preiss 1998). On the other hand, plant

enzymes that irreversibly cleave nucleotide sugars have been described that may effectively interrupt the flow of glycosyl moieties toward the biosynthesis of end products such as starch, cell wall polysaccharides or sucrose (Feingold and Avigad 1980). It is conceivable that in conjunction with AGPase, UDP-glucose pyrophosphorilase (UGPase), sucrose synthase, and starch synthase, among others, these enzymes will participate in controlling the levels of nucleotide sugars engaged in starch formation. Among a few enzymes reported to date that can hydrolyze nucleotide sugars in plants, AGPase is shown to catalyze the phosphorolityc breakdown of ADPG (Murata 1977). UGPase is known to split UDPG in the presence of a pyrophosphate and its activities are greatly stimulated by some signal metabolites, indicating that it may play an important role in the control of photosynthate partitioning (Gibson and Shine 1983).

We presume that the events mentioned above could be happening in cpLa and the high expression of AGPase could be leading to a high breaker activity of the starch chain toward the fatty acid formation or AGPase activity could hydrolyze nucleotide sugars to direct this carbon source to the fatty acid biosynthesis. Possibly, having the *N. oleoabundans* cells in a prolonged nutritional stress takes the carbon destined to the starch formation toward the fatty acid synthesis.

Pozueta-Romero et al. (1999) have proposed the operation of synthesis/breakdown metabolic cycles to control the rate of starch formation. According to this hypothesis, the balance between enzymatic activities catalyzing the synthesis of gluconeogenic intermediates and those activities catalyzing their breakdown can determine the net rate of starch synthesis (Rodríguez-López et al. 2000). Based in this possibility, Rodríguez-López et al. (2000) found a phosphodiesterasic activity that

catalyzes the hydrolytic breakdown of ADPG in a variant of AGPase and control the intracellular levels of ADGP linked to starch biosynthesis. Furthermore, studies of leaves and potato tubers have demonstrated that diurnally changed expression of genes involved in starch metabolism is not followed by the same diurnal fluctuation in protein levels in most cases and that the stimulation of starch synthesis depending on sugar level occurs in a short time window and involves the posttranslational redox activation of AGPase. The posttranslational mechanism should be key in the regulation of starch synthesis in response to diurnal changes in the sugar level (Yu et al. 2011).

In cpLa was obtained an overexpression of proteins involved in the glucose catabolism through glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. This indicates that the central metabolism pathways are highly activated to direct the carbon flow to the metabolic precursors synthesis that the cell needs under the stress condition. Probably these pathways were highly activated to consume the carbon source at a rate greater than the rate of cell generation, which would promote the conversion of the carbon excess into lipids (Pérez-Garcia et al. 2011).

This process is often accomplished in two steps: exponential cell division leading to decreased growth from limits of nutrients, thereby leading to accumulation of lipids (Leman 1997). It might not be only related to higher lipid-synthesizing enzymes under nitrogen starvation, but to the cessation of other enzymes associated with cell growth and proliferation and operation of enzymes specifically related to accumulation of lipids (Pérez-Garcia et al. 2011).

Synthesis of fatty acids needs a high amount of energy, 7 ATP and 14 NADPH are required for the formation of palmitate. All this energy could be obtained from the

tricarboxylic acid cycle and pentose phosphate pathway, which would explain these high proteins expression in these metabolic routes. The proteins present in these catabolic pathways are still in cpCa but in a lower expression than in cpLa, presumably because the carbohydrate synthesis needs less energy, 4 ATP and 2 NADH.

Moreover, the high expression of pyruvate dehydrogenase in cpLa suggests that the carbon flow can be directed (as far as possible) to the synthesis of acetyl-CoA or malonyl-CoA, which are biosynthetic precursors that provide of carbon skeleton to the fatty acid synthesis, among other pathways. This was also observed by Rismani-Yazdi and coworkers (2012) in a transcriptomic level in which genes associated with the pyruvate dehydrogenase complex for converting pyruvate to acetyl-CoA and lipases involved in the release free fatty acid from cell wall glycerophospholipids were overexpressed under nitrogen starvation conditions.

In **figure 7**, it can be seen that two of the enzymes acting in the TCA, isocitrate dehydrogenase and a-ketoglutarate dehydrogenase were underexpressed in cpLa, accordingly, two proteins involved in the glyoxylate cycle were highly expressed, malate synthase and isocitrate lyase indicating that, for this condition, the carbon flow was bypassed through the glyoxylate cycle. It is known that the glyoxylate cycle is activated when the concentration of intracellular fatty acid is high (Nogales et al. 2004). It is likely that these glyoxylate cycle proteins were overexpressed after later in the growth the fatty acid accumulation.

In cpLa, proteins related with the photosynthesis process were found in a higher expression compared with cpCa. This fact is intriguing because both, cpLa and cpCa, were cultured in strict dark conditions. These proteins include the ones presents in the

photosystems I (apolipoprotein 4Fe-4S) and II (CP43 chlorophyll apoprotein) and in the Calvin cycle (RuBisCo). However, it is known that under nitrogen starvation, accumulation of lipids is attributed to mobilization of lipids from chloroplast membranes, as a source of chloroplastic nitrogen, and is relocated by RuBisCo (Pérez-Garcia et al. 2011). This hypothesis is supported by the fact that development of chloroplasts is dependent on nitrogen. Chloroplast breakdown for the internal supply of nitrogen under nitrogen reduction in dark conditions leads to cell survival and growth in the face of prolonged nitrogen shortage (Pérez-Garcia et al. 2011).

It is known that under heterotrophic microalgal growth the photosynthetic pathways remain quite active without an important participation in the process of glucose metabolism (Pérez-Garcia et al., 2011).

Studies on the effect of repeated (and long-term) propagation in the absence of light and using an organic carbon/energy source such as glucose, ethanol and glycerol, for the production of substances involved in the photosynthetic apparatus of microalgae like pigments are not yet conclusive (Chen and Chen 2006). Some of these substances continue to be synthesized in the dark, for example, the light-harvesting pigment phycocyanin from *Galdieria sulphuraria* (Bumbak et al. 2011). It appears that the proteins involved in the photosynthesis to produce some of these light-dependent metabolites are constitutively produced in the cell despite the dark conditions. This is consisted with our observation that our dark cultures are still colored and the presence of photosynthesis related proteins in our proteomics experiments.

In cpLa we identified an overexpression of the protein GDP-mannose pyrophosphorylase (GMPP) which catalyzes the synthesis of GDP-mannose. GMPP is

involved in morphogenesis and cell wall organization (Jiang et al. 2008). It has been reported that the repression of GMPP in yeast leads to phenotypes including cell lysis, defective cell wall, and failure of polarized growth and cell separation (Jiang et al. 2008). It is possible that this protein has been synthesized to keep the granules of lipids within the cells by reinforcing the cell wall.

In addition it was found that proteins involved in the cell division cycle have a higher relative abundance in cpCa. The cell division cycle proteins have a kinase activity and it has been suggested that these proteins are essential for initiation of DNA replication and they play a role in regulating cell cycle progression (Berg et al. 2002). Since cpCa obtained a higher biomass in terms of cell number it makes sense that this protein was found in a higher expression than in the other condition.

Proteins related to the cell protection under stress condition were highly expressed in both conditions, cpLa and cpCa. It is suggested that these stress related proteins were strongly expressed to deal with nutritional stress and other kind of stresses possibly present in the system. However, only cpLa showed a higher expression of catalase and super oxide dismutase that are involved in oxidative stress (de Jesus et al. 1989). The same oxidative stress could explain why we found higher expression levels of proteins that are involved in the synthesis of antioxidants such as tocopherol. The main antioxidant functions that tocopherols plays in photosynthetic organisms are thought to be in the scavenging of lipid peroxy radicals and in quenching O_2 (Sakuragi et al. 2006). Perhaps these kinds of proteins were overexpressed in cpLa to avoid the lipid oxidation.

It is important to point out that our proteome results are in agreement with the transcriptomic studies done by Rismani-Yazdi and coworkers (2012) in phototrophic cultures of *N. oleoabundans* under nitrogen limiting conditions. All those experiments were performed with phototrophic cultures, as compared to the heterotrophic dark condition carried out in our work. There is a great similarity between the expression levels of genes involved in the fatty acid and carbohydrate synthesis and in the central metabolism, and the protein expression found in cpLa for the same metabolic pathways although these two studies can be complementary and usefully to understand more about the metabolism of the unsequenced *N. oleoabundans.*

5. Conclusions

Proteomics studies of unsequenced algae can provide a broad overview of the important metabolic processes from which we can design hypotheses that can guide future detailed studies on improving lipid or carbohydrate accumulation.

Our results have started to shed light on how coordinated metabolic processes redirect the flow of fixed carbon toward biosynthesis and storage of lipids under nitrogen starvation.

Under long periods of nitrogen starvation (cpLa), the lipid synthesis pathway is highly activated. To supply this pathway precursor, pyruvate dehydrogenase was overexpressed. Under these conditions, the central metabolic pathways were highly active to redirect the carbon, if possible, to the synthesis of pyruvate dehydrogenase and ATP synthesis. Additionally, the pentose phosphate pathway, specifically glucose

6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were overexpressed to supply reducing power in the form of NADPH to lipid synthesis and inorganic nitrogen assimilation.

Enzymes associated with the carbohydrate synthesis such as UDP-glucose pyrophosphorylase and starch synthase were overexpressed to contend with short periods of nitrogen limitation (cpCa), as in the case of plants. However, the enzyme ADP-glucose pyrophosphorylase was overexpressed under conditions of preferential lipid accumulation, indicating that under prolonged nitrogen starvation, this enzyme acts hydrolyzing starch chains to channel the carbon flow towards the lipid synthesis, double function that is already known of this protein.

This proteomic study is a first approach to unravel the lipid and carbohydrate metabolism in an unsequenced microalgae, such as *N. oleoabundans*. Comparing the differential protein expression under two different metabolite accumulation conditions, it was possible to identify key proteins that appear to regulate the pathways of lipid and carbohydrate synthesis as well as the central pathways of carbon and the energy flow in *N. oleoabundans.*

Only a fraction of algal species have been sequences to date, however the proteomics results described in this paper illustrate that a significant amount of insight in metabolic pathway regulation can be obtained from a proteomic study on an algal species for which no genome sequence and annotation is available. So far, this is the first proteomic study of the microalgae *N. oleoabundans* however this work is complementary and confirmatory to a transcriptomics study of the metabolism of this

microalga under nitrogen starvation, even though the latter was performed under phototrophic conditions.

Acknowledgements

We thank Andrea Hunt and George Tsaprailis from the Proteomics Facility of the University of Arizona for their technical support with proteomics experiments. We thank Mario Caro for technical support in the Fermentation Pilot Plant of the Biotechnology Institute – UNAM. This work was supported by the University of Arizona, grant XXXXX XXXX XXXX; and the Universidad Nacional Autónoma de México (UNAM), grant DGAPA/PAPIIT/UNAM IT200312. DMS held a scholarship from The Mexican Council of Science and Technology CONACyT.

References

Arcondéguy T, Jack R, Merrick M. 2001. PII signal transduction proteins, pivotal players in microbial nitrogen control. Microbiol. Mol. Biol. Rev. **65**(1):80-105.

Arnér ES, Holmgren A. 2000. Physiological functions of thioredoxin and thioredoxin reductase. Eur. J. Biochem. **267**: 6102-6109.

Berg JM, Tymoczko JL, Stryer L. 2002. Biochemistry. Freeman WH ed. 5th ed, New York.

Bradfor MM. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.

Bumbak F, Cook S, Zachleder V, Hauser S, Kovar K. 2011. Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. App. Microbiol. Biotechnol. **91**:31-46.

Chen GQ, Chen F. 2006. Growing phototrophics cells without light. Biotechnol. Lett. **28**:607-616.

de Jesus MD, Tabatabai F, Chapman DJ. 1989. Taxonomic distribution of copper-zinc superoxide dismutase in green algae and its phylogenetic importance. J. Phycol. **25**(4): 767-772.

Dunahay TG, Jarvis EE, Roessler PG. 1995. Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. J. Phycol. **31**:1004-1011.

Feingold DS, Avigad G. 1980. *in* The biochemistry of plants. Preiss J. ed. Academic New York. **3**: 101-170.

Garibay-Hernández A, Vázquez-Duhalt R, Serrano-Carreón L, Martinez A. 2013. Nitrogen limitation in *Neochloris oleoabundans*: A reassessment of its effect on cell growth and biochemical composition**.** Appl. Biochem. Biotechnol. 10.1007/s12010-013-0454-1.

Gibson DM, Shine WE. 1983. Uridine disphosphate glucose breakdown is mediated by a unique enzyme activated by fructose 2,6-bisphosphate in *Solanum tuberosum*. Proc. Natl. Acad. Sci. USA 1983, **80**:2491-2494.

Giovanardi M, Ferroni L, Baldisserotto C, Tedeschi P, Maietti A, Pantaleoni L, Pancaldi S. 2012. Morphophysiological analyses of Neochloris oleoabundans (Chlorophyta) grown mixotrophically in a carbon-rich waste product. Protoplasma. DOI 10.1007/s00709-012-0390-x

Gouveia L, Oliveira A. 2009. Microalgae as a raw material for biofuels production. J. Ind. Microbiol. Biotechnol. **36**: 269-274.

Gouveia L, Marques A, da Silva T, Reis A. 2009. *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. J. Ind. Microbiol. Biotechnol. **36**:821-826

Guarnieri MT, Nag A, Smolinski SL, Darzins A, Seibert M, Pienkos PT. 2011. Examination of triacylglycerol biosynthetic pathway via De novo transcriptomic and proteomic analyses in an unsequenced microalga. PLoS ONE. **6**(10): e25851.

Jiang H, Ouyang H, Zhou H, Jin C. 2008. GDP-mannose pyrophosphorylase is essential for cell wall integrity, morphogenesis and viability of *Aspergillus fumigatus*. Microbiol. **154:** 2730-2739.

Kawata M, Nanba M, Matsukawa R, Chihara M, Karube I. 1998. Isolation and characterization of a green alga *Neochloris sp.* for CO_2 fixation. Stud. Surf. Sci. Catal. **114**:637-640.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

Lehninger A, Nelson DL, Cox MM. 2013. Lehninger principles of biochemistry. Freeman WH ed. 6th ed, New York.

Leman J. 1997. Oleaginous microorganisms: and assessment of the potential. Adv. Appl. Microbiol. **43**:195-243.

Levine RB, Constanza-Robinson MS, Spatafora GA. 2010. *Neochloris oleoabundans* grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production. Biomass. Bioenerg. **35**(1): 40-49.

Li YQ, Horsman M, Wang B, Wu N, Lan C. 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl. Microbiol. Biotechnol. **81**(4): 629-636.

Mata TM, Martinis AA, Caetano NS. 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sust. Energ. Rev. **14**: 217-232.

Moellering ER, Benning C. 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii.* Eukaryot. Cell. **9**:97-106.

Morales-Sánchez D, Tinoco-Valencia R, Kyndt J, Martínez A. 2013. Heterotrophic growth of *Neochloris oleoabundans* using glucose as a carbon source. Biotechnol. Biofuels. **6**: 100.

Murata T. 1977. Partial purification and some properties of ADP-glucose phosphorylase from potato tubers. Agric. Biol. Chem. **41**:1995-2002.

Murray KE, Shields JA, Garcia ND, Healy FG. 2012. Productivity, carbon utilization, and energy content of mass in scalable microalgae systems. Biores. Tech. **114**: 499-506.

Nguyen HM, Baudet M, Cuine S, Adriano JM, Barthe D, Billon E, Bruley C, Beisson F, Peltier G, Ferro M, Li-Beisson Y. 2011. Proteomic profiling of oil bodies from the unicellular green microalga *Chlamydomonas reinhardtii* with focus on proteins involved in lipi metabolism. Proteomics. **11**:4266-4273.

Nogales J, Guijo MI, Quesada A, Merchan F. 2004. Functional analysis and regulation of the malate synthase from *Chlamydomonas reinhardtii.* Planta. **219**:325-331.

Patil V, Tran KQ, Giselrod HR. 2008. Towards sustainable production of biofuels from microalgae. Int. J. Mol. Sci. **9**:1188-1195.

Pereira H, Barreira L, Mozes A, Florindo C, Polo C, Duarte CV, Custódio L, Varela J. 2011. Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae. Biotechnol. Biofuels. **4**:61.

Perez-Garcia O, Escalante FME, de-Bashan LE, Bashan Y. 2011. Heterotrophic cultures of microalgae: metabolism and potential products. Water. Res. **45**(1):11-36.

Pozueta-Romero J, Peralta P, Akazawa T. 1999. Sucrose-starch conversion in heterotrophic tissues. Crit. Rev. Plant. Sci. **18**:489-525.

Preiss J. 1998. *in* The biochemistry of plants. Preiss J ed. Academic New York. **14**:181-254.

Pruvost J, Van Vooren G, Cogne G, Legrand J. 2010. Investigation of biomass and lipids production with *Neochloris oleabundans* in photobioreactor. Biores. Technol. **100**(23): 5988-5995.

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC. 2010. Genetic engineering of algae for enhanced biofuel production. Eukaryotic. Cell. **9**(4): 486-501.

Rismani-Yazdi H, Haznedaroglu BZ, Hsin C, Peccia J. 2012. Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation. Biotechnol. Biofuels. **5**:74.

Rodríguez-López M, Baroja-Fernández E, Zandueta-Criado A, Pozueta-Romero J. 2000. Adenosine diphosphate glucose pyrophosphatase: a plastidial phosphodiesterase that prevents starch biosynthesis. PNAS. **97**(15): 8705-8710.

Rodríguez-Moyá M, Gonzalez R. 2010. Systems biology approaches for the microbial production of biofuels. Biofuels. **1**(2):291-310.

Sakuragi Y, Maeda H, DellaPenna D, Bryant DA. 2006. α-tocopherol plays a role in photosynthesis and macronutrient homeostasis of the cyanobacterium *Synechocystis* sp. PCC 6803 that is independent of its antioxidant function. Plant. Physiol. **14**(2):508-521.

Santos AM, Lamers PP, Janssen M, Wijffels RH. 2013. Biomass and lipid productivity of *Neochloris oleoabundans* under alkaline-saline conditions. Algal Res. **2**: 204-2011.

Schroda M, Blöcker D, Beck CF. 2000. The HSP70A promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. Plant J. **21**(2):121-131.

Sheehan JT, Dunahay T, Benemann J, Roessler P. 2010. A look back at the US Department of Energy's Aquatic Species Program-biodiesel from algae. Report no. NREL/TP-580-24190. National Renewable Laboratory, Golden, Colorado.

Sibold L, Henriquet M, Possot O, Aubert JP. 1991. Nucleotide equence of nifH regions from Methanobacterium ivanovii and Methanosarcina barkeri 227 and characterization of glnB-like genes. Res. Microbiol. **142**(1): 5-12.

Simpson RJ. 2003. Introduction to proteomics. *In*: Proteins and proteomics. A laboratory manual. Cold Spring Harbor Laboratory Press. ISBN 0-87969-553.

Sousa C, Compadre A, Vermuë MH, Wijffel RH. 2013. Effect of oxygen at low and high light intensities on the growth of *Neochloris oleoabundans***.** Algal Res. **2**:122-126.

Streb S, Zeeman SC. 2012. Starch metabolism in Arabidopsis. *In*: The Arabidopsis book.10: e0160. doi: 10.1199/tab.0160.

Tornabene T, Holzer G, Lien S, Burris N. 1983. Lipid composition of the nitrogen starved green alga *Neochloris oleabundans.* Enzyme. Microb. Tech. **5**: 435-440.

Wang B, Lan CQ. 2011. Biomass production and nitrogen and phosphorous removal by the green alga *Neochloris oleoabundans* in simulated wastewater and secondary municipal wastewater effluent. Bioresour. Technol. **102**(10): 5639-5644.

Wu N, Li Y, Lan C. 2011. Production and rheological studies of microalgal extracellular biopolymer from lactose using the green alga *Neochloris oleobundans*. J. Polym. Environ. **19**: 935-942.

Yu HT, Xu SB, Zheng CH, Wang T. 2012. Comparative proteomic study reveals the involvement of diurnal cycle in cell division, enlargement, and starch accumulation in developing endosperm of *Oryza sativa.* J. Proteome. Res. **11**: 359-371.

Zheleznova NN, Yang C, Ryan RP, Halligan BD, Liang M, Greene AS, Cowley Jr. AW. 2012. Mitochondrial proteomic analysis reveals deficiencies in oxygen utilization in medullary thick ascending limb of Henle in the Dahl salt-sensitive rat. Physiol. Genomics. **44**:829-842.

Zybailov B, Mosley A, Sardiu M, Coleman M, Florens L, Washburn M. 2006. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. J. Proteome Res**. 5**:2339-2347**.**

Figure legends

Figure 1. Physiological response of *N. oleoabundans* under two strategies of heterotrophic cultivation. A) Cultures that promotes the lipid accumulation (cpLa): Batch cultures with 50 g/L of glucose and 0.5 g/L of sodium nitrate (C/N = 278). B) Cultures that promotes the carbohydrate accumulation (cpCa): Fed-batch cultures with intermitten nitrate additions and an initial C/N ratio of 278.

Figure 2. Overview of the proteomic LC-MS/MS experimental setup.

Figure 3. A) Distribution of proteins indentified using the inidicated databases and B) top-hit distribution of proteins from a total of 398 differentially expressed proteins found in the proteme of *N. oleoabundans* from cpLa and cpCa using Uniprot and BLAST-NCBI matches.

Figure 4. A) The acetyl-CoA carboxylase reaction. Acetyl-CoA carboxylase has three functional regions: biotin carrier protein (blue); biotin carboxylase, which activates CO_2 by attaching it to a nitrogen in the biotin ring in an ATP-dependent reaction; and transcarboxylase which transfers activated CO_2 from biotin to acetyl-CoA, producing malonyl-CoA. The long, flexible biotin arm carriers the activated CO_2 from the biotin carboxylase region to the transcarboxylase active site. The active enzyme in each step is shaded in green (adapted from Lehninger et al. 2013). B) Overview of the fatty acid biosynthesis pathway in green algae. The numbers represent the fold change in the

protein expression. Numbers in red are for cpLa and numbers in blue for cpCa. PHD: pyruvate dehydrogenase; ACCase: Acetyl CoA carboxylase; MAT: Malonyl-CoA transacylase; KAS: β-ketoacyl-ACP synthase; KAR: β-ketoacyl-ACP reductase; HD: β-hydroxyacyl-ACP dehydrase; ENR: Enoyl-ACP reductase.

Figure 5. Nitrogen regulation (Ntr) system. The activities of both GS and NtrC are regulated in response to the intracellular nitrogen status. UTase (*glnD* product) catalyzes the uridylylation and deuridylylation of PII (*glnB* product). ATase catalyzes the adenylylation and deadenylylation of GS. NtrB catalyzes the phosphorylation and dephosphorylation of NtrC (taken from Arcondéguy et al. 2001).

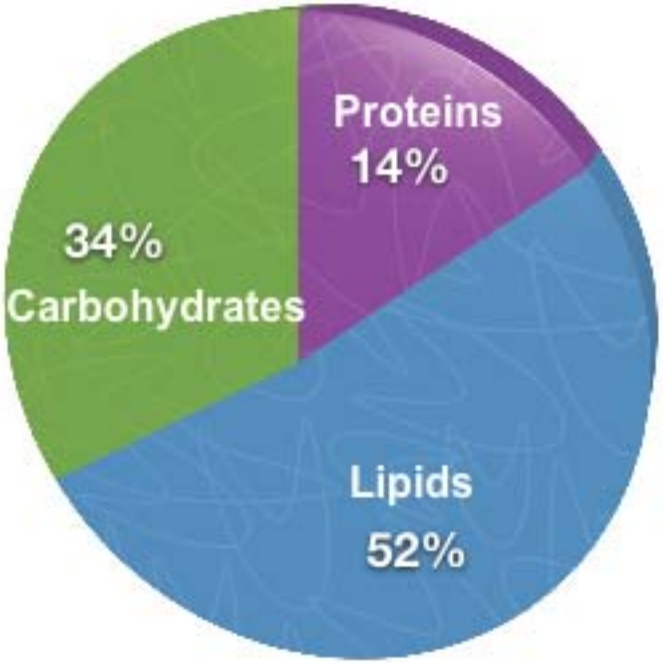
Figure 6. Overview of the carbohydrate biosynthesis. The numbers represent the fold change in the protein expression. Numbers in red are for cpLa and numbers in blue for cpCa. CS: Cellulose synthase; UGPase: UDP-glucose pyrophosphorylase; MUT: Mutase; AGPase: ADP-glucose pyrophosphorylase; SS: Starch synthase; BE: Branching enzyme; GBI: Glucan branching enzyme.

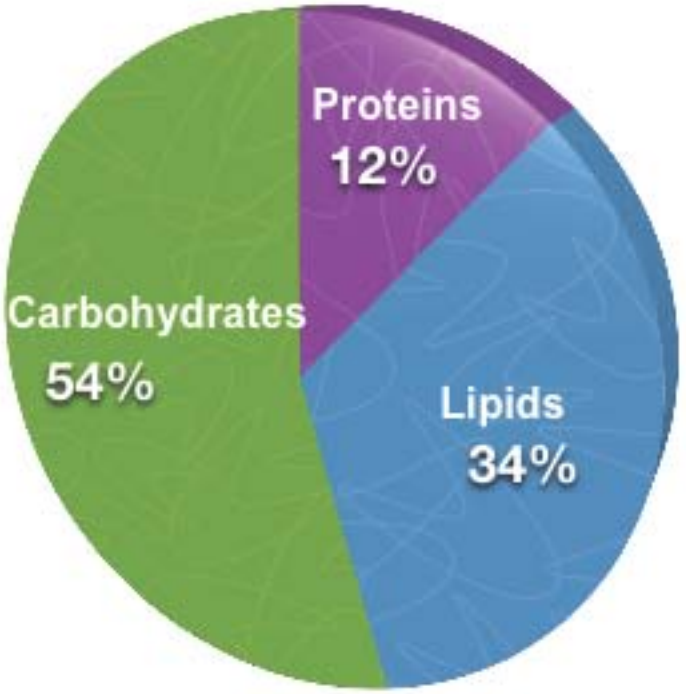
Figure 7. Overview of the central metabolism. The numbers represent the fold change in the protein expression. Numbers in red are for cpLa and numbers in blue for cpCa. NR: Nitrate reductase; NiR: Nitrite reductase; HK: Hexokinase; GPI: Glucose 6-phosphate isomerase; PFK: Phosphofructokinase; ALDO: Aldolase; TPI: Triose phosphate isomerase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PGK: Phosphoglycerate mutase; PGM: Phosphoglycerate mutase; EN: Enolase; PK:

Pyruvate kinase; PHD: Pyruvate dehydrogenase complex; GDH: Glucose 6-phosphate dehydrogenase; PGL: 6-Phosphogluconolactonase; PGD: 6-Phosphogluconate dehydrogenase; RPI: Ribulose 5-phosphate isomerase; RPE: Ribulose 5-phosphate epimerase; TK: Transketolase; MS: Malate synthetase; ICL: Isocitrate lyase; CITS: Citrate synthase; ACT: Aconitase; ID: Isocitrate dehydrogenase; KD: α-ketoglutarate dehydrogenase; SUS: Succinyl-CoA synthetase; SUD: Succinic dehydrogenase; FUM: Fumarase; MD: Malate dehydrogenase.

Figure 1

A) Cultures that promotes the lipid accumulation (cpLa). Batch cultures with 50 g/L glucose and 0.5 g/L sodium nitrate (C/N = 278).





B) Cultures that promotes the carbohydrate accumulation (cpCa). Fed-batch cultures with intermittent nitrate additions and an initial C/N ratio of 278, 50 g/L glucose.

Figure 2

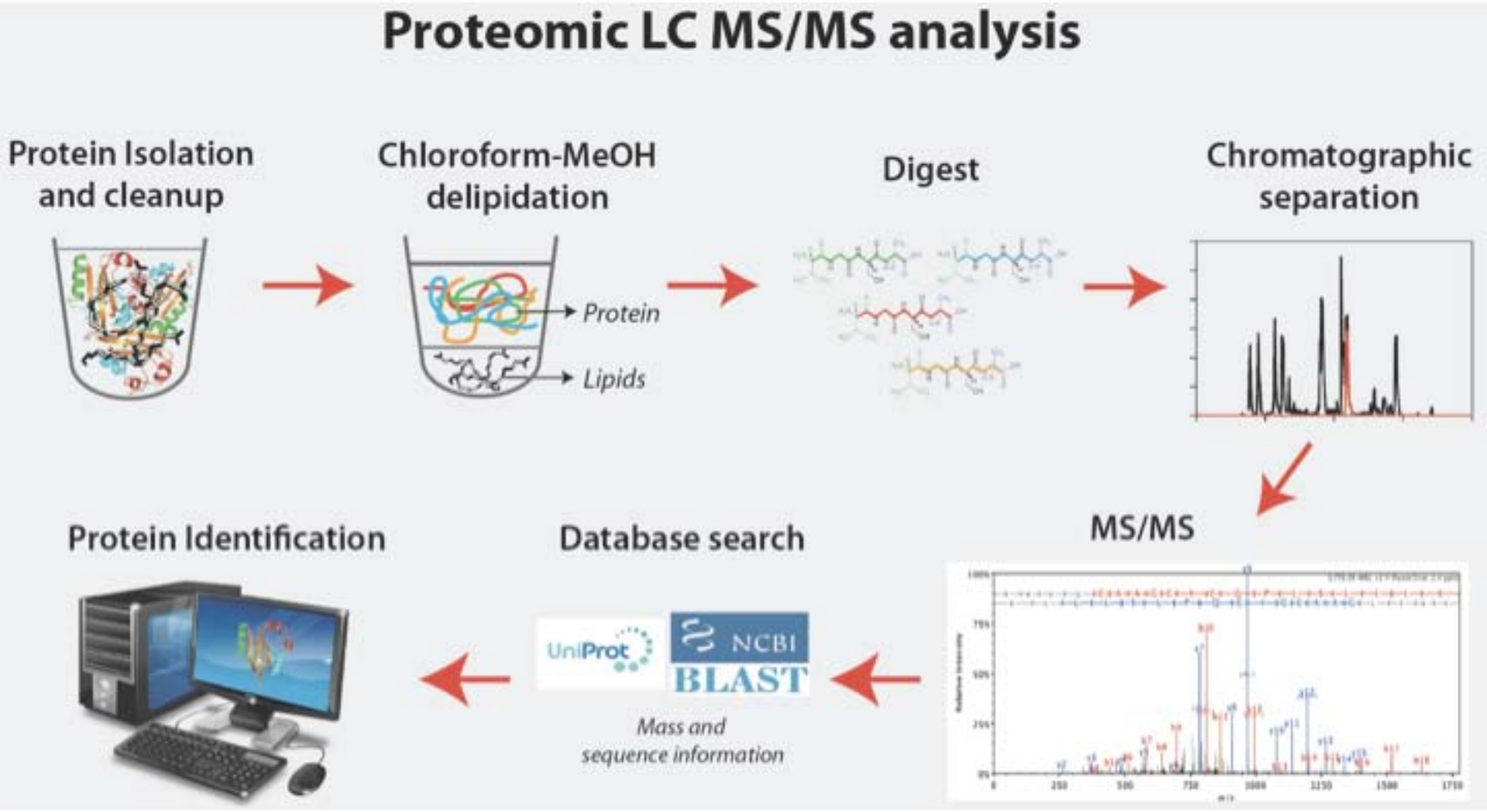

Proteomic LC MS/MS analysis
Protein Isolation and cleanup
Chloroform-MeOH delipidation
Protein
Lipids
Digest
Chromatographic separation
MS/MS
Database search
UniProt
NCBI
BLAST
Mass and sequence information
Protein Identification

Figure 3

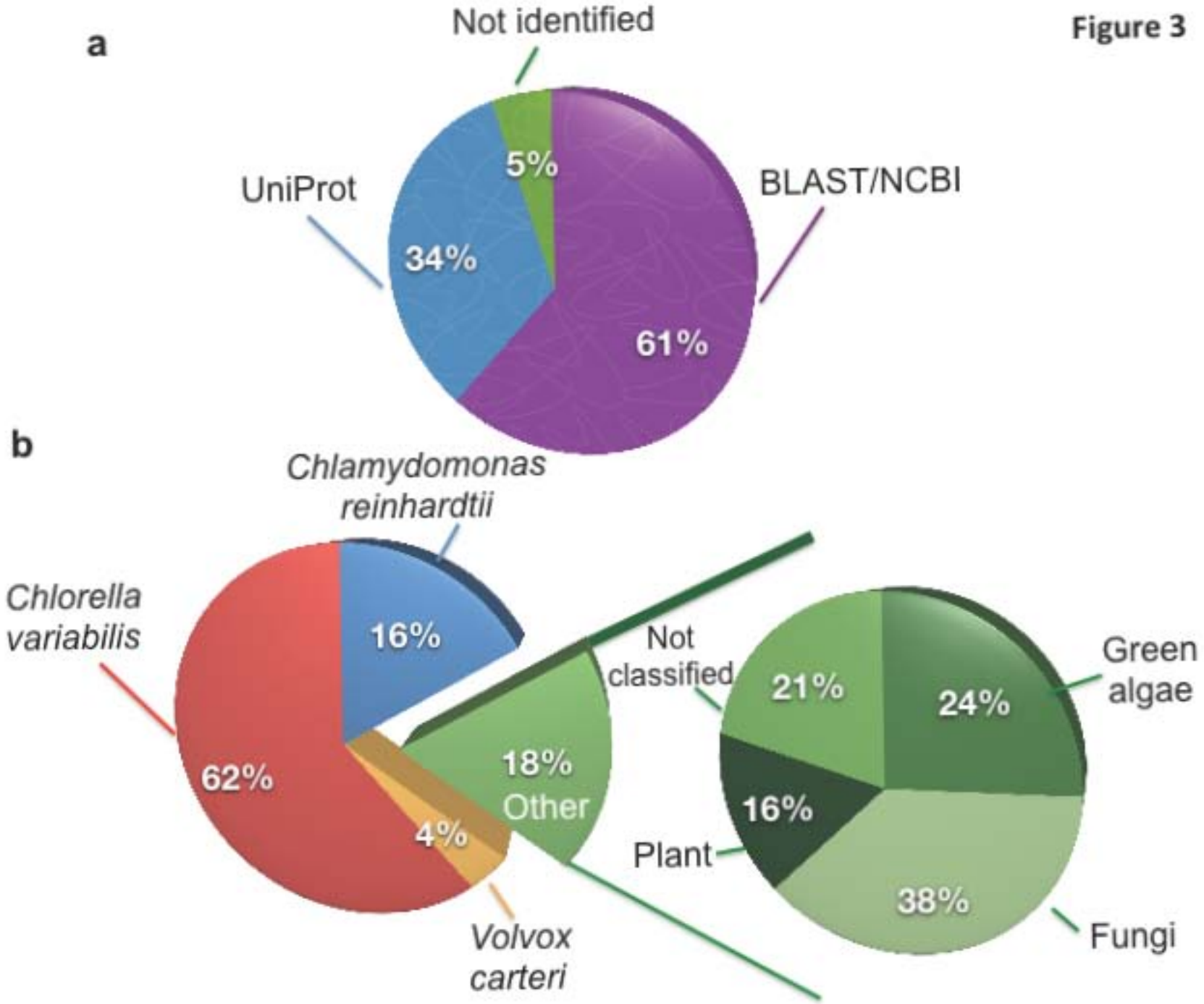

a
Not identified
5%
UniProt
34%
BLAST/NCBI
61%
b
Chlamydomonas reinhardtii
16%
Chlorella variabilis
62%
4%
Volvox carteri
18% Other
Not classified
21%
24%
Green algae
16%
Plant
38%
Fungi

Figure 4a

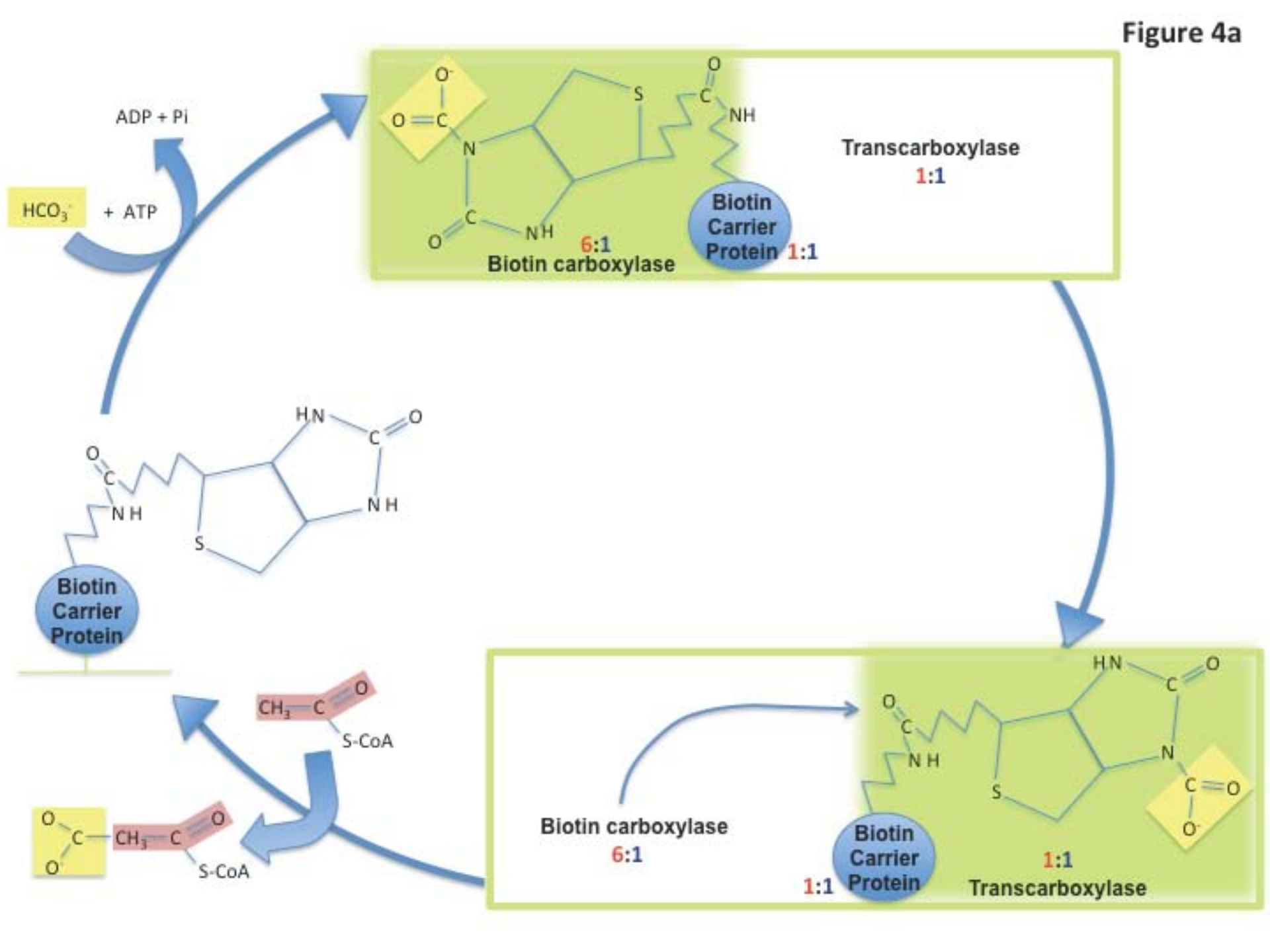

ADP + Pi
HCO3- + ATP
Biotin carboxylase
6:1
Biotin Carrier Protein 1:1
Transcarboxylase
1:1
Biotin Carrier Protein
S-CoA
S-CoA
Biotin carboxylase
6:1
1:1
Biotin Carrier Protein
1:1
Transcarboxylase

Figure 4b

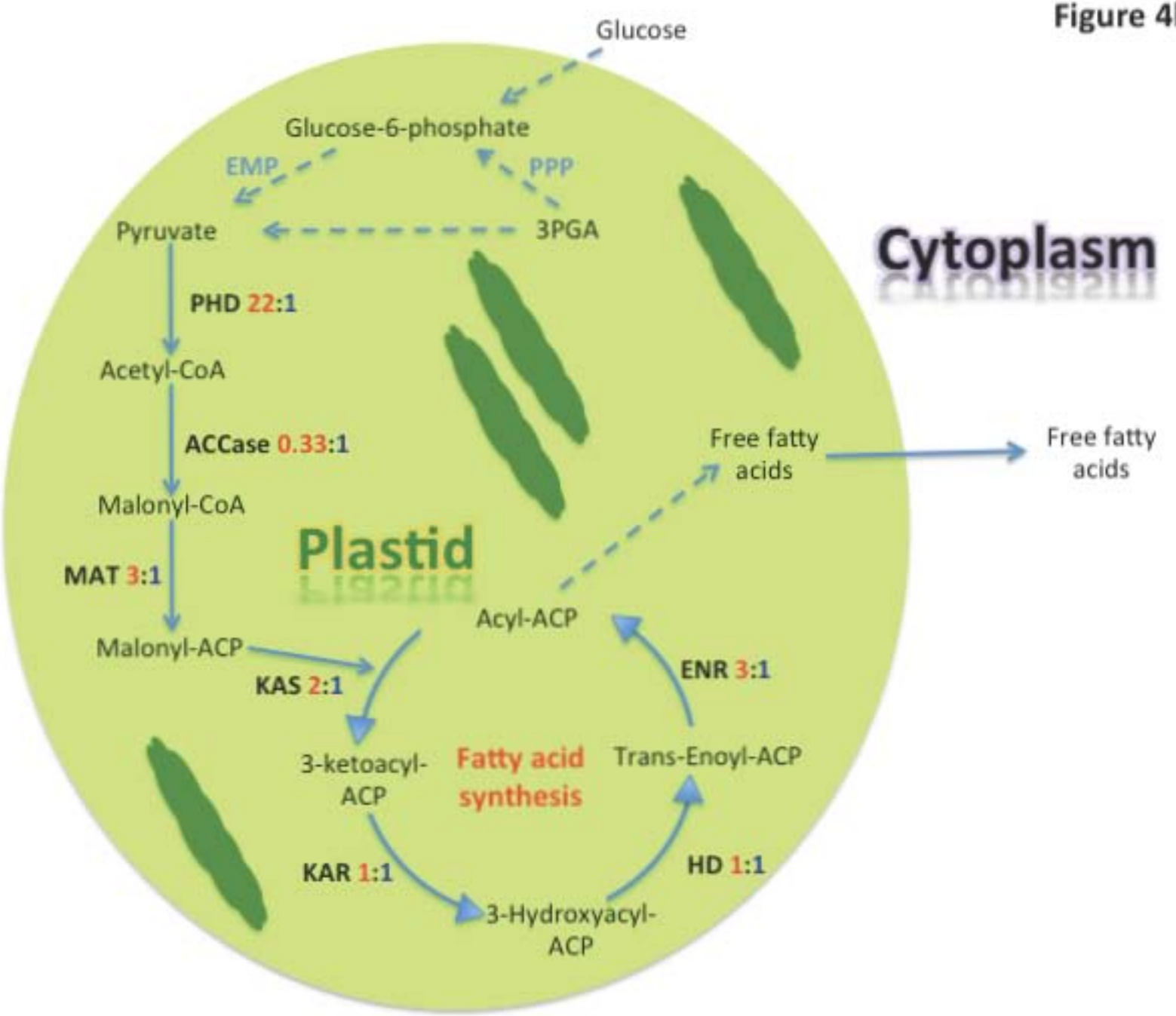

Glucose
Glucose-6-phosphate
EMP
PPP
Pyruvate
3PGA
Cytoplasm
PHD 22:1
Acetyl-CoA
ACCase 0.33:1
Malonyl-CoA
Free fatty acids
Free fatty acids
Plastid
MAT 3:1
Acyl-ACP
Malonyl-ACP
KAS 2:1
ENR 3:1
3-ketoacyl-ACP
Fatty acid synthesis
Trans-Enoyl-ACP
KAR 1:1
HD 1:1
3-Hydroxyacyl-ACP

Figure 5

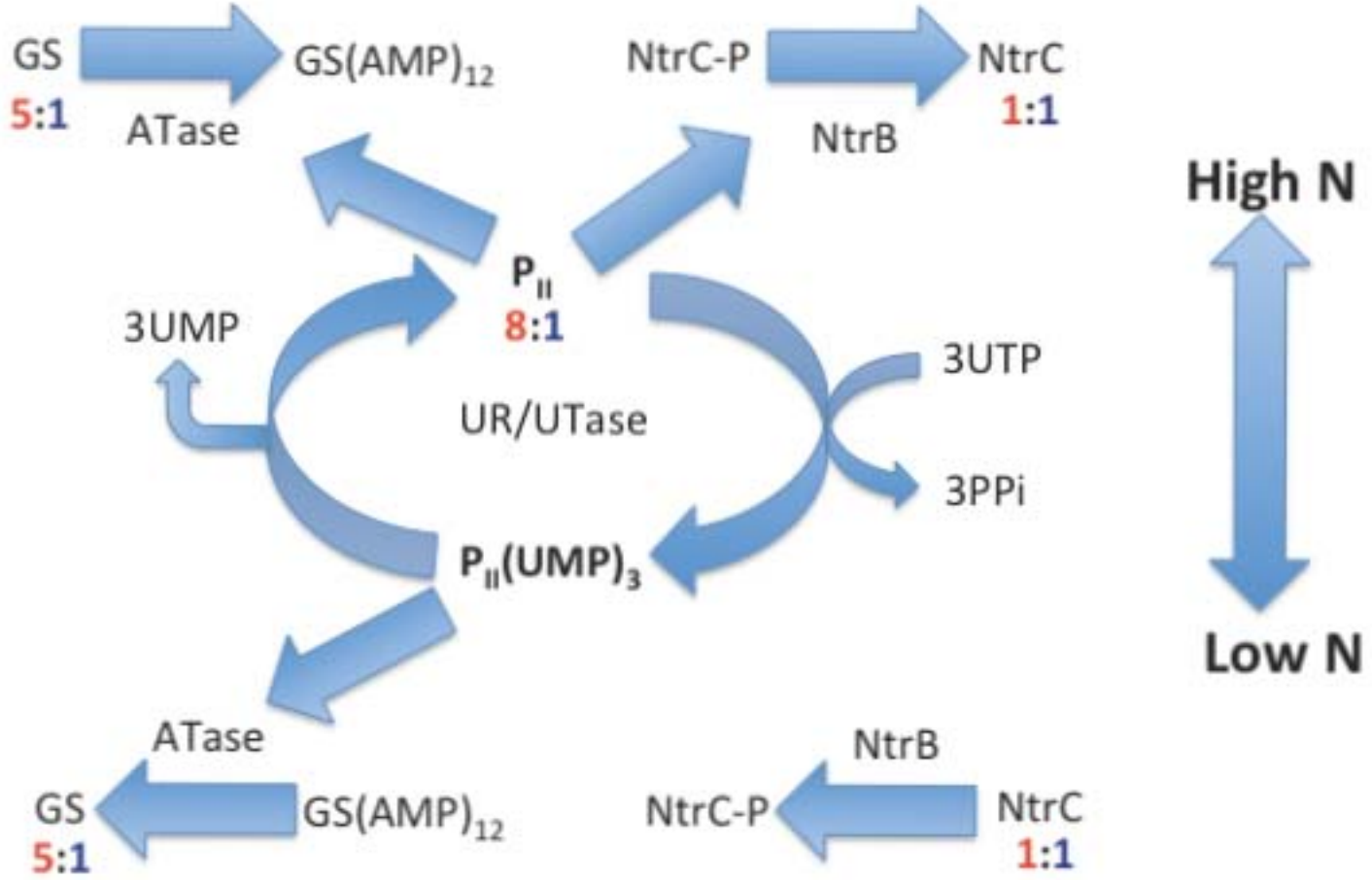

GS
5:1
ATase
GS(AMP)12
NtrC-P
NtrB
NtrC
1:1
High N
PII
8:1
3UMP
3UTP
UR/UTase
3PPi
PII(UMP)3
Low N
ATase
GS
5:1
GS(AMP)12
NtrB
NtrC-P
NtrC
1:1

Figure 6

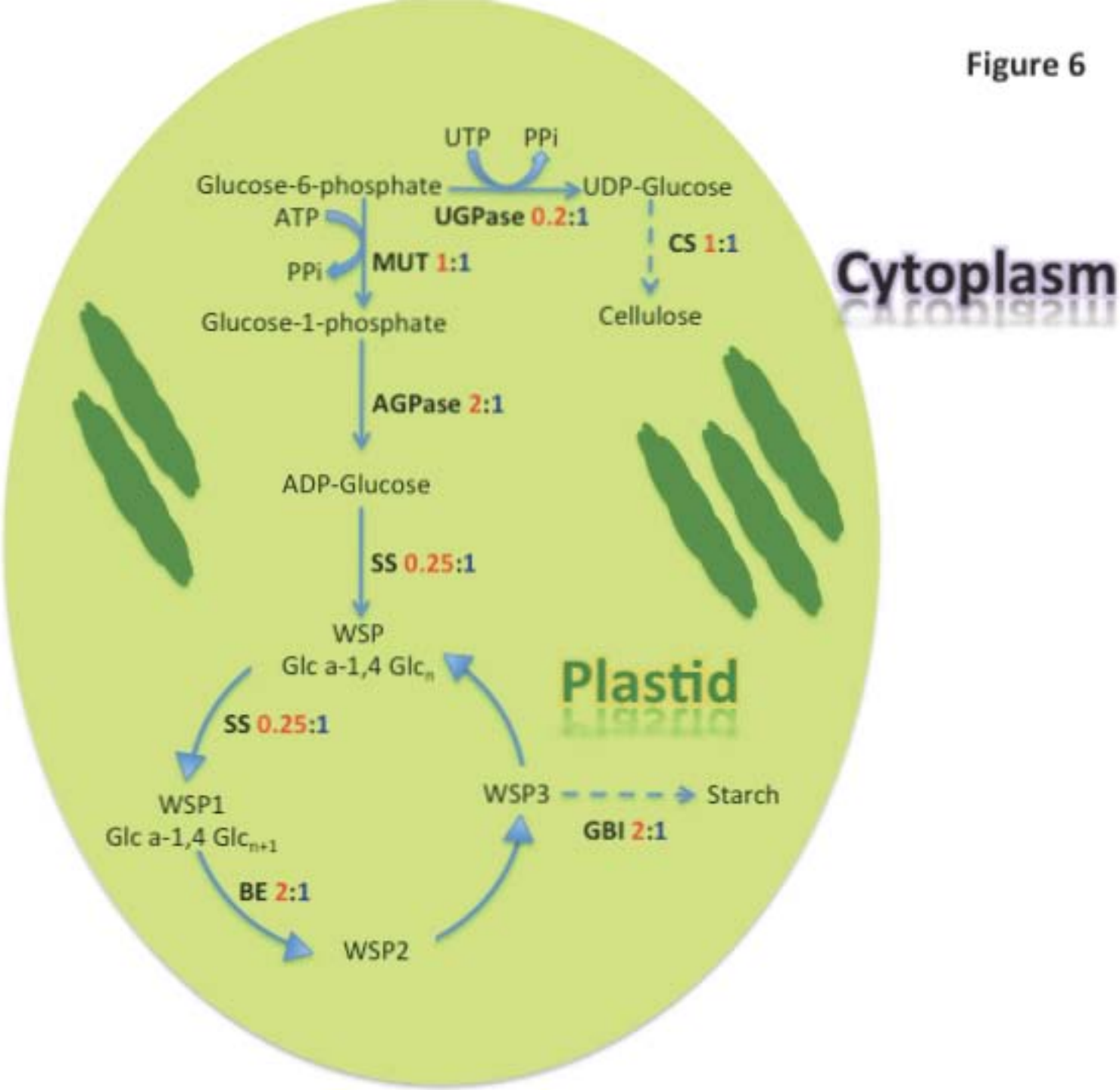

UTP
PPi
Glucose-6-phosphate
UDP-Glucose
UGPase 0.2:1
ATP
CS 1:1
PPi
MUT 1:1
Cytoplasm
Glucose-1-phosphate
Cellulose
AGPase 2:1
ADP-Glucose
SS 0.25:1
WSP
Glc a-1,4 Glc$_n$
Plastid
SS 0.25:1
WSP1
Glc a-1,4 Glc$_{n+1}$
WSP3
Starch
GBI 2:1
BE 2:1
WSP2

Figure 7

Membrane

Nitrate

Glucose

Nitrate

NADH
NAD+
NR 2:1

Nitrite

NiR 1:1

Import to chloroplast for nitrogen assimilation

Glucose

GLK 1:1

Glucose 6-P

GDH 6:1

NADP+ NADPH

6-P-glucono g-lactone

PGL 1:1

6-P-gluconate

PGD 6:1

NADP+ NADPH

Ribulose 5-P

RPI 2:1

RPE 1:1

GPI 2.5:1

Fructose 6-P

PFK 1:1

TK 3:1

Xylulose 5-P

Ribose 5-P

Fructose 1,6-diP

FBA 2.3:1

TK 3:1

Dihydroxyacetone-P

TPI 3:1

Glyceraldehyde 3-P

Erythrose 4-P

Sedoheptulose 7-P

GAP 2.4:1

1,3-diP-glycerate

PGK 6:1

3-P-phoglycerate

PGM 6:1

P-Enolpyruvate

EN 12:1

Pyruvate

PFK 2:1

Cytoplasm

Oxalacetate

ATP CO_2

Citrate

MAS 3:1

Glyoxylate

ICL 3:1

Malate

Isocitrate

Glyoxysome

Fumarate

Succinate

Glutamine

Asparagine

Aspartate

Aspartate family aminoacid synthesis

Glutamate

CO_2

Ribulose 1,5-P

3-P-glicerate

Ribose 5-P

Ribulose 5-P

Gliceraldehide 3-P

Sedoheptulose 7-P

Xylulose 5-P

Erythrose 4-P

Fructose 6-P

Pyruvate

CoASH + NAD+

PDH 22:1

CO_2 + NADH + H

Acetyl-CoA

CIS 1:1

CoASH

Oxalacetate

NADH + CO_2

NAD+

MDH 3:1

Malate

Citrate

ACH 3:1

Mitochondrion

Isocitrate

Glutamate family aminoacid family and chloroplast synthesis

Glutamate

Ammonium

FUM 5:1

Fumarate

IDH 0.5:1

NAD+

NADH + CO_2

Glutamine

Nitrite

α-ketoglutarate

Fatty acid synthesis

FADH2

FAD

SDH 2:1

OGD 0.14:1

NAD+ + CoASH

NADH + H + CO_2

Succinate

SCLA 3:1

Succinyl-CoA

MME 1:1

PFL 1:1

Malate

Pyruvate

Acetyl-CoA

CoASH + GTP

GDP + Pi

NADP+ NADPH + CO_2

Plastid

VIII. RESUMEN DE RESULTADOS

VIII.1 Determinación de las fuentes de carbono que pueden ser metabolizadas bajo condiciones estrictamente heterotróficas por *N. oleoabundans*

Los estudios realizados en matraces indicaron que *N. oleoabundans* es capaz de crecer bajo condiciones heterotróficas estrictas con glucosa o celobiosa como únicas fuente de carbono, llegando a concentraciones de masa celular de 2.3 y 1.56 g_{DCW}/L y productividades de 0.47 y 0.3 g_{DCW}/L/día, respectivamente, a los 5 días de cultivo. La microalga no es capaz de crecer utilizando xilosa, arabinosa, fructosa, sacarosa, lactosa, glicerol o ácido acético bajo condiciones heterotróficas.

VIII.2 Transporte de glucosa transmembranal

El transporte de glucosa transmembranal fue investigado en células provenientes de un cultivo lote con 3 g_{GLC}/L y [^{14}C]-glucosa como marcador. El ionóforo carbonil cianida m-clorofenil hidrazona (CCCP) fue incorporado como un desacoplador del gradiente de protones transmembranal. Los resultados mostraron que cuando se añadió el CCCP a las células de *N. oleoabundans* se inhibió el transporte de glucosa a través de la membrana, indicando que las células pueden transportar la glucosa solamente en presencia de un gradiente de protones, por lo tanto esta microalga utiliza un sistema de transporte de tipo simporte para internalizar la glucosa a la célula. Los cálculos basados en las velocidades de consumo y transporte de glucosa sugieren que el consumo de glucosa depende de la capacidad de transporte de la célula debido a que las velocidades de consumo y transporte son similares (8 µmol/g/min y 5 µmol/g/min, respectivamente).

VIII.3 Efecto de la relación C/N y la adición de pulsos de nitrato de sodio sobre la composición macromolecular de *N. oleoabundans*

Utilizando diferentes estrategias de cultivo con diferentes relaciones C/N, bajo condiciones ambientales controladas (pH, temperatura, aireación) en bioreactores de 4-litros, se encontró que promueve un cambio en la composición macromolecular de *N. oleoabundans* bajo condiciones heterotróficas. En cultivos lote con una relación C/N balanceada (C/N = 17; 3 g/L de glucosa y 0.5 g/L de nitrato de sodio) conducen a la acumulación de proteínas (44% p/p) como el componente celular mayoritario, con una concentración de masa celular de 1.72 g_{DCW}/L. A una relación C/N alta (inanición de nitrógeno durante la fase estacionaria y 50 g/L de glucosa iniciales) se incrementa significativamente la masa celular (9.2 g_{DCW}/L) y resulta en alto contenido lipídico (52% p/p) con una productividad lipídica de 528.5 mg/L/día. También, se lograron altas densidades celulares utilizando cultivos alimentados (50 g/L de glucosa inicial y adición intermitente de nitrato de sodio), promoviendo la acumulación de carbohidratos (54% p/p), principalmente en forma de almidón. El contenido de lípidos y proteínas para este cultivo fue de 33.7 y 11.6% p/p, respectivamente. Bajo esta condición se obtuvo una masa celular máxima de 14.2 g_{DCW}/L con una productividad de lípidos de 1,420 mg_{DCW}/L/día.

VIII.4 La estrategia de la alimentación exponencial en cultivos alimentados limitados por nitrógeno promueve la acumulación de metabolitos de reserva

La estrategia de la alimentación exponencial en los cultivos heterotróficos de *N. oleoabundans* permitió obtener una alta densidad celular (hasta 26.5 g/L). El comportamiento fisiológico de la microalga a dos diferentes tasas de dilución fue reflejado en el tipo de metabolito de reserva acumulado.

La primera etapa en lote con suficiencia de nutrientes permitió acumular preferencialmente proteínas (44% p/p). Durante la etapa de alimentación, en el

cultivo a la mayor velocidad de crecimiento, 0.042 h^{-1} (EFBC 0.042) (una velocidad similar a la obtenida en crecimiento sin limitación de nutrientes en cultivo lote), el nitrato y carbono fueron utilizado para sintetizar proteínas en proporciones similares a las obtenidas en cultivos lote , por lo que probablemente bajo estas condiciones no existió estrés nutricional por nitrato. En el cultivo a la menor velocidad de crecimiento, 0.035 h^{-1} (EFBC 0.035), los rendimientos de lípidos y carbohidratos en nitrato y los rendimientos de lípidos y carbohidratos en glucosa fueron 1.7 y 4 veces mas altos, respectivamente, que en el EFBC 0.042. Es decir, disminuyendo 20% la velocidad de crecimiento, el flujo de carbono fue preferentemente dirigido hacía la síntesis de metabolitos de reserva con una mayor eficiencia que la síntesis de proteínas.

Durante la etapa posterior a la alimentación, cuando las células estuvieron en inanición por de nitrógeno, habiendo todavía una alta concentración de glucosa en el medio (hasta 29.8 g/L), en el cultivo precedido por la mayor velocidad de crecimiento (EFBC 0.042) se promovió la acumulación preferencial de lípidos hasta el 54% del peso seco, y el contenido de carbohidratos fue de 26.9%. En esta condición el flujo de carbono fue dirigido hacía la síntesis de lípidos con un rendimiento de lípidos en glucosa y una productividad de lípidos 1.5 y 1.3 veces superior, respectivamente, al EFBC 0.035. La alimentación de la fuente de nitrógeno en el EFBC 0.035 condujo a un decremento en la acumulación de lípidos (44.6%) pero el contenido de carbohidratos resultó 1.5 veces mayor (38.1%) que el EFBC 0.042 (26.9%).

La reducción de la velocidad de crecimiento ocasiona el incremento del contenido de los ácidos grasos saturados en comparación con el EFBC 0.042. La estrategia de alimentar la fuente de nitrógeno a una alta velocidad fue adecuada para obtener una alta productividad de masa celular (1,900 mg_{DCW}/L/día) y una alta productividad global de lípidos (1,020 mg_{LIP}/L/día), los cuales son los valor más altos reportados hasta ahora para *N. oleoabundans*, ya sea en cultivos auto, mixo o heterotróficos. Por otro lado, el EFBC 0.035 empezó a sintetizar preferencialmente carbohidratos desde la etapa de alimentación y probablemente

esa fue la razón por la que acumuló menos lípidos que el EFBC 0.042. A pesar de esto, la productividad global de lípidos fue similar en ambos EFBC y la estrategia de imponer un estrés metabólico a través de la limitación por nitrógeno a una baja velocidad de alimentación resultó en un incremento de la acumulación de materiales de reserva (carbohidratos + lípidos) y una mayor productividad de masa celular (2,040 mg_{DCW}/L/día).

Estos resultados indican que existen mecanismos de regulación complejos en la microalga que cambian de forma significativa el contenido lipídico, y en general de materiales de reserva, en función de la velocidad de crecimiento cuando la microalga es limitada y/o sometida a condiciones de limitación por nitrógeno.

El contenido de energía que puede ser obtenido a partir de la masa celular total producida con *N. oleoabundans* a partir de 50 g/L de glucosa es comparable con el contenido de energía que puede ser obtenido al metabolizar la misma cantidad de glucosa a etanol producido usando levaduras (**Table 4, sección VII.2**). Sin embargo, la energía que potencialmente puede ser obtenida de combustibles líquidos como el etanol y el biodiesel a partir de los carbohidratos y lípidos producidos por *N. oleoabundans* es 5 y 2 veces menor a la energía obtenida solamente de transformar 50 g/L de glucosa en etanol, por ej. usando levaduras. Desde este punto de vista, los cultivos heterotróficos alimentados utilizando glucosa (u otros azúcares fermentables) como fuente de carbono no resultan favorables desde el punto de vista del contenido de energía para la producción de biocombustibles en comparación con la producción de etanol por métodos fermentativos.

Sin embargo, este tipo de estudios es útil para entender la fisiología de esta especie de microalga y con la ayuda de estos conocimientos, mejorar algunos parámetros de producción.

Evaluaciones técnico-económicas recientes [25], de producción de bio-crudo a partir de cultivos de microalgas, indican que los costos de producción con las tecnologías actuales son de 109 dólares EUA por galon utilizando

estanques abiertos y de 77 dólares EUA por galón para fotobioreeactores cerrados. Estos costos son demasiado elevados y es probable que con los reultados generados en la presente tesis, y usando la metodología reportada por Richardson et al. [25] el costo de producción sea mucho menor al reportado en el citado trabajo.

VIII.5 La acumulación de lípidos es provocada por la alta actividad de la vía de síntesis de lípidos y por el incremento en los niveles de piruvato deshidrogenasa y de poder reductor en forma de NADPH obtenido a través de la vía de las pentosas fosfato: estudios de proteómica

Por otro lado, los estudios de proteómica indicaron que bajo largos períodos de inanición de nitrógeno (condición de acumulación preferencial de lípidos), algunas reacciones o pasos de la vía de síntesis de lípidos se encuentran altamente activos. Particularmente en las etapas iniciales de la síntesis de ácidos grasos, donde se compromete el flujo de carbono hacia la síntesis de lípidos, las enzimas biotina carboxilasa (BC), la proteína acarreadora de acilos (ACP), y tres enzimas que forman parte del complejo multienzimático de la ácido graso sintetasa, enoil-ACP reductasa (ENR), malonil-CoA:ACP transacilasa (MAT) y la ß-cetoacil-ACP sintetasa (KAS) resultaron con un mayor abundancia. Para suministrar precursores a esta vía, la enzima piruvato deshidrogenasa resultó también altamente sobreproducida. Bajo estas condiciones algunos pasos de las vías del metabolismo central resultaron altamente activas para dirigir el carbono, dentro de lo posible, hacia la síntesis de piruvato y ATP. Específicamente, la mayoría de las enzimas que dan lugar a las reacciones glucolíticas y a la vía de las pentosas fosfato, especialmente las enzimas glucosa 6-fosfato deshidrogenasa y la 6-fosfogluconato deshidrogenasa, resultaron sobreproducidas. Estas últimas, indicando que se utilizan para generar una mayor cantidad de poder reductor, en forma de NADPH, para la síntesis de lípidos y a la asimilación de nitrógeno inorgánico.

En contraposición, las enzimas relacionadas con la síntesis de carbohidratos como la UDP-glucosa pirofosforilasa y la almidón sintetasa fueron sobreproducidas para contender con los períodos cortos de limitación de nitrógeno: condición de acumulación preferencial de carbohidratos. Además, la enzima ADP-glucosa pirofosforilasa se sobreprodujo bajo condiciones de acumulación preferencial de lípidos, indicando que bajo condiciones de inanición prolongada de nitrógeno, esta enzima actúa hidrolizando las cadenas de almidón para canalizar el flujo de carbono hacia la síntesis de lípidos, doble función que ya es conocida en esta proteína en células de plantas.

Todos estos estudios, incluyendo las estrategias de cultivo heterotrófico y la proteómica, son un primer acercamiento para develar el metabolismo de los lípidos y de los carbohidratos de una microalga no secuenciada como es *N. oleoabundans*. Comparando la expresión diferencial de proteínas bajo dos diferentes condiciones de acumulación de metabolitos de reserva, fue posible identificar a las proteínas clave que, al parecer, regulan las vías metabólicas de la síntesis de lípidos y de carbohidratos, así como las vías centrales de carbono y el flujo de energía en *N. oleoabundans*. Además, ahora se conocen las estrategias de cultivo heterotrófico en las que se pueden favorecer preferencialmente la formación de proteínas o de materiales de reserva (lípidos y carbohidratos) sugiriendo que existen mecanismos de regulación que son gobernados por las condiciones de limitación o inanición de nitrato.

IX. CONCLUSIONES

1. *N. oleoabundans* es capaz de crecer de manera heterotrófica estricta utilizando glucosa o celobiosa como fuente de carbono.
2. *N. oleoabundans* no consume xilosa, arabinosa, sacarosa, fructosa, lactosa, glicerol o ácido acético como fuente de carbono en estricta oscuridad.
3. La glucosa es transportada a través de la membrana por un sistema tipo simporte.
4. Una relación C/N balanceada (17) promueve la acumulación preferencial de proteínas (44% w/w) y se obtiene una masa celular de 1.72 g/L con 3 g/L de glucosa.
5. Una alta relación C/N (inanición de nitrógeno durante la fase estacionaria) promueve la acumulación preferencial de lípidos (52% w/w), 2 veces más que en cultivos sin limitación por nitrógeno, obteniéndose una masa celular de 9.2 g/L a partir de 50 g/L de glucosa.
6. Cultivos alimentados (50 g/L de glucosa inicial y adición intermitente de nitrato de sodio) condujeron al incremento de la masa celular (14.2 g/L) y provocaron la acumulación preferencial de carbohidratos (54% w/w; 2 veces más que en cultivos sin limitación por nitrógeno), debido a que las enzimas relacionadas con la síntesis de carbohidratos como la UDP-glucosa pirofosforilasa y la almidón sintetasa fueron sobreproducidas para contender con los períodos cortos de limitación de nitrógeno.
7. La estrategia de la alimentación exponencial permitió obtener cultivos de alta densidad celular de hasta 26.5 g_{DCW}/L, el cual es el más alto reportado a la fecha para cultivos de *N. oleoabundans*.
8. Manteniendo una velocidad de crecimiento de 0.042 h^{-1} se promovió la acumulación preferencial de lípidos hasta el 54% del peso seco y se obtuvo una alta productividad de masa celular (1.9 g_{DCW}/L/día) y de lípidos (1.02 g_{LIP}/L/día), la cual también es la mayor reportada a la fecha.

9. La alimentación de la fuente de nitrógeno para obtener una velocidad de crecimiento de 0.035 h^{-1} condujo a un decremento en la acumulación de lípidos (44.6%), pero el contenido de carbohidratos resultó 1.5 veces mayor (38.1%) que el EFBC 0.042 (26.9%), logrando una productividad de masa celular de2.04 g_{DCW}/L/día. La limitación de la fuente de nitrógeno desde la etapa de alimentación condujo a que se la acumulación de lípidos y carbohidratos fuese similar durante la etapa posterior a la alimentación.
10. La reducción de la velocidad de crecimiento a 0.035 h^{-1} ocasionó el incremento del contenido de los ácidos grasos saturados en comparación con cultivos a la máxima velocidad de crecimiento de la microalga, indicando una regulación compleja del metabolismo de lípidos bajo diferentes condiciones fisiológicas.
11. El contenido de energía que puede ser obtenido a partir de la masa celular total obtenida a partir de 50 g/L de glucosa en los cultivos alimentados exponencialmente es comparable con el contenido de energía que puede ser obtenido del etanol producido por levaduras también a partir de 50 g/L de glucosa.
12. La energía que se puede generada a partir del etanol y del biodiesel obtenidos de carbohidratos y lípidos sintetizados por *N. oleoabundans* , usando 50 g/L de glucosa, es 5 y 2 veces menor, respectivamente, a la energía obtenida solamente del etanol a partir de levaduras.
13. Bajo largos períodos de inanición de nitrógeno. condición que favorece la acumulación preferencial de lípidos, las enzimas que resultaron sobreproducidas y permiten un mayor flujo de carbono hacía esta vía son:
    - ✓ En la vía de síntesis de lípidos: biotina carboxilasa (BC), la proteína acarreadora de acilos (ACP), y tres enzimas que forman parte del complejo multienzimático de la ácido graso sintetasa: enoil-ACP reductasa (ENR), malonil-CoA:ACP transacilasa (MAT) y la B-cetoacil-ACP sintetasa (KAS).

- ✓ La piruvato deshidrogenasa, para suministrar precursores a la vía de síntesis de lípidos.
- ✓ En la vía de las pentosas fosfato: glucosa 6-fosfato deshidrogenasa y 6-fosfogluconato deshidrogenasa, para generar una mayor cantidad de poder reductor, en forma de NADPH, el cual es utilizado en reacciones biosintéticas, la síntesis de lípidos y asimilación de nitrógeno inorgánico.

14. Las enzimas relacionadas con la síntesis de carbohidratos como la UDP-glucosa pirofosforilasa y la almidón sintetasa fueron sobreproducidas para contender con los períodos cortos de limitación de nitrógeno y promover la acumulación de material de reserva (carbohidratos) con menor gasto de energía y poder reductor que la síntesis de lípidos.
15. La enzima ADP-glucosa pirofosforilasa se sobreprodujo bajo condiciones de acumulación preferencial de lípidos, lo cual sugiere que funciona, en este contexto, para hidrolizar las cadenas de almidón y canalizar el flujo de carbono hacia la síntesis de lípidos.

X. PERSPECTIVAS

1. Realizar cultivos mixotróficos con las fuentes de carbono que no pudieron ser metabolizadas por *N. oleoabundans* bajo condiciones heterotróficas estrictas, para determinar la viabilidad de utilización de estas fuentes de carbono y el potencial uso de los residuos agroindustriales como materia prima y de ser factible a futuro realizar ingeniería metabólica para proveer a la microalga con la capacidad de metabolizar un mayor número de fuentes de carbono.
2. Para maximizar la producción de la masa celular, de los lípidos y de los carbohidratos, se recomienda realizar cultivos alimentados exponencialmente a una tasa de dilución menores a $0.042h^{-1}$, prolongando la fase de alimentación de nitrato hasta que la glucosa se agote en el medio.
3. En microalgas, tanto oleaginosas como no oleaginosas, las siguientes estrategias podrían incrementar la velocidad de la vía de síntesis de lípidos y por consiguiente su producción-acumulación:
   - Sobreexpresión de genes que codifica para la biotina carboxilasa, la malonil-CoA transacilasa, la β-cetoacil-ACP sintetasa y la enoil-ACP reductasa.
   - Sobreexpresión el gen que codifica para la piruvato deshidrogenasa para incrementar la poza de acetil-CoA o malonil-CoA.
   - Sobreexpresión de los genes que codifican para la glucosa 6-fosfato deshidrogenasa y 6-fosfogluconato deshidrogenasa, enzimas que catalizan la formación de NADPH en la vía de las pentosas fosfato, para suministrar poder reductor en forma de NADPH a la síntesis de ácidos grasos.
16. Eliminar los genes que codifican para la UDP-glucosa pirofosforilasa y la almidón sintetasa de tal manera que se suprima la síntesis de almidón.

PRESENTATION OF THIS THESIS

This section describes how this thesis was structured. It is important to point out that these sections are presented in both Spanish and English due to the heterogeneity of the judges of the PhD defense.

Chapter XI shows a general abstract of this investigation. In chapter XII an introduction to the work topic and the background information that led to this work are presented. Chapters XIII and XIV describe the justification and hypotheses that were raised. Chapter XV displays the general and specific objectives that were established for the development of the experimental work.

Chapter VII presents the publications arising from this investigation. This section includes the results of this work, shown in detail in three manuscripts, one of them already published, another one submitted for publication and the last one in preparation to be submitted. In order to facilitate the reading and the sequence of the thesis, chapter XVI presents a summary of the results reported in the three manuscripts mentioned above, and a general discussion is made about the same.

Chapter XVII shows the main conclusions and contributions generated from this work, while Chapter XVIII exhibits the suggestions for future work from ideas produced in the present work.

Chapter XIX shows the literature cited in the chapter XII (introduction), excluding those that are presented in the manuscripts.

Finally, in the appendices chapter (XX) two divulgation notes published in a local newspaper in Cuernavaca, Morelos are displayed. Furthermore, abstracts and posters presented at national and international conferences are included as well.

XI. ABSTRACT

In this study we assessed the ability of *N. oleoabundans* to grow under heterotrophic conditions. The effect of the C/N ratio and the cell growth rate on the macromolecular composition, the kinetic and stoichiometric parameters, the fatty acid profile and the proteomic profile in heterotrophic cultures was analyzed. Through flask cultures, using 9 carbon sources (glucose, fructose, sucrose, xylose, arabinose, glycerol, acetic acid, cellobiose and lactose) and a mineral medium (BBM), it was determined that *N. oleoabundans* was able to grow using glucose or cellobiose as sole carbon source under strict darkness. Under a balanced C/N ratio of 17 and using bioreactor batch cultures containing 3 g/L of glucose, a maximal cell mass of 1.72 g/L was found, with protein being the major cell component (43.7% w/w; 30.9% of carbohydrates and 24.0% of total lipids). At a C/N ratio of 278 the cell mass concentration increased threefold (to 9.2 g/L) and lipid accumulation was doubled (51.7% w/w) through N-limitation, resulting in high lipid productivity (0.53 g/L/day), with a protein and carbohydrate content of 14.4 and 33.3%, respectively. Fed-batch cultures were performed at a C/N ratio of 278 and with nitrate pulse additions. This condition allowed a maximal cell mass of 14.2 g/L (1.5-fold higher) to be achieved and switched the metabolism to carbohydrate synthesis (54.2% of DCW), mainly in the form of starch, with a protein and carbohydrate content of 11.6 and 33.7%, respectively.

Also, the effect of the growth rate in the macromolecular composition of *N. oleoabundans* was studied in a system consisting of three stages. During the first stage, the batch cultivation, the major cellular components were proteins. In the second stage, a specific growth rate of 0.042 and 0.035 h^{-1} was controlled using an exponential nitrate-fed profile. After the fed stage and exhaustion of sodium nitrate, the cultures were in a nitrogen-starving condition and therefore the number of cells did not rise, however there was a 2-fold increase in the cell

mass at both growth rates, with the culture at 0.035 h^{-1} being the one with the highest biomass productivity (2.04 g_{DCW}/L/day). At 0.042 h^{-1} lipid accumulation was promoted up to 50% of the DCW with a cell mass productivity of 1.9 g_{DCW}/L/day. The protein and carbohydrate content was 17.5 and 26.9% of the DCW, respectively. However, at the slower growth rate of 0.035 h^{-1}, the lipid accumulation decreased to 44.6% and the carbohydrate levels increased to 38.1%.

Proteomic studies were carried out to compare the protein synthesis under two different scenarios, preferential lipid accumulation and preferential carbohydrate accumulation conditions. These results have started to shed light on how coordinated metabolic processes redirect the flow of fixed carbon toward biosynthesis and storage of lipids and carbohydrate in response to the time of nitrogen starving conditions.

Under long periods of nitrogen starvation, the first steps of the lipid synthesis pathway were found to be highly activated. To supply precursors for lipid production, the level of pyruvate dehydrogenase was significantly increased. Under these conditions, the central metabolism pathways became highly activated to redirect the carbon flow towards the pyruvate dehydrogenase and ATP synthesis. Furthermore, the pentose phosphate pathway, specifically the glucose 6-phosphate dehydrogenase and the 6-phosphogluconate dehydrogenase enzymes were synthesized in high abundance to supply reducing power in the form of NADPH for lipid synthesis and inorganic nitrogen assimilation.

Carbohydrate synthesis related enzymes that channel carbon to starch and sugar synthesis such as UDP-glucose pyrophosphorylase and starch synthase were overexpressed when short durations of nitrogen limitation were encountered, as in the case of plants. However, the ADP-glucose pyrophosphorylase enzyme was overexpressed under preferential lipid accumulation conditions, indicating that under prolonged nitrogen starvation conditions, this enzyme hydrolyzes the starch chains to channel the carbon flow to the lipid synthesis, a dual function that is already known in this protein.

XII. INTRODUCTION

Several photosynthetic microalgae have been identified as a rich source of ω-3 and ω-6 fatty acids. In addition, depending on the microalgae species, various high-value chemical compounds can be extracted from them such as pigments, antioxidants, b-carotenes, polysaccharides, triglycerides, vitamins, and biomass, which are largely used as bulk commodities in different industrial sectors (e.g. pharmaceuticals, cosmetics, nutraceuticals, functional foods, biofuels). Also algae hydrocolloids, like alginate and carrageenan are produced from seaweeds (specially macroalgae) and are largely used as viscosity modifying agents in foods and pharmaceuticals [1-3]. More recently several processes have introduced nutritional stress during cultivation, mainly through the limitation and starvation of the nitrogen source, to increase the production of reserve materials such as carbohydrates or lipids, which can be used for biofuel production or to obtain "oils" with similar quality and composition to edible oils. Consequently, several processes have been developed to obtain some of these compounds on a commercial scale; most of these developments are based on photoautotrophic growth using CO_2 as a carbon source [4, 5].

Although certain species are obligate photoautotrophs, numerous microorganisms currently classified as microalgae are in fact obligate heterotrophs and others are capable of both heterotrophic and photoautotrophic metabolism either sequentially or simultaneously [4]. The heterotrophic growth of microalga depends on the strain and culture conditions, and the consumption of the carbon source depends on the transport or diffusion of the carbon source across the membrane, and the enzymatic processes required for its incorporation into the central carbon metabolism [6].

Compared to photoautotrophic growth, heterotrophic cultivation of microalgae eliminates light requirements, can significantly increase growth rates and cell mass, protein, carbohydrate and lipid productivities [7-9]; bioreactor operation and maintenance is relatively simple and can be performed under

strict axenic conditions; also cell masses obtained under heterotrophic conditions are higher because the energy density of the carbon source is higher in comparison with carbon dioxide [9] and cell densities can be increased using some culture strategies like fed-batch cultures, leading to decrease the cost of biomass harvesting [10-12]. A fed-batch culture is essentially a batch culture supplied with nutrient medium continuously or intermittently during cultivation [4]. Specific growth rate can be controlled at or near the maximum specific growth rate by continuous exponential substrate addition. This could allow having a limiting substrate concentration in the medium during the feed [13], which is advantageous because some microalgal species can accumulate lipids and/or carbohydrates when some nutrient is limited in the culture media [2, 4, 6, 8, 9, 12].

Heterotrophic cultures also have drawbacks: there is a limited number of microalgal species that can grow heterotrophically; energy expenses and costs by adding an organic substrate are higher; growth can be inhibited by an excess of organic substrate; and light-induced metabolites cannot be produced [9, 14]. One of the most notable advantages of the phototrophic cultivation is that under such conditions microalgae fix carbon dioxide and produce oxygen, contributing to the reduction of carbon emissions to the atmosphere [15]. Microalgae can grow heterotrophically using the same media components used in phototrophic cultures, but with an organic carbon source instead of using a continuous flow of carbon dioxide and light. However, the cost of the organic carbon source –that is often high– and the production of CO_2 are major commercial and environmental concerns of heterotrophic cultures [15]. The price of the glucose (obtained from starch that is produced from plants that are cultivated under phototrophic conditions, *e. g.* corn) is in the order of 0.6 US dollars per kg, while the use of carbon dioxide from flue gases is favorable due to the reduction of emissions to the atmosphere [15]; although, additional cleanup steps of the flue gas are likely required, which can intrinsically be expensive as well.

A microalgae suitable for heterotrophic culture should have the following physiological abilities: divide and metabolize without light, grow on easily sterilized culture media, adapt rapidly to environmental changes and withstand the hydrodynamic stresses generated in stirred tank bioreactors and peripheral equipment [4,6,14]. Several strains of algae, including *Chlorella protothecoides, Galdieria sulphuraria, Nitzschia laevis* and *Crypthecodinium cohnii* have been studied under heterotrophic growth conditions to achieve high quantities of dry cell weight (DCW) and fatty acids, or high productivity of valuable chemicals [7,16,17,18].

N. oleoabundans is a microalgae of biotechnological and commercial interest because it has the ability to accumulate lipids under phototrophic nutritional stress condition, which can be used for different purposes [19]. From the point of view of the commercial production of biofuels from lipids (such as biodiesel) and high-value products (such as pigments), it is desirable to obtain high productivities of microalgal biomass and metabolites of interest. This could theoretically be achieved through heterotrophic cultures designed for this purpose, using cultures strategies such as the manipulation of C/N ratio in the medium and/or the growth rate in fed-batch cultures.

One of the obstacles that limit the knowledge of the microalgal metabolism is the lack of available genome sequence information of most of the microalgal strains [20]. Rapidly developing post-genomic, system biology approaches such as transcriptomics, proteomics, and metabolomics have become essential for understanding how microorganisms respond and adapt to changes in their physical environment [20]. The implementation of these approaches, such as proteomics, can start to shed light on how metabolic processes redirect the flow of fixed carbon toward biosynthesis of reserve metabolites –such as lipids and carbohydrates– and carbon central pathways overexpressing proteins that are key in those pathways.

XIII. JUSTIFICATION

N. oleoabundans is an oleaginous microalgae that can produce 80% of triacylglycerols (TAG) of total lipids. Most of the fatty acids are saturated having 16-20 carbon atoms [21], suitable for use as an edible oil or biodiesel production [22].

At the beginning of this thesis, there was almost no research related to the use of this microalga for production of reserve metabolites, and none under heterotrophic conditions. However, in preliminary studies (unpublished data) in our working group, it was observed that this microalga was able to grow under heterotrophic conditions using glucose as carbon source.

Previous reports have shown that the C/N ratio in microalgal cultures has significant effects on the reserve metabolism. Thus, at a high C/N ratio preferential lipid accumulation is promoted [23]. In the case of *N. oleoabundans* it has been observed that the nitrogen source growth-limiting strategy (high C/N ratio) is effective for the preferential lipid accumulation [15, 19, 22]. A process of controlled nitrogen limitation may be carried out through exponentially fed-batch cultures controlling the growth rate specifically. Thus, in addition to promoting the accumulation of reserve metabolites, it could be possible to obtain high cell densities in a single process.

N. oleoabundans is a microalgae that does not have a sequenced genome. This limits the understanding of its metabolism and therefore, the development of genetic engineering tools that may increase the capacity of this microalgae to produce reserve metabolites at a growth rate comparable with bacteria or yeast. However, the 'omic' tools such as transcriptomics, proteomics or metabolomics have been used whenever this limitation exists. Specifically, proteomics may help to understand why some key proteins in microalgal metabolic pathways are synthesized when the microalgae is under specific culture conditions. This could

be considered as the beginning of understanding the metabolism of not sequenced microalgae.

XIV. HYPOTHESIS

Manipulating the C/N ratio and the cell growth rate in heterotrophic cultures of *N. oleoabundans* leads to an increased concentration of the cell mass and the variable accumulation of reserve materials, such as carbohydrates and lipids, furthermore the analysis of the proteome under these different culture strategies disclose key points in the synthesis of proteins involved in the central metabolism and in the pathways of reserve metabolites synthesis.

XV. OBJECTIVES

OVERALL OBJECTIVE

Study the effect of C/N ratio and the cell growth rate on the macromolecular composition and the proteome profile of *N. oleoabundans* under strict heterotrophic growth conditions.

SPECIFIC AIMS

1. Determine the carbon sources that can be metabolized by *N. oleoabundans* under strict heterotrophic conditions.

2. Study the effect of balanced and high C/N ratio on the accumulation of reserve metabolites in *N. oleoabundans*.

3. Carry out fed-batch cultures limited by the nitrogen source using two feed strategies: intermittent and exponential, both with sodium nitrate to maximize the biomass and reserve metabolites production by *N. oleoabundans*.

4. Identify and analyze the proteins that are synthesizing differentially between lipid accumulation and carbohydrate accumulation conditions in *N. oleoabundans* heterotrophic cultures.

XVI. SUMMARY OF RESULTS

XVI.1 Determination of carbon sources metabolized under heterotrophic conditions by *N. oleoabundans*

Results from shake flasks cultures indicate that *N. oleoabundans* was able to grow under strict heterotrophic cultures conditions with glucose or cellobiose as the only carbon source; however, no growth was obtained using xylose, arabinose, fructose, sucrose, lactose, glycerol or acetic acid. Final dry cell weights of 2.3 g/L (dry cell mass productivity of 0.47 g/L/day) and 1.56 g/L (dry cell mass productivity of 0.3 g/L/day) were obtained at 5 days of cultivation of *N. oleoabundans* with 10 g/L of glucose or cellobiose, respectively.

XVI.2 Transmembrane glucose transport

Transmembrane glucose transport was investigated in cells growing in batch cultures containing 3 g/L glucose and [^{14}C]-glucose tracer; the ionophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was incorporated as a proton gradient decoupler. When added to *N. oleoabundans*, CCCP inhibits the glucose transport across the membrane, indicating that the cells can only transport glucose in the presence of a proton gradient; hence suggesting that this microalga uses a glucose symporter to transport this sugar into the cell. Calculations based on the rates of consumption and uptake suggest that glucose consumption depends on the transport capacity of the cell because the glucose consumption and transport rates are very similar (8umol/g/min and 5 umol/g/min, respectively).

XVI.3 Effect of the C/N ratio and the nitrate pulses addition on the macromolecular composition of *N. oleoabundans*

Using different cultivation strategies with variable C/N ratios under environmental controlled conditions (pH, temperature and aeration rate) in a 5-L bioreactor allows metabolic switching. Batch cultures with balanced C/N ratios (17) accumulate proteins (up to 44 % w/w) as the major cell component with a maximal cell mass of 1.72 g/L; a high C/N ratio (nitrogen limitation during the stationary phase) significantly increases the dry cell mass (9.2 g/L) and yields a high lipid content (up to 52 % w/w) with a lipid productivity of 528.5 mg/L/day. Also, it was possible to obtain a high cell density and carbohydrate accumulation (up to 54 % (w/w), mainly starch) by using fed-batch cultures (50 g/L initial glucose and the addition of nitrate pulses). This condition allowed a maximal cell mass of 14.2 g/L with a productivity of 1.42 g_{DCW}/L/day.

XVI.4 The exponentially fed strategy in N-limited fed-batch cultures promotes the preferential accumulation of reserve metabolites

The exponentially fed-batch strategy resulted in a high cell mass production of *N. oleoabundans*. The physiological behavior of the microalgae at two different growth rates was reflected by the kind of reserve metabolite accumulated.

The first batch stage with sufficient nutrients allowed accumulating proteins (up to 44% w/w) in both EFBC's. During the fedbatch stage in the EFBC 0.042 the nitrate was used to build protein instead of reserve metabolites and the carbon flow was directed to the cellular duplication and probably there was no nutrient or environmental stress under this condition. In the EFBC 0.035 the yields of lipid and carbohydrate on nitrate consumed ($Y_{LIP\text{-}CARB/NIT}$) and the yields of lipid and carbohydrate on glucose consumed ($Y_{LIP\text{-}CARB/GLC}$) were 1.7 and 4-times higher, respectively, than in the EFBC 0.042. This means that under the lower growth rate the carbon and nitrogen flow were preferably directed toward the synthesis of reserve metabolites with a higher efficiency than the protein synthesis accordingly with the nitrate stress condition.

During the post fedbatch stage when the nitrate concentration was exhausted and there still was a high glucose concentration, at the maximum growth rate (EFBC 0.042) the lipid accumulation was promoted to 53.8% of the dry cell weight (DCW) and carbohydrate and protein content were 26.9 and 17.5% w/w, respectively. At this condition the carbon flow was directed to lipid biosynthesis and the yield of lipid in glucose and the lipid productivity were about 1.5 and 1.3-times higher, respectively, than the EFBC 0.035.

Feeding the nitrogen source at a lower growth rate (EFBC 0.035) led to a decrease in the lipid accumulation (44.6%) but the carbohydrate content was 1.5-fold higher (38.1%) than the EFBC 0.042 (26.9%). Reducing the growth rate to half caused a few changes on relative content of every fatty acid, in which the saturated fatty acid content was increased compared to that in EFBC 0.042.

The strategy to feed the nitrogen source at a high growth rate works to obtain a high cell mass productivity (1.9 g_{DCW} $L^{-1}day^{-1}$) and a high global lipid productivity (1.02 $g_{LIP}L^{-1}day^{-1}$), which is the highest value, reported so far for this microalgae. On the other hand, the EFBC 0.035 started producing carbohydrate from the fed-batch stage and this was probably the reason for having a decreased lipid accumulation when compared to EFBC 0.042. Nevertheless, the overall lipid productivity was equally high and the strategy of imposing a metabolic stress by nitrogen limitation at a lower growth rate results in an increased accumulation of reserve materials and a higher biomass productivity (2.04 g_{DCW} $L^{-1}day^{-1}$).

Assuming that the total amount of carbohydrates and lipids are extracted and can be converted to ethanol and biodiesel and using the factors indicated in **table 4** of the **section V.3**, a total amount of 483.6 MJ/m^3 can be reached. As a comparison the conversion of the 1,500 g of glucose to ethanol by yeast (maximum theoretical yield of 0.51 $g_{EtOH}/_{GLC}$ and a fermentation efficiency of 95%), 649.57 MJ/m^3 yield can be obtained. Furthermore, assuming that the microalgae can accumulate up to 80% (w/w) of lipid content (no carbohydrate accumulation), it is hypothetically possible to obtain 616.02 MJ/m^3. Using the total biomass (lipids, proteins and carbohydrates, 795.9 g of DCW) with the

energy content obtained before, it could be feasible to obtain 662.5 MJ/m^3, which is similar to the energy values obtained from yeast. From this analysis, it is clear that the conversion of glucose or other types of sugars to microalgal cell mass containing a high amount of reserve materials (including only lipids) is not favorable in terms of energy content in comparison with the conversion of such sugars to ethanol. Therefore, from the perspective of biofuel production it is better to produce low cell density cultures of yeast with ethanol as an end-product than high cell density cultures with microalgae with lipids and/or carbohydrates as end-products.

However, this work is useful to understand the physiology of our microalgal species and with this knowledge it will be possible to improve these important production parameters. Moreover, to our knowledge, these are the highest lipid and carbohydrate productivities reported until now for cultures of *N. oleoabundans*.

XVI.5 Lipid accumulation triggered by pyruvate dehydrogenase accumulation and high pentose phosphate pathway activation was revealed through proteomic studies in heterotrophic N-limited cultures of *Neochloris oleoabundans*

Proteomic studies indicated that under long periods of nitrogen starvation (preferential lipid accumulation condition), some reaction steps in the lipid synthesis pathway are highly activated. Specifically, the enzymes biotin carboxylase (BC), the acyl carrier protein (ACP) and three enzymes that are part of the multienzymatic complex of the fatty acid synthase, the enoyl-ACP reductase (ENR), the malonyl-CoA:ACP transacylase (MAT), and the β-ketoacyl-ACP synthase (KAS) appear to be key regulated enzymes. To supply this elevated lipid pathway, the precursor pyruvate dehydrogenase was overproduced by the cells. Under these conditions, some steps in the central metabolic pathways were highly active to redirect the carbon to pyruvate

dehydrogenase and ATP synthesis. Specifically, most of the enzymes that lead to the glycolytic reactions, and to the pentose phosphate pathway, especially glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were overproduced. These latter to supply reducing power in the form of NADPH to lipid synthesis and inorganic nitrogen assimilation.

Enzymes associated with the carbohydrate synthesis such as UDP-glucose pyrophosphorylase and starch synthase were overproduced to contend with short periods of nitrogen limitation (preferential carbohydrate accumulation condition), as in the case of plants. Furthermore, the enzyme ADP-glucose pyrophosphorylase was overproduced under conditions of preferential lipid accumulation, indicating that under prolonged nitrogen starvation, this enzyme hydrolyzes starch chains to channel the carbon flow towards the lipid synthesis, a double function that is already known of this protein in other photosynthetic organisms, such as plants.

These studies of heterotrophic cultures and proteomic follow-on analysis are a first approach to unravel the lipid and carbohydrate metabolism in an unsequenced microalgae, such as *N. oleoabundans*. By comparing the differential protein expression under two different metabolite accumulation conditions, it was possible to identify key proteins that appear to regulate the pathways of lipid and carbohydrate synthesis as well as the central pathways of carbon and the energy flow in *N. oleoabundans.*

XVII. CONCLUSIONS

1. *N. oleoabundans* is able to grow under strict heterotrophic conditions using glucose or cellobiose as carbon source.
2. *N. oleoabundans* doesn't use xylose, arabinose, sucrose, fructose, lactose, glycerol or acetic acid as a carbon source under dark conditions.
3. Glucose is transported through the membrane by a symporter system.
4. Batch cultures with a balanced C/N ratio (17) accumulate proteins (44% w/w) as the major cell component producing1.72 g/L of DCW from 3 g/L of glucose.
5. A high C/N ratio (nitrogen starvation during the stationary phase) promotes the accumulation of lipids content (52%), 2-fold more than in cultures without nitrogen limitation, obtaining a DCW of 9.2 g/L form 50 g/L of glucose.
6. High cell density (14.2 g/L) and carbohydrate accumulation (54%; 2-fold more than in cultures without nitrogen limitation) were achieved using fed-batch cultures (50 g/L initial glucose and the addition of nitrate pulses), this behavior was due to increased levels of the enzymes involved in the synthesis of carbohydrates, such as UDP-glucose pyrophosphorylase and starch synthase, to contend with short periods of nitrogen limitation.
7. The exponential feeding strategy allowed obtaining high cell density cultures, up to 26.5 g_{DCW}/L, which is the highest value reported so far for cultures of *N. oleoabundans*.
8. The preferential accumulation of lipids (up to 54% of DCW) and high cell mass (1.9 g_{DCW}/L/day) and lipid (1.02 g_{DCW}/L/day) productivities were obtained in exponential fedbatch cultures at a cell growth rate of 0.042 h^{-1}. Also, these values are the highest reported so far.

9. Feeding the nitrogen source at a lower growth rate (0.035 h^{-1}) led to a decrease in the lipid accumulation (44.6%), but the carbohydrate content was 1.5-fold higher (38.1%) than the EFBC 0.042 (26.9%), reaching a cell mass productivity of2.04 mg_{DCW}/L/day. The nitrogen limitation since the feed-batch stage promoted the accumulation of lipids and carbohydrates also during the post fed-batch stage.
10. Reducing the growth rate to 0.035 h^{-1} causes an increase in the content of saturated fatty acids as compared to EFBC 0.042, indicating a complex regulation of lipid metabolism under different physiological conditions.
11. The energy content that can be obtained from the total cell mass in cultures containing 50 g/L of glucose is similar to the energy content that can be obtained fermenting 50 g/L of glucose to ethanol with yeast.
12. The energy that can be obtained, as liquid fuels such as ethanol and biodiesel, from carbohydrates and lipids produced by *N. oleoabundans* with 50 g/L glucose, is 5 and 2-times lower, respectively, than the energy obtained only from ethanol by yeast.
13. Under extended periods of nitrogen starvation (preferential lipid accumulation condition), the overproduced enzymes, which allowed a higher carbon flux to the fatty acid pathway, were:
    - ✓ In the lipid biosynthesis pathway: biotin carboxylase (BC), the acyl carrier protein (ACP) and three enzymes that are part of the multienzymatic complex of the fatty acid synthase: the enoyl-ACP reductase (ENR), the malonyl-CoA:ACP transacylase (MAT), and the β-ketoacyl-ACP synthase (KAS).
    - ✓ Pyruvate dehydrogenase, to provide precursors to the lipid pathway.
    - ✓ In the pentose phosphate pathway: glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, to generate a higher amount of reducing power, as NADPH, which is

used in biosynthetic reactions, for lipid synthesis and the assimilation of inorganic nitrogen.

14. Enzymes involved in the carbohydrate synthesis such as UDP-glucose pyrophosphorylase and starch synthase were overexpressed to contend with short periods of nitrogen limitation and promoted the preferential carbohydrate accumulation condition; which, in comparison with the lipid synthesis, uses less energy and reducing power.
15. The ADP-glucose pyrophosphorylase was overproduced under preferential lipid accumulation conditions, this fact suggests that in such conditions starch is hydrolyzed and the carbon flow is channeled to the synthesis of lipid.

XVIII. PERSPECTIVES

1. It is suggested to perform mixotrophic cultures with the carbon sources that couldn't be metabolized by *N. oleoabundans* under strict heterotrophic conditions and determine the feasibility of using these sources and the potential use of agroindustrial wastes or, in the future, perform metabolic engineering with the microalga to metabolize a wide array os carbon sources.
2. To maximize cell mass, lipids and carbohydrate production it is recommended to carry out exponentially fed-batch cultures at a dilution rates lower that 0.042 h^{-1}, thereby extending the nitrate feed phase until glucose in the medium is depleted.
3. The following strategies could increase the rate of lipid synthesis and therefore its production-accumulation on microalgae that normally do not accumulate lipids (no oil) and in oleaginous ones:
   - Overexpression of the genes encoding biotin carboxylase, malonyl-CoA transacylase, β-ketoacyl-ACP reductase and enoyl-ACP reductase.
   - To increase the acetyl-CoA and malonyl-CoA pool, overexpression of the gene encoding pyruvate dehydrogenase.
   - In order to provide reducing power in the form of NADPH for the fatty acid synthesis, overexpress the genes coding for the glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, enzymes that catalyze the formation of NADPH in the pentose phosphate pathway.
   - To suppress the starch synthesis, delete the genes encoding UDP-glucose pyrophosphorylase and starch synthase.

XIX. REFERENCIAS ADICIONALES (additional references)

1. Guil-Guerrero JL, Navarro-Juarez R, Lopez-Martinez JC, Campra-Madrid P, Rebolloso-Fuentes MM: **Functional properties of the biomass of three microalgal species.** *J Food Eng* 2004, **65:511-517.**
2. Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M: **Biodiesel production from oleoaginous microorganism.** *Renew Energ* 2009, **34**: 1-5.
3. Barrow C, Shahidi F: **Marine nutraceuticals and functional foods.** *CRC Press, Taylor & Francis Group,* 2008.
4. Chen GQ, Chen F: **Growing phototrophics cells without light**. *Biotechnol Lett* 2006, **28**:607-616.
5. Olaizola M: **Commercial development of microalgal biotechnology from the test tube to the marketplace**. *Biomol Eng* 2003, **20**:459-466.
6. Mojtaba A, Mohd S, Rosfarizan M, Raha A, Arbakariya B: **Improvement of medium composition for heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, using response surface methodology.** *Biochem Eng J* 2011, **53:**187-195.
7. Cheng Y, Zhou W, Gao C, Lan K, Gao Y, Wu Q: **Biodiesel production from Jerusalem artichoke (Helianthus Tuberosus L.) tuber by heterotrophic microalgae Chlorella protothecoides**. *Soc Chem Ind* 2009, **84**:777-781.
8. O´Grady J, Morgan J: **Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol**. *Bioprocess Biosyst Eng* 2010, **34** (Short communication 1):121-125.
9. Perez-Garcia O, Escalante FME, de-Bashan LE, Bashan Y: **Heterotrophic cultures of microalgae: metabolism and potential products.** *Water Res* 2011, **45**(1):11-36.
10. Eriksen N: **the technology of microalgal culturing.** *Biotechnol Lett* 2008, **30**(9): 1525-1536.

11. Bumbak F, Cook S, Zachleder V, Hauser S, Kovar K: **Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations**. *App Microbiol Biotechnol* 2011, **91**:31-46.
12. Wen Z Y, Chen F: **Heterotrophic production of eicosapentaenoic acid by microalgae.** *Biotechnol Adv* 2003, **21**(4):273-294.
13. Martínez A, Ramírez OT, Valle F: **Effect of growth rate on the production of ß-galactosidase from Escherichia coli in Bacillus subtilis using glucose-limited exponentially fed-batch cultures**. *Enzyme Microb Technol* 1998, **22**:520-526.
14. Chen F: **High cell density culture of microalgae in heterotrophic growth.** *Trends Biotechnol* 1996, **14**:412-426.
15. Gouveia L, Oliveira L: **Microalgae as a raw material for biofuels production**. *J Ind Microbiol Biotechnol* 2009, **36**:269-274.
16. Graverholt O, Eriksen N: **Heterotrophic high cell-density fed-batch and continuous flow cultures of *Galdieria sulphuraria* and production of phycocyanin.** *Appl Microbiol Biotechnol* 2007, **77**:69-75.
17. Wen Z Y, Chen F**: A perfusion-cell bleeding culture strategy for enhancing the productivity of eicosapentaenoic acid by *Nitzschia laevis*.** *Appl Microbiol Biotechnol* 2001, **57**:316-322.
18. de Swaaf M, Sijtsma L, Pronk J: **High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii.*** *Biotechnol Bioeng* 2003, **81**:666-672.
19. Pruvost J, Van Vooren G, Cogne G, Legrand J: **Investigation of biomass and lipids production with *Neochloris oleabundans* in photobioreactor**. *Bioresour Technol* 2009, **100**(23):5988-5995.
20. Guarnieri MT, Nag A, Smolinski SL, Darzins A, Seibert M, Pienkos PT: **Examination of triacylglycerol biosynthetic pathway via De novo transcriptomic and proteomic analyses in an unsequenced microalga**. *PLoS ONE* 2011, 6(10): e25851.

21. Morales-Sánchez D, Tinoco R, Kyndt J, Martínez A: **Heterotrophic growth of *Neochloris oleoabun*dans using glucose as a carbon source**. *Biotechnol Biofuels* 2013, 6:100.
22. Tornabene T, Holzer G, Lien S, Burris N: **Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans***. *Enzyme Microb Technol* 1983, 5: 435-440.
23. Li YQ, Horsman M, Wang B, Wu N, Lan C: **Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans***. *Appl Microbiol Biotechnol* 2008, 81(4): 629-636.
24. Eriksen N: **The technology of microalgal culturing**. *Biotechnol Lett* 2008, 30(9): 1525-1536.
25. Richardson JW, Johnson MD, Zhang X, Zemke P, Chen W, Hu Q: **A financial assessment of two alternative cultivation systems and their contributions to algae biofuel economic viability**. *Algal Research* 2013 (in press).

XX. APÉNDICES (Apendix)

XX.1 Congresos Nacionales e Internacionales

XX.1.1 VII Reunión Nacional de la Red Mexicana de Bioenergía A. C., Cuernavaca, Morelos, México. Octubre 25-28, 2010.

XX.1.2 2nd International Conference on Algal Biomass, Biofuels and Bioproducts. San Diego CA, USA. June 10-13, 2012.

XX.1.3 2º Congreso Iberoamericano sobre biorrefinerias. Jaen, España. Abril 10-12, 2013.

XX.1.4 3rd International Conference on Algal Biomass, Biofuels and Bioproducts. Toronto, Canada. June 16-19, 2013.

XX.1.5 3rd International Conference on Algal Biomass, Biofuels and Bioproducts. Toronto, Canada. June 16-19, 2013.

XX.2 Artículos de divulgación de la ciencia

XX.2.1 Unión de Morelos. Periódico local, 26 de Agosto de 2013.

XX.2.2 Unión de Morelos. Periódico local, 2 de Septiembre de 2013.

XX.1.1 ***VII Reunión Nacional de la Red Mexicana de Bioenergía A. C., Cuernavaca, Morelos, México. Octubre 25-28, 2010*****:**

Panorama de la Producción de Biodiesel a partir de Microalgas en Zonas Áridas de México

(Oral)

Daniela Morales Sánchez, María del Refugio Trejo Hernández, Rafael Vazquez Duhalt y Alfredo Martínez Jiménez

Panorama de la Producción de Biodiesel a partir de Microalgas en Zonas Áridas de México

[1]Daniela Morales Sánchez, [2]María del Refugio Trejo Hernández, [1]Rafael Vazquez Duhalt y [1]Alfredo Martínez Jiménez

[1]Instituto de Biotecnología - Universidad Nacional Autónoma de México. Av. Universidad 2001, Col. Chamilpa. C.P. 62210. Cuernavaca, Morelos. Apdo. Postal 510-3. Tel. (+52) 777 3291630.

[2]Centro de Investigación en Biotecnología - Universidad del Estado de Morelos. Av. Universidad 1001. Col. Chamilpa. C.P. 62209. Cuernavaca, Morelos. Tel. (+52) 777 3297000 ext. 3520.

dmorales@ibt.unam.mx; alfredo@ibt.unam.mx; mtrejo@uaem.mx; vazqduh@ibt.unam.mx

Una alternativa potencial, que asegura satisfacer o reemplazar la demanda global de petrodiesel, es la obtención de lípidos microalgales para la producción de biodiesel. Esta tecnología, en contraste con las plantas oleaginosas, ofrece ventajas como: mayor eficiencia fotosintética; eficacia superior en la asimilación de nutrientes; y periodos cortos de producción sostenida durante todo el año. Los cultivos microalgales son independientes de la estacionalidad inherente a los cultivos agrícolas y de la fertilidad del suelo, condición que posibilita prescindir de herbicidas y pesticidas y además, permite emplear territorios no arables, incluyendo áreas marginales no aptas para agricultura (ej. desiertos y playas). En este sentido, las zonas áridas del norte de México, y otras, representan una opción promisoria para la instalación de sistemas de cultivo con microalgas; es posible aprovechar tierras que no son útiles para la agricultura, y/o alguna otra actividad económica, generando un valor agregado que también permite contribuir a la creación de empleos. En este proyecto se desarrolló una revisión de trabajos enfocados a los sistemas de producción de biodiesel utilizando microalgas, incluyendo selección de microalgas, el lugar y los modos de cultivo, y el estado del arte actual en la aplicación de estos cultivos a nivel comercial. Aunque se concluye que el potencial es enorme, la revisión también permite determinar que es necesario mayor investigación (tanto básica como aplicada), desarrollo tecnológico e innovación para lograr el éxito económico y comercial de cultivos microalgales enfocados a la producción de biodiesel.

XX.1.2 2nd International Conference on Algal Biomass, Biofuels and Bioproducts. San Diego CA, USA. June 10-13, 2012:

Batch Heterotrophic Culture of Neochloris oleoabundans Using Glucose as Carbon Source

(Póster)

Morales-Sánchez D., Tinoco R. and Martínez A.

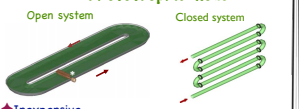
Batch heterotrophic culture of *Neochloris oleoabundans* using glucose as carbon source

Daniela Morales-Sánchez, Raunel Tinoco, Alfredo Martínez

Universidad Nacional Autónoma de México, Instituto de Biotecnología. A. P. 510-3. Cuernavaca, Mor. 62250, México. Email: dmorales@ibt.unam.mx

Introduction

Phototrophic mode



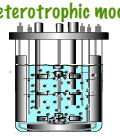
- Inexpensive construction and operation
- Evaporation losses
- Poor CO_2 transfer
- Easy to contamination

- Minimum loss of CO_2
- Low contamination risk
- Expensive construction

➡ LOW CELL DENSITY

➡ HIGH CELL MASS PRODUCTION COSTS

Heterotrophic mode

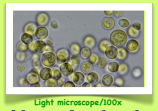


- Light is not needed
- Low contamination risk
- Culture conditions can be controlled

➡ HIGH CELL DENSITY

➡ HIGH LIPID PRODUCTIVITY

In comparison with phototrophic growth, heterotrophic conditions can significantly increase growth rates, final cell number and cell mass[1].

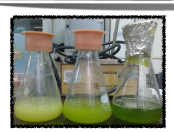


Neochloris oleoabundans is a green edaphic microalgae of biotechnological interest that accumulate lipids (rich in triacylglycerides) under phototropic and N-limited coditions.

The aim of this work was to characterize the performance of *Neochloris oleoabundans* growing in batch heterotrophic cultures with glucose as the only carbon source.

Materials and Methods

Cell and mass production of *N. oleoabundans* in batch heterotrophic culture



Shake flasks: Initial tests and Inocula development

Stirred tank 4L

- ☐ Glucose
- ☐ Bolds Basal Mineral Medium
- ☐ N source: Nitrate
- ☐ Dark conditons (shake flasks and bioreactors)

Evaluated conditions

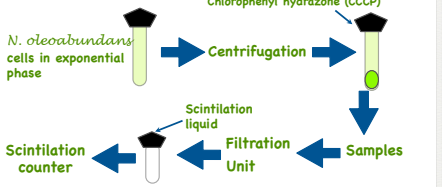
C/N ratio 17

C/N ratio 278

Glucose transport rate

a) [^{14}C]-glucose

b) [^{14}C]-glucose + Carbonyl Cyanide m-Chlorophenyl hydrazone (CCCP)



The glucose transport rate was determined by the incorporation of [14C]-glucose through the cell membrane.

Analytical procedures

- ☐ Cell growth: dry cell weight and cell number.
- ☐ Substrate concentration: Glucose (enzymatic analyzer), nitrate (ion selective electrode).
- ☐ Biochemical composition: Proteins (Lowry), Carbohydrates (phenol-sulphuric acid), Total Lipids (solvent extraction) and Fatty Acid Profile (Gas Chromatography – Mass Spectrometry).

Conclusion

N. oleoabundans heterotrophic cultures with glucose as the only carbon source allow obtaining 10-fold cell mass in comparison with phototropic cultures. Under nutrient sufficiency proteins were the major cell component. High C/N ratio increased significantly the cell mass and prolonged N-limitation promoted a high lipid content. *N. oleoabundans* uses a symporter system for glucose transporter.

These results allow proposing heterotrophic batch cultures of *N. oleoabundans* as an alternative for protein or lipid production.

Acknowledgments

This work was supported by:
The "Universidad Nacional Autónoma de México": Grant DGAPA/PAPIIT/UNAM IT200312-2.

Results

Shake flasks and mineral media allowed to determine that *N. oleoabundans* is able to grow using glucose as the only carbon source under dark conditions.

4-L heterotrophic cultures (C/N=17; Fig. 1A) showed a duplication time (t_D) of 13 h, that correspond to half the value of phototrophic cultures[2]; also, the cell number increased 3.5-times; and the cell mass yield on glucose was high ($Y_{X/S}$=0.57 g_{DCW}/g_{GLC}). Under this condition, there was no nutrient limitation (glucose or nitrate) and the major cellular component was protein (figure 1A).

N. oleoabundans could grow using high glucose concentration (50 g_{GLC}/L; C/N=278; Fig. 1B), also with a t_D of 13 h and $Y_{X/S}$=0.62 g_{DCW}/g_{GLC}. When these cultures entered into the phase of N-limitation accordingly the cell number didn't increase, but there was a 4-fold increase in cell mass (Fig. 1B); the cell mass productivity was 1.03 g_{DCW}/L/day; and lipid accumulation was promoted up to 50% of the cell content.

The major components of lipids in both cultures were oleic, palmitic, linoleic and stearic acid, respectively.

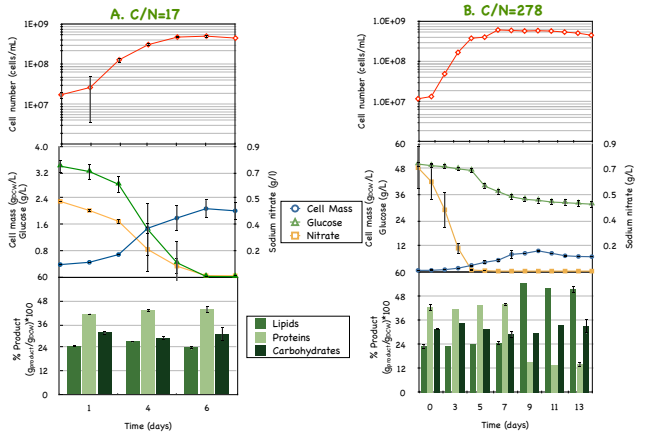
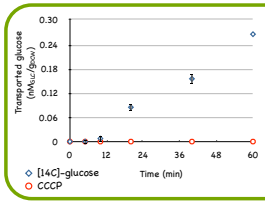


Fig. 1 Cell number, cell mass, nutrients uptake (glucose and nitrate) and biochemical composition kinetics of N. oleoabundans cultures with (A) C/N=17 and (B) C/N=278.



The glucose transmembranal transport rate of *N. oleoabundans* was 5 $\mu mol_{GLC}/g_{DCW}$/min and the glucose uptake rate was 8 $\mu mol_{GLC}/g_{DCW}$/min.

The CCCP inhibited the glucose transport, therefore the transmembrane transport was dependent of a proton-motive force indicating that the glucose transporter is a symporter system.

Fig. 2 Transmembrane glucose transport kinetic for cells obtained from a batch culture with C/N=17.

References

1. O'Grady, J., Morgan, J., 2010. Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. Bioprocess and Biosystems Engineering, Short communication 34(1), 121-125.
2. Garibay, A. 2009.(In spanish) Evaluación de condiciones de cultivo en la producción de lípidos con el alga Neochloris oleoabundans para su uso potencial como biodiesel. Mc S Thesis in Biochemistry Sciences. Instituto de Biotecnología/UNAM, México.

XX.1.3 2º Congreso Iberoamericano sobre biorrefinerias. Jaen, España. Abril 10-12, 2013:

Heterotrophic Growth of *Neochloris oleoabundans* Using Glucose as Carbon Source

(Póster)

Morales-Sánchez D., Tinoco R., Kyndt J. and Martínez A.

Heterotrophic growth of Neochloris oleoabundans usign glucose as carbon source

Daniela Morales-Sánchez, Raunel Tinoco, Alfredo Martínez*

Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México. A. P. 510-3, Cuernavaca, Mor. 62250, México.
*Corresponding author. *E-mail*: alfredo@ibt.unam.mx

Introduction

Phototrophic

Open system

- Inexpensive construction and operation
- Evaporation
- Poor CO_2 transfer
- Easy to contaminate

Closed system

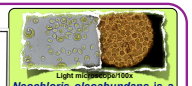
- Minimum loss of CO_2
- Low contamination risk
- Expensive construction

➡LOW CELL DENSITY
➡HIGH CELL MASS PRODUCTION COSTS

Heterotrophic

- Light is not needed
- Low contamination risk
- Culture conditions can be controlled

➡HIGH CELL DENSITY
➡HIGH LIPID PRODUCTIVITY



***Neochloris oleoabundans* is a green edaphic microalgae of biotechnological interest that accumulate lipids (rich in triacylglycerides) under phototropic and N-limited conditions.**

The aim of this work was to determine the carbon sources that can be metabolized by *N. oleoabundans* in heterotrophic cultures and to perform batch and fed-batch cultures supplemented with glucose to study the macromolecular composition of the microalgae under strict heterotrophic conditions and using balanced and high glucose/nitrate (C/N) ratios.

In comparison with phototrophic growth, heterotrophic conditions can significantly increase growth rates, final cell number and cell mass[1].

Materials and Methods

Cell and mass production of *Neochloris oleoabundans* in batch and fedbatch heterotrophic cultures

1. Carbon source study

Shake flasks

- Glucose
- Fructose
- Lactose
- Xylose
- Arabinose
- Glycerol
- Acetic acid
- Sucrose
- Cellobiose
- Dark conditions
- 300 rpm
- 25 ºC

2. Batch cultures

Bioreactor 4 L

- Glucose and sodium nitrate
- C/N = 17
- C/N = 278
- pH 7
- 25 ºC
- Dark conditions
- 300 rpm
- 1 vvm

3. Fedbatch cultures

Bioreactor 4 L

(A)Nitrate pulses feeding
(B)Exponentially feeding profile

- Glucose and sodium nitrate
- C/N = 278 initial
- pH 7
- 25 ºC
- Dark conditions
- 300 rpm
- 1 vvm

Analytical procedures

- **Cell growth:** dry cell weight and cell number.
- **Substrate concentration:** Glucose (enzymatic analyzer), nitrate (ion selective electrode).
- **Biochemical composition:** Proteins (Lowry), Carbohydrates (phenol-sulphuric acid), Total Lipids (solvent extraction).

Conclusion

N. oleoabundans **was able to grow under strict heterotrophic conditions with glucose or cellobiose as the only carbon source. No growth was obtainded usign xylose, arabinose, fructose, sucrose, lactose, glycerol or acetic acid. Batch cultures with balanced C/N ratios (17) accumulate proteins (up to 44 % w/w) as the major cell component; a high C/N ratio (nitrogen limitation during the stationary phase) significantly increases the dry cell mass and yields a high lipid content (up to 52 % w/w). High cell density and carbohydrate accumulation (up to 54 % w/w, mainly starch) were achieved using fed-batch cultures with the intermitent additions of nitrate. A exponentially feed-batch strategy controlling the growth rate with the nitrate feed rate allow maintain low levels of nitrate and promote the lipid accumulation (up to 52 % w/w).**

These results suggest heterotrophic batch and exponentially fedbatch cultures of *N. oleoabundans* as an alternative for the production of proteins or lipids with simple culture strategies and mineral media supplemented with glucose.

References

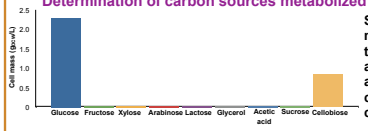
1. O'Grady, J., Morgan, J., 2010. Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. Bioprocess and Biosystems Engineering, Short communication 34(1), 121-125.

Acknowledgments

This work was supported by:
The "Universidad Nacional Autónoma de México": Grant DGAPA/PAPIIT/UNAM IT200312.

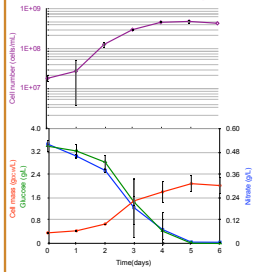
Results

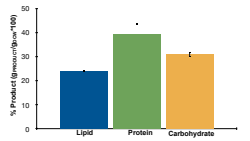
Determination of carbon sources metabolized by *N. oleoabundans*



Shake flasks and mineral media allowed to determine that *N. oleoabundans* is able to grow using glucose and cellobiose as the only carbon source under dark conditions.

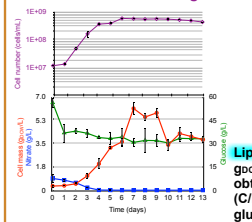
Batch cultures with an equilibrated C/N ratio allow protein accumulation

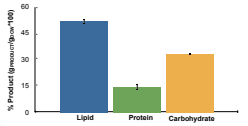




Batch cultures under a balanced C/N ratio of 17 (3 g/L of initial glucose) allowed a maximal cell mass of 1.72 g_{DCW}/L, with a high cell mass yield on glucose ($Y_{X/S}$=0.57 g_{DCW}/g_{GLC}). This condition produced protein as the major dry cell weight (DCW) component (0.44 g_{PROT}/g_{DCW}).

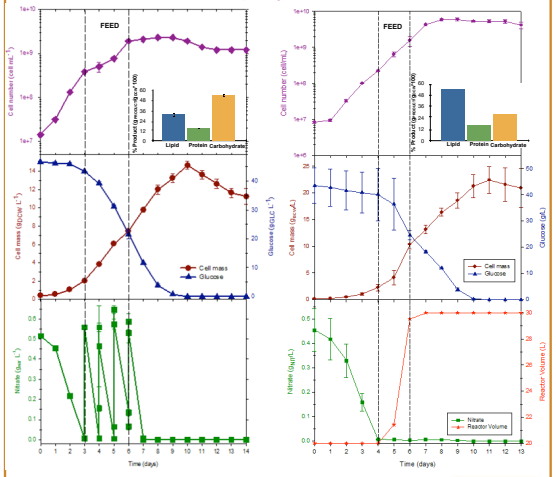
Batch cultures with high C/N ratio promote the accumulation of lipids





Lipid accumulation was stimulated (0.52 g_{LIP}/g_{DCW}) and a maximal cell mass of 9.2 g_{DCW}/L was obtained when nitrogen limited bacth cultures (C/N = 278) were performed with 50 g/L of intial glucose.

A fed-batch strategy allow to control the metabolism to the carbohydrate or lipid synthesis.



FBC allowed to reach a maximal cell mas of 14.2 g_{DCW}/L and with carbohydrates (mainly starch >80%) as the major cellular component (0.54 g_{CARB}/g_{DCW}). When *N. oleoabundans* was cultured using a exponential feed of nitrate during 2 days and then was maintaned in a batch stage under nitrogen depletion, a maximal cell mass of 22.1 g_{DCW}/L was obtained and the major cellular component were lipids (0.5 g_{LIP}/g_{DCW}).

XX.1.4 3rd International Conference on Algal Biomass, Biofuels and Bioproducts. Toronto, Canada. June 16-19, 2013:

Nitrogen-limited heterotrophic fedbatch cultures of the microalgae *Neochloris oleoabundans* to enhance lipid production

(Póster)

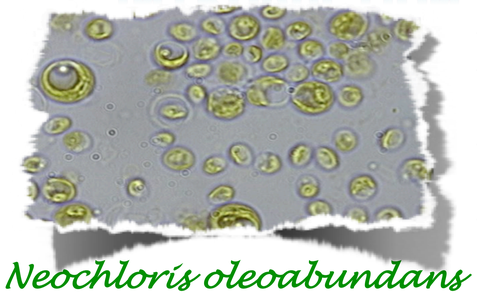
Morales-Sánchez D., Tinoco R., Caro M. and Martínez A.

NITROGEN-LIMITED HETEROTROFIC FEDBATCH CULTURES OF THE MICROALGAE *Neochloris oleoabundans* TO ENHANCE LIPID PRODUCTION

Daniela Morales-Sánchez, Mario Caro, Raunel Tinoco, Alfredo Martinez

Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional autónoma de México
A. P. 510-3. Cuernavaca, Mor. 62210. México. Email: dmorales@ibt.unam.mx

INTRODUCTION

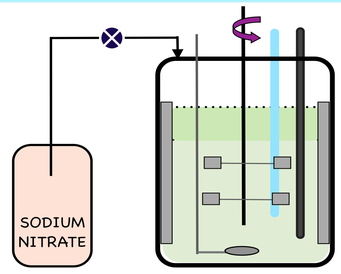


Neochloris oleoabundans

- Green edaphic microalgae
- Oleaginous microalgae
- Assimilation and metabolism of glucose in dark conditions
- N-limitation promote the accumulation of lipids under phototrophic conditions[1]
- Exponentially fedbatch cultures can increase the cell density and associated products[2]

The aim of this work was to evaluate the lipid accumulation in a system consisting of three stages of heterotrophic cultures of *N. oleoabundans*: starting with a nutrient sufficient stage; the second, a nitrogen fed stage was designed to increase the cell mass; and the last, a Nitrogen-starving stage was used to promote the accumulation of lipids.

MATERIALS AND METHODS



Fedbatch cultures

- Initial C/N ratio 278
- Glucose as carbon source
- Sodium Nitrate as nitrogen source and exponential feed during the 2nd stage
- A fixed specific growth rate (μ) of 0.05 h^{-1} was used for the 2nd stage, this μ is the maximum obtained in batch cultures without nutrient limitations

Analytical procedures

- **Cell growth:** dry cell weight and cell number.
- **Substrate concentrations:** Glucose (enzymatic analyzer), nitrate (ion selective electrode).
- **Biochemical composition:** Proteins (Lowry), carbohydrates (phenol-sulphuric acid), total lipids (extraction with solvent) and fatty acid profile (gas chromatography – mass spectrometry).

RESULTS

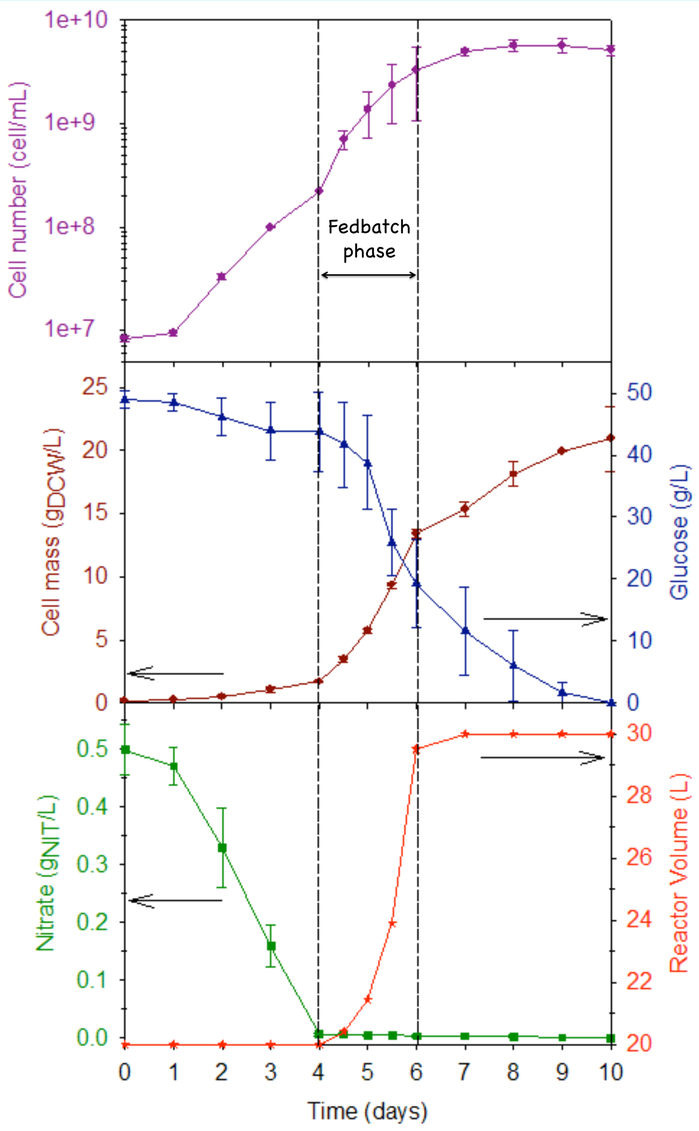


Figure 1. Exponentially fedbatch cultivation of *N. oleoabundans* (DCW: Dry Cell Weight).

In the first stage, a batch phase (Fig. 1), the microalga was grown without nitrogen or glucose limitation and the major cellular component was protein (Fig. 2). In the second-stage (Fig. 1), a fedbatch phase, with a controlled specific growth rate (μ) of 0.05 h^{-1} was set using an exponential nitrate-fed profile:

$$F = \frac{\mu X_0 V_0 \exp(\mu t)}{Y_{X/S} S_i}$$

where S_i is the substrate concentration in the inlet stream, V_0 and X_0 are the volume and biomass at the beginning of the fedbatch phase, t is the elapsed time of the fedbatch phase, and the $Y_{X/S}$ is the cell yield on nitrate. The fedbatch phase was designed to allow one cell duplication. In the third stage (Fig. 1), a second batch phase, the nitrate was depleted and the residual glucose (19.3 g_{GLC}/L) was used to promote the accumulation of reserve metabolites. At this nitrogen-starving condition accordingly the number of cells did not increase, but there was a 2-fold increase in the cell mass (Fig. 1).

At the end of the culture, when the glucose was depleted, the lipid accumulation was promoted up to 50% of the dry cell weight (Fig. 2); being the oleic (55.3%), linoleic (22.7%) and palmitic (13.3%) acids the main components. The protein and carbohydrate content was 17.9 and 25.1% of the DCW, respectively. Overall this strategy allowed obtaining a cell mass of 20.72 g_{DCW}/L, yields of 0.42 g_{DCW}/g_{GLC}, 0.68 g_{DCW}/g_{NIT} and 0.22 g_{LIP}/g_{GLC}, with a high lipid production: 11.02 g_{LIP}/L.

Table 1. Kinetic and stoichiometric parameters evaluated in this work.

Parameter	1st Batch phase	Fedbatch phase	2nd Batch phase	Global
μ (1/h)	0.024 ±0.002	0.043 ±0.001	0.0033 ±0.002	0.019 ±0.0005
ΔX (g_{DCW}/L)	1.51 ±0.095	11.73 ±0.250	7.47 ±2.20	20.72 ±2.500
$Y_{DCW/GLC}$ (g_{DCW}/gGLC)	0.24 ±0.045	0.50 ±0.050	0.40 ±0.03	0.42 ±0.040
$Y_{DCW/NITRATE}$ (g_{DCW}/g_{NIT})	3.07 ±0.006	0.39 ±0.008	NA	0.68 ±0.08
$Y_{LIPID/GLC}$ (g_{LIP}/g_{GLC})	0.04 ±0.004	0.15 ±0.020	0.4 ±0.18	0.22 ±0.008
$Y_{LIPID/NITRATE}$ (g_{LIP}/g_{NIT})	0.50 ±0.010	0.11 ±0.008	NA	0.36 ±0.004
Q_{DCW} (g_{DCW}/L/day)	0.380 ±0.020	5.87 ±0.120	1.87 ±0.55	2.07 ±0.250
Q_{LIPID} (g_{LIP}/L/day)	0.060 ±0.006	1.7 ±0.120	1.82 ±0.09	1.09 ±0.010
$Q_{PROTEIN}$ (g_{PROT}/L/day)	0.130 ±0.008	2.82 ±0.010	0.82 ±0.07	0.29 ±0.030
$Q_{CARBOHYDRATE}$ (g_{CARB}/L/day)	0.100 ±0.004	2.12 ±0.140	0.14 ±0.01	0.52 ±0.030

μ: growth rate; ΔX: increase in DCW; $Y_{DCW/GLC/NITRATE}$: cell yield on glucose/nitrate; $Y_{LIPID/GLC/NITRATE}$: lipid yield on glucose/nitrate; $Q_{DCW/LIPID/PROTEIN/CARBOHYDRATE}$: cell/lipid/protein/carbohydrate productivity; NA: Not Applicable

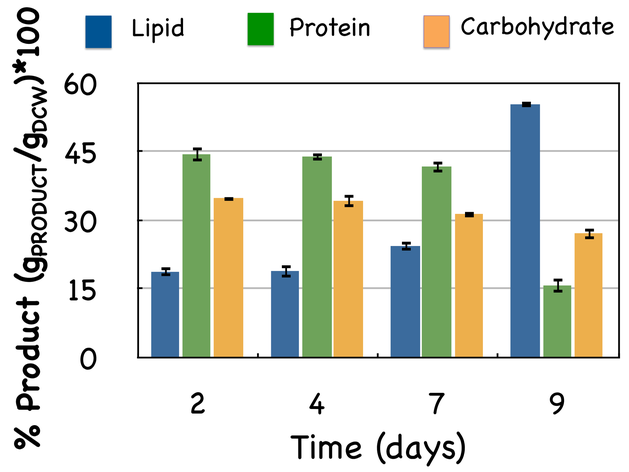


Figure 2. Biochemical composition of *N. oleoabundans* during cultivation.

CONCLUSION

The exponentially fedbatch strategy allowed to obtain a high cell mass production of *N. oleoabundans*. Maintaining an actual growth rate of 0.043 h^{-1} during the feed and a concentration of the limiting substrate close to zero allowed to increase the number of cells up to $5x10^9$ cells/mL. Lipid and cell mass productivities of 1,090 mg_{LIPID}/L/day and 2,070 mg_{DCW}/L/day were obtained in this work. To our knowledge, these are the highest lipid and cell mass productivities reported until now for cultures of *N. oleoabundans*.

REFERENCES

[1]Gouveia L, Marques A, da Silva T, Reis A: *Neochloris oleoabundans* UTEX 1185: a suitable renewable lipid source for biofuel production. J Ind Microbiol Biotechnol 2009, 36:821-826.

[2]De Swaaf M, Sijtsma L, Pronk J: High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnni*. Biotechnol Bioeng 2003, 81:666-672.

ACKNOWLEDGMENTS

This work was supported by: Universidad Nacional Autónoma de México
Grant: DGAPA / PAPIIT / UNAM IT200312.

XX.1.5 3rd International Conference on Algal Biomass, Biofuels and Bioproducts. Toronto, Canada. June 16-19, 2013:

A proteomic study of *Neochloris oleoabundans* grown under heterotrophic conditions

(Póster)

Kyndt J., Morales-Sánchez D., Ogden K. and Martínez A.

A Proteomic Study of *Neochloris oleoabundans* Grown Under Heterotrophic Conditions

J. Kyndt*[1], D. Morales-Sánchez[2], K. Ogden[3], A. Martinez[2]
1.College of Science and Technology, Bellevue University, USA;
2.Instituto de Biotecnología, UNAM, Mexico;
3.Department of Chemical and Environmental Engineering, University of Arizona, USA.

Abstract:
Neochloris oleoabundans is an oleaginous microalgae of biotechnological and commercial interest. Our previous work under strict heterotrophic cultivation indicated that this microalgae can accumulate lipids under nitrogen starvation in batch cultures, but is also able to accumulate carbohydrates under fedbatch cultures with short nitrate pulses. The aim of this work was to compare the proteomes in both lipid and carbohydrate accumulating conditions and to identify the metabolic switches as potential targets for future mutagenesis studies.
This proteomic study is a first approach to unveiling the lipid and carbohydrate metabolism of unsequenced *N. oleoabundans.* By comparing differentially expressed proteins under two metabolite accumulating conditions we revealed the regulation of key proteins in essential pathways involved in lipid synthesis, carbohydrate production and energy flux in *N. oleoabundans*.

Introduction:

Our previous results indicated that *Neochloris oleoabundans*, a microalgae of biotechnological and commercial interest, can accumulate lipids under long period of nitrogen starvation (-N condition) and is able to switch its metabolism to accumulate carbohydrates under heterotrophic fed-batch cultures with sodium nitrate pulses, which induces short periods of nitrogen limitation (*1*; Figure 1). The genome of *N. oleoabundans* has not been sequenced yet. In order to identify the metabolic switches involved, a proteomic study was carried out comparing cells grown under these two conditions.
Our understanding of algal metabolism is still largely inadequate. The lack of available genome sequence information limits the development of biological tools that are required for strain improvement of unsequenced microalga. However, rapidly developing systems biology approaches such as transcriptomics, proteomics, and metabolomics have become very effective and essential for understanding how microorganisms respond and adapt to changes in their physical environment (*2, 3*).

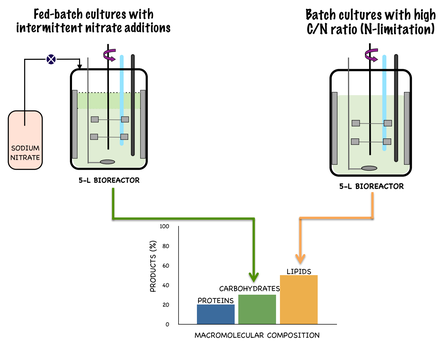


Figure 1. Physiological response of *Neochloris oleoabundans* under two strategies of heterotrophic cultivation.

Materials and Methods:

The protein extractions were performed using a mechanical lysis and acetone cleanup steps and a final 2D Cleanup Kit (GE Healthcare). The proteomic analysis was performed using liquid chromatography to separate peptides prior to MS/MS analysis in an LTQ Orbitrap Velos mass spectrometer (Figure 2). The MS/MS spectra were searched against all the theoretical peptide MS/MS spectra from Chlorophyta proteins downloaded from UniProt. Unidentified peptides were further analyzed by BLAST (NCBI) against plant and algal databases. A total of 398 proteins were identified (Figure 3).

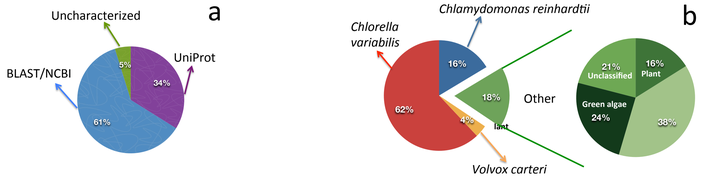


Figure 3. Protein distribution found in the databases (a) and top-hit species distribution for UniProt and BLAST-NCBI matches for the *N. oleoabundans* proteome (b).

References:
1. Morales-Sánchez, D., Tinoco-Valencia, R., Kyndt, J., and Martinez, A., 2013, Heterotrophic growth of *Neochloris oleoabundans* using glucose as carbon source, *Biotechnology for Biofuels*, submitted.
2. Guarnieri MT, Nag A, Smolinski SL, Darzins A, Seibert M, Pienkos PT, 2011, Examination of triacylglycerol biosynthetic pathway via De novo transcriptomic and proteomic analyses in an unsequenced microalga. *PLoS ONE* 2011 6(10): e25851.
3. Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C., and Peccia, J., 2012, Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insight into triacylglyceride accumulation, *Biotechology for Biofuels 5*, 74.

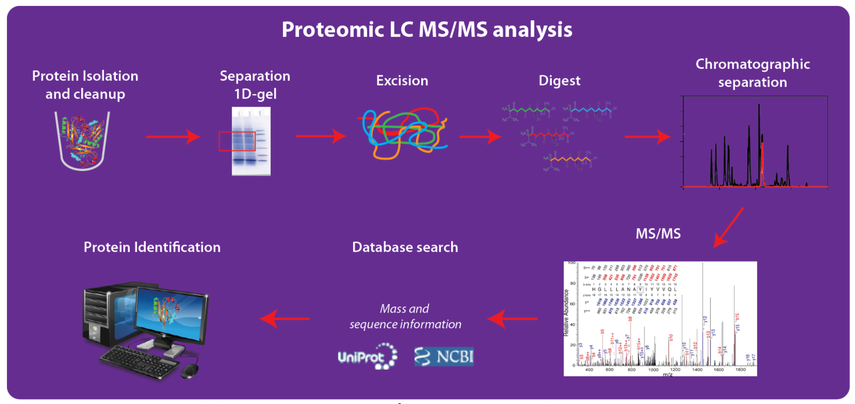


Figure 2. Overview of the proteomic LC MS/MS experimental setup.

Results:

Among the differentially expressed proteins, 14 were involved in the metabolism of lipids and nitrogen with a higher level of abundance in the –N condition (Figure 4). In the carbohydrate accumulation condition 5 differentially expressed proteins were found with a higher level of abundance and involved in starch and sugar biosynthesis (Figure 5a).
To supply precursors for lipid production, proteins involved in the glycolysis (Figure 5b) and mainly the protein pyruvate dehydrogenase complex -for converting pyruvate to acetyl-CoA- was highly abundant in the -N condition (Figure 5d). To supply the necessary biological energy and reducing power (ATP and NADPH) for fatty acid production, cells increased production under the –N scenario of proteins in the tricarboxylic acid cycle (Figure 5d) and the pentose phosphate pathway (Figure 5c). These proteins are primarily involved in supplying reducing equivalents for anabolic metabolism, including the production of fatty acids and assimilation of inorganic nitrogen.

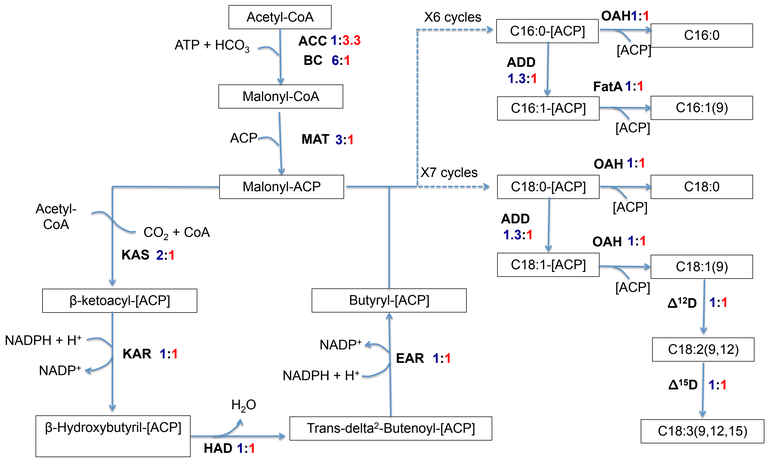
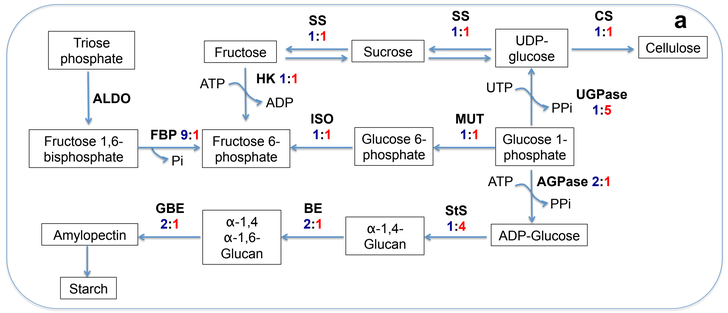
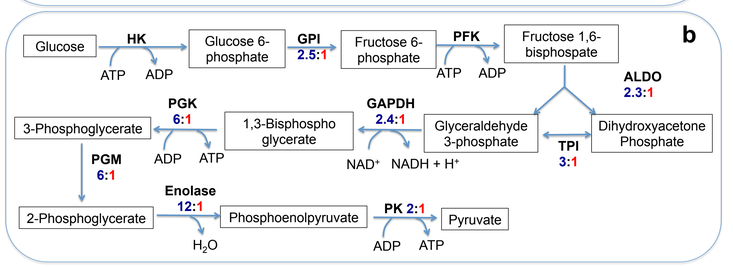


Figure 4. Overview of the fatty acid biosynthesis pathway in green algae. The numbers represent the relative ratio of protein abundance. Numbers in blue are for the N-deprivation condition and numbers in red the carbohydrate accumulation condition. ACC: Acetyl CoA carboxylase; **BC**: Biotin carboxylase; **MAT**: Malonyl-CoA transacylase; **KAS**: β-ketoacyl-ACP synthase; **KAR**: β-ketoacyl-ACP reductase; **HAD**: β-hydroxyacyl-ACP dehydrase; **EAR**: Enoyl-ACP reductase; **AAD**: Acyl-ACP desaturase; **OAH**: oleoyl-ACP hydrolase; **FatA**: Acyl-ACP thioesterase A; **Δ12D**: $\Delta^{12}D(\omega^6)$-desaturase; **Δ15D**: $\Delta^{15}D(\omega^3)$-desaturase.





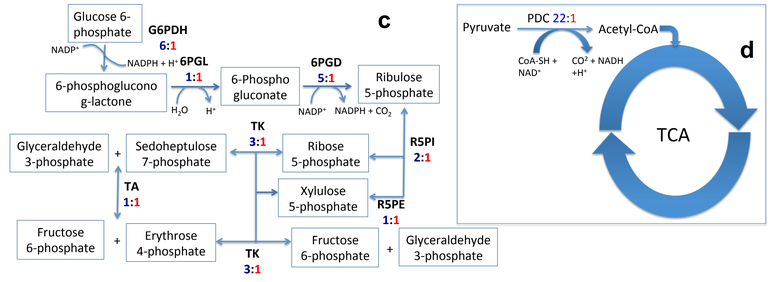


Figure 5. Overview of the carbohydrate biosynthesis (a), glycolysis (b), pentose phosphate pathway (c), and tricarboxylic acid cycle (d). SS: Sucrose synthase; **CS:** Cellulose synthase; **ALDO:** Aldolase; **HK**: Hydroxykinase; **FBP**: Fructose bisphosphatase; **ISO:** Isomerase; **MUT:** Mutase; **UGPase**:UDP-glucose pyrophosphorylase; **AGPase**: ADP-glucose pyrophosphorylase; **St S:** Starch synthase; **BE:** Branching enzyme; **GBI:** Glucan branching enzyme; **GPI:** Glucose 6-phosphate isomerase; **PFK:** Phosphofructokinase; **PGK:** Phosphoglycerate mutase; **GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase; **PGM:** Phosphoglycerate mutase; **TPI:** Triose phosphate isomerase; **PK:** Pyruvate kinase; **G6PDH:** Glucose 6-phosphate dehydrogenase; **6PGL:** 6-Phosphogluconolactonase; **6PGD:** 6-Phosphogluconate dehydrogenase; **TK:** Transketolase; **R5PI:** Ribulose 5-phosphate isomerase; **TA:** Transaldolase; **R5PE:** Ribulose 5-phosphate epimerase; **PDC:** Pyruvate dehydrogenase complex.

Conclusions:

- Key enzymes in the fatty acid synthesis pathway are upregulated under N starvation conditions, thereby increasing carbon flux to lipid synthesis.
- Carbohydrate synthesis related enzymes that channel carbon to starch and sugar synthesis are highly upregulated when short durations of N limitation are encountered.
- The cells are optimizing the energy flux and reducing power production in response to higher energy requirements to synthesize lipids versus carbohydrate production.

XX.2 Articulos de divulgación de la ciencia

XX.2.1 Unión de Morelos. Periódico local, 26 de Agosto de 2013: "Pintando de verde el futuro de los combustibles: Primera parte: Importancia de las algas en la generación de los combustibles fósiles y de biorefinerias en el presente"

XX.2.2 Unión de Morelos. Periódico local, 2 de Septiembre de 2013: "Pintando de verde el futuro de los combustibles: Segunda parte: Cultivos de algas, características y ventajas"

XX.2.1 Unión de Morelos. Periódico local, 26 de Agosto de 2013:

Pintando de verde el futuro de los combustibles: Primera parte: importancia de las algas en la generación de los combustibles fósiles y de biorefinerias en el presente

Daniela Morales-Sánchez y Alfredo Martínez Jiménez

ACADEMIA DE CIENCIAS DE MORELOS, A.C.

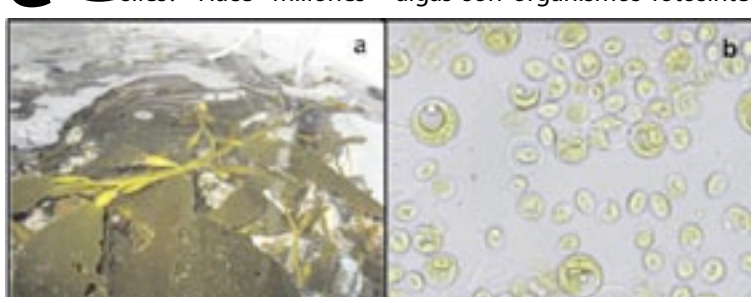
¿Comentarios y sugerencias?, ¿Preguntas sobre temas científicos? CONTÁCTANOS: editorial @acmor.org.mx

Pintando de verde el futuro de los combustibles:

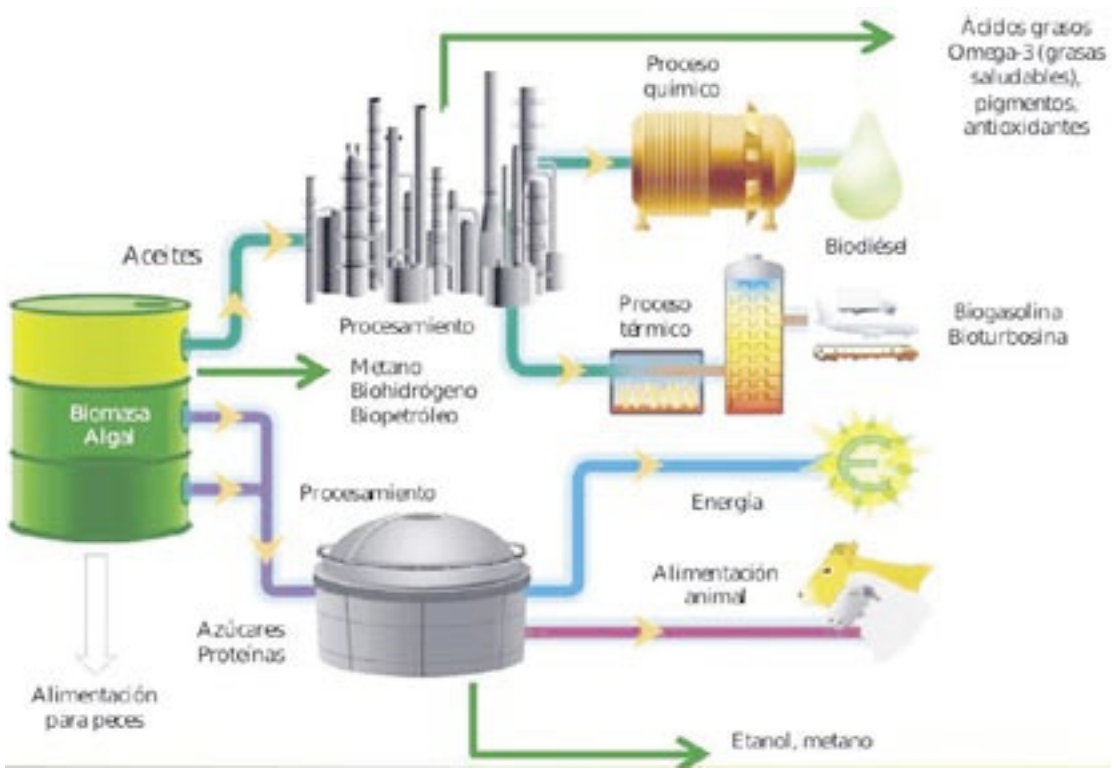
Primera parte: importancia de las algas en la generación de los combustibles fósiles y de biorefinerias en el presente

M. en C. Daniela Morales Sánchez, Estudiante de Doctorado, Instituto de Biotecnología de la UNAM.
Dr. Alfredo Martínez Jiménez, Miembro de la Academia de Ciencias de Morelos, Investigador Titular del Instituto de Biotecnología de la UNAM Campus Morelos.

¿Conoces el origen de los combustibles fósiles? Hace millones de años los combustibles fósiles, tales como el petróleo, carbón y gas natural, se formaron a partir de la lenta descomposición de organismos como peces, plantas y algas marinas que quedaron sepultados por la arena y la arcilla bajo temperaturas y presiones muy altas. Es decir, que una parte o una gran parte de los combustibles tienen su origen en las algas. Pero ¿qué son las algas? Las algas son organismos fotosintéticos, que necesitan del bióxido de carbono y la luz del sol para poder vivir, crecer y reproducirse, y están presentes en todos los ecosistemas existentes de la tierra, tanto acuáticos como terres-



1.Macrocystis pyrifera (a) y Neochloris oleoabundans (b), macro y microalga de interés biotecnológico y comercial, ya que pueden ser utilizadas para alimentación (animal y humana) por su contenido proteico y para la generación de biocombustibles por su contenido de aceites y carbohidratos.



2.Esquema general de procesos para la producción de biocombustibles, ácidos grasos, pigmentos y alimentos para ganado a partir de cultivos microalgales, entre otros. Al concepto de generar varios productos a partir de material biológico, similares a los obtenidos a partir del petróleo en una refinería, se le conoce con el nombre de *'Biorefinería'* (Adaptado de Olivares, 2011).

ACADEMIA DE CIENCIAS DE MORELOS, A.C.

¿Comentarios y sugerencias?, ¿Preguntas sobre temas científicos? CONTÁCTANOS: editorial @acmor.org.mx

tres, representando una gran variedad de especies y tamaños, algunas visibles a simple vista como las macroalgas (figura 1a) y otras de tamaño microscópico como las microalgas (figura 1b).
La hipótesis más aceptada para la generación de petróleo indica que este combustible fósil en efecto se originó en gran parte a partir de algas, en escalas geológicas de tiempo; fue un proceso muy lento, que requirió millones de años. Pero ahora, gracias a los avances en biotecnología, podemos obtener combustibles a partir de algas en tan sólo días. Pues sí, resulta que cada célula de alga (o en otras palabras, el 'cuerpo' del alga que en adelante llamaremos biomasa) está formada por proteínas, aceites y carbohidratos, entre otros muchos compuestos. El contenido de aceites y carbohidratos dentro de la *biomasa* es dependiente de la especie de alga que se cultive. Algunos tipos de algas tienen una alta preferencia por almacenar aceites (40-70% dentro de su biomasa) y otras son ricas en carbohidratos (40-80%), la mayoría contienen entre 15 y 50% de proteínas.
El aceite y los carbohidratos de las algas pueden ser utilizados como materias primas para la elaboración de diversos biocombustibles. Mediante el proceso de 'fotosíntesis' las microalgas y macroalgas utilizan la energía del sol para crecer y reproducirse mientras capturan el dióxido de carbono que utilizarán para almacenar energía en forma de aceites y carbohidratos dentro de su biomasa; este proceso es similar al que realizan las plantas como el maíz y la soya. Posteriormente, esos aceites y carbohidratos pueden ser extraídos de la biomasa de las algas (también puede utilizarse su biomasa completa) para ser convertidos en una amplia variedad de biocombustibles renovables a partir de diferentes procesos. Tales biocombustibles incluyen: el metano (o biogás), el cual es un gas combustible que se genera por la descomposición de la biomasa del alga mediante la acción de microorganismos como bacterias, en ausencia de oxígeno (sin aire); el biodiésel, producido a partir del aceite que se extrae de la biomasa de las algas y que posteriormente es transformado en un biocombustible con propiedades similares al diésel a partir de procesos químicos; el biohidrógeno, que se obtiene mediante un proceso natural que realizan algunos tipos de algas verdes cuando crecen en presencia de luz, ausencia de oxígeno y se activa una enzima (catalizador biológico que produce la misma alga) llamada hidrogenasa, encargada de convertir la energía derivada de la fotosíntesis en hidrógeno. Las algas también contienen diferentes tipos de carbohidratos dentro de su biomasa. Existen procesos tecnológicos para aprovechar estos carbohidratos y convertirlos en biocombustibles como el etanol (ver por ejemplo, I. Muñoz y A. Martínez, *"El alcohol como biocombustible: El ejemplo brasileño"* en La Unión de Morelos del 26 de diciembre de 2011, disponible en http://bit.ly/19EcvFN). Los carbohidratos se extraen mediante la ayuda de procesos químicos y biológicos, y posteriormente se transforman en azúcares simples como la glucosa, tal y como se hace con el almidón del maíz. Posteriormente estos azúcares simples son aprovechados por microorganismos como las levaduras para producir el etanol mediante un proceso llamado fermentación, el mismo tipo de proceso utilizado para la elaboración de cerveza. Existen otros componentes dentro de la biomasa llamados *isoprenoides* que son hidrocarburos (compuestos por carbono e hidrógeno) que pueden producir biogasolina y un combustible para aviación denominado bioturbosina. En otro tipo de procesos, toda la biomasa "cosechada" de los estanques de cultivo de las algas se somete a altas presiones y temperaturas y se genera un producto parecido al petróleo, el cual se denomina biopetróleo o biocrudo.
Como se ha resumido, se pueden obtener muchos biocombustibles a partir de algas mediante procesos que se pueden integrar como se ilustra en la figura 2, donde se muestra que también se pueden adaptar estas tecnologías a la generación de otras sustancias con alto valor, tales como antioxidantes o pigmentos y a la producción de alimento para ganado o para la acuacultura.
Es necesario recordar que los recursos energéticos fósiles no son eternos y que debemos contribuir con el cuidado del medio ambiente para las próximas generaciones. El uso de algas para la producción de combustibles alternos contribuye con el cuidado del medio ambiente y puede proveer de energía, y otros productos, incluyendo proteínas, para llevar a cabo las actividades de la vida cotidiana. Por esto, es necesario multiplicar los esfuerzos en investigación y desarrollo para que estas tecnologías sean viables en México y para que podamos pintar de verde el futuro de los combustibles de las próximas generaciones. Aún queda mucho por hacer en el campo de investigación de las algas, ya que se desea optimizar la producción de biomasa algal y/o el contenido dentro de la biomasa de aceite, carbohidratos y en su caso proteínas y otros compuestos de mayor valor comercial como los antioxidantes; el tópico de producción de biomasa algal es tema de una siguiente entrega de "Lunes en la Ciencia".

Referencias

Olivares J. A. (2011). Innovations in algal biofuels within NAABB. Trabajo presentado en: "International Algae Congress". 1 y 2 de Diciembre de 2011. Berlín, Alemania.

Para actividades recientes de la Academia y artículos anteriores puede consultar: www.acmor.org.mx

XX.2.2 Unión de Morelos. Periódico local, 2 de Septiembre de 2013:

Pintando de verde el future de los combustibles: Segunda parte: cultivos de algas, características y ventajas

Daniela Morales-Sánchez, Mitsue León Saiki y Alfredo Martínez Jiménez

ACADEMIA DE CIENCIAS DE MORELOS, A.C.

¿Comentarios y sugerencias?, ¿Preguntas sobre temas científicos? CONTÁCTANOS: editorial @acmor.org.mx

Pintando de verde el futuro de los combustibles

Segunda parte: cultivos de algas, características y ventajas

***M en C Daniela Morales Sánchez**, Estudiante de Doctorado, Instituto de Biotecnología de la UNAM.*
***M en C. Mitsue León Saiki,** Exestudiante de Maestría, Instituto de Biotecnología de la UNAM.*
***Dr. Alfredo Martínez Jiménez**, Miembro de la Academia de Ciencias de Morelos, Investigador Titular del Instituto de Biotecnología de la UNAM Campus Morelos.*

Sistema abierto al ambiente para el cultivo de microalgas y macroalgas en estanques tipo "pista de carreras".

Adicionalmente a lo presentado la semana pasada en este espacio (ver referencia [1]), las microalgas pueden emplearse para resolver problemas de tipo ambiental, alimentándolas con carbono proveniente del dióxido de carbono generado por las industrias y/o propagándolas en aguas residuales. Uno de los principales efluentes de las plantas termoeléctricas es un gas con elevado contenido de dióxido de carbono que, de no ser capturado, se dispersaría en la atmósfera como parte de las emisiones de gases de efecto invernadero. En cultivos *fototróficos*, es decir, que obtienen su energía de la luz, como los cultivos de algas que llevan a cabo la fotosíntesis, fijando de forma natural el dióxido de carbono y transformándolo mediante reacciones bioquímicas en carbono orgánico que forma aproximadamente el 40% en peso de la masa celular de las algas. De esta manera, el cultivo de algas puede contribuir a capturar el carbono que, de otra forma, contribuiría al efecto invernadero. Otro problema ambiental para el cual se ha propuesto el uso de las algas es el tratamiento de aguas residuales. Las aguas residuales provenientes del hogar, industrias y/o agricultura son una fuente rica en nitrógeno y fósforo, 'alimentos' indispensables para el crecimiento de las algas. Adicionalmente al beneficio ambiental, el uso de estas estrategias ayuda a reducir los costos de producción, ya que no es necesario comprar los nutrientes que requieren las algas para crecer.

Para obtener una cantidad considerable de biomasa de algas, éstas se tienen que "cultivar" de forma similar a los vegetales, pero, en lugar de usar una parcela de tierra, el cultivo de micro y macroalgas se realiza en grandes recipientes con un medio de cultivo consistente de agua en la que se disuelven los componentes que requiere el alga para crecer. Éstos son nutrientes idénticos a los que se usan como fertilizantes para el cultivo de plantas. Tomemos como ejemplo a las algas microscópicas o microalgas. Éstas son comúnmente cultivadas en largos estanques de poca profundidad, que, desde arriba, parecen pistas de carrera y que se encuentran abiertos al medio ambiente (figura 1). Se cultivan también en "fotobiorreactores", recipientes transparentes cerrados, cuando se requiere evitar el contacto directo entre el ambiente y el cultivo (figura 2), permitiendo el paso de la luz y con la "alimentación" de una corriente de gas que contiene dióxido de carbono. El cultivo de algas en estanques abiertos es más económico y sencillo en cuanto al diseño y material de construcción (generalmente concreto, resinas o diferentes tipos de plástico) y son relativamente fáciles de operar. Sin embargo, este sistema tiene las desventajas de depender de las condiciones climatológicas (por ej. variaciones de temperatura durante el día, dilución de los cultivos por acumulación de agua cuando llueve o bien perdida de agua evaporación de agua cuando la temperatura es elevada) y de que puede ser invadido por microorganismos e incluso por insectos y/u otros animales. Por su parte, los fotobiorreactores (figura 2) no sufren los problemas de contaminación de los estanques abiertos, ya que el cultivo no tiene contacto directo con el ambiente exterior, pero tienen la desventaja de ser más caros debido al diseño y modo de operación. Por esta razón, el uso de este tipo de sistemas se limita actualmente a la producción de biomasa para alimentación en la acuacultura y a la investigación para definir las mejores condiciones de cultivo, es decir aquellas que permiten encontrar estrategias y condiciones para lograr altas productividades de biomasa algal, para después usarlas en cultivos abiertos.

Desde los años 50's se había tenido la idea de producir combustibles a partir de algas, aunque en ese tiempo no resultaba económicamente viable, principalmente, porque los combustibles fósiles eran muy baratos y abundantes. En los años 70's la idea volvió a tomar importancia debido al encarecimiento de los combustibles derivados del petróleo. Sin embargo, por muchos años las algas continuaron siendo utilizadas especialmente para alimentación humana, como las algas que nos comemos en el sushi o la "spirulina" que se producía en el lago de Texcoco, usada como complemento alimentico los deportistas, en la acuacultura y en la obtención de algunos productos especializados como el polímero biológico conocido como "alginato" que usan los dentistas para tomar impresiones de nuestros dientes o el agar que se utiliza en los laboratorios de microbiología para generar una base semisólida para el crecimiento de muchos microorganismos. No obstante, fue hasta el 2008 que a las algas se les consideró nuevamente como una opción viable de cultivo para obtener biocombustibles, cuando los precios de los combustibles fósiles empezaron a elevarse hasta llegar a más de 130 dólares por barril de petróleo. A consecuencia de esto, en varias partes del mundo, la iniciativa privada y/o los gobiernos han estado invirtiendo muchos recursos para desarrollar biocombustibles a partir de algas. Tanto ha sido el interés por las algas que una de las empresas más importantes, la productora más grande de aviones –Boeing– ha realizado inversiones del orden de millones de dólares apostando por los biocombustibles a partir de algas. Esa inversión se debe al convencimiento de que el desarrollo de los combustibles de nueva generación a partir de algas es inaplazable. En el Instituto de Biotecnología de la UNAM, nuestro grupo de trabajo realiza investigación para desarrollar aplicaciones del cultivo de microalgas dentro del concepto de la biorefinería (ver la referencia [1]) y la figura 2 ejemplifica algunos de los sistemas de cultivo que hemos empleado en las pesquisas.

En realidad, el uso de las algas en la producción de biocombustibles es bastante promisorio y ventajoso, dado que otros biocombustibles que actualmente se desarrollan en varios países provienen de cultivos vegetales empleados en la alimentación, como es el caso del bioetanol a partir de la caña de azúcar o del almidón de maíz. El cultivo de algas, a diferencia de estos recursos vegetales, no requiere del uso de tierras fértiles, no necesita lluvia, no requiere de grandes cantidades de agua además de que ésta se puede reciclar, no se trata de un sistema de producción estacional como el de los vegetales, y, lo más importante en estos tiempos de escasez alimentaria, no compite por materias primas con los sistemas actuales de producción de alimentos. Frecuentemente, al pasar junto a un estanque de algas consideramos que sólo es agua sucia; sin embargo, las ventajas del cultivo de algas hará que la población se dé cuenta gradualmente de que constituye una opción atractiva en el mediano y largo plazos para la generación de biocombustibles en biorefinerías y para la resolución de problemas ambientales.

Actualmente, se utilizan diversas estrategias para seleccionar especies de algas con un mayor contenido de aceites, carbohidratos y/u otros componentes, y se mejoran las estrategias de cultivo y los procedimientos de cosecha y extracción de productos. Hay que recordar que adaptar a una especie a las necesidades de la humanidad no es tarea fácil. La domesticación de cultivos como el maíz y la soya llevaron cientos de años para poder producirse en la forma masiva que permite la alimentación de cientos de millones de personas. En el caso de las algas, el objetivo es la 'biorefinería'. Un proceso sustentable y amigable con el medio ambiente que permitirá la obtención de biocombustibles, así como de varios productos de interés (pigmentos, alimento para ganado y peces, suplementos alimenticios, entre otros), a partir de la biomasa de diferentes algas.

Sistemas cerrados al ambiente para el cultivo de microalgas en fotobiorreactores.

Referencias
1. Daniela Morales Sánchez y Alfredo Martínez Jiménez, *Pintando de verde el futuro de los combustibles. Primera parte: importancia de las algas en la generación de los combustibles fósiles y de biorefinerias en el presente*, Unión de Morelos, 26 de agosto de 2013, p. 30, http://bit.ly/197ZjYK.

Para actividades recientes de la Academia y artículos anteriores puede consultar www.acmor.org.mx

CARTELERA CINES

VIGENCIA: DEL VIERNES 30 DE AGOSTO AL JUEVES 05 DE SEPTIEMBRE DEL 2013.

DIANA
LOS ILUSIONISTAS DIG ING 11:30 / 13:50 / 18:40
EL CONJURO DIG ESP 16:00 / 21:00
CAZADORES DE SOMBRAS DIG ING 12:00 / 14:40 / 17:20 / 20:00 / 22:30
EL CONJURO DIG ING 12:10 / 14:30 / 17:00 / 19:40 / 22:00
CAZADORES DE SOMBRAS DIG ESP 11:00 / 13:40 / 16:20 / 19:00 / 21:40
EL CONJURO DIG ESP 12:30 / 14:50 / 17:10 / 19:30 / 22:10
ONE DIRECTION DIG ASI SOMOS 15:30 / 19:50 / 21:50
ONE DIRECTION DIG 3D ASI SOMOS 11:10 / 13:20 / 17:40
ARMADAS Y PELIGROSAS DIG 12:50 / 15:15 / 17:40 / 20:10 / 22:40
PERCY JACKSON 2 DIG ESP V-D 11:40 / 14:10 L-V 11:40
NO SE SI CORTARME LAS VENAS DIG V-D 16:40 / 19:20 / 21:30 L-V 14:10 / 16:40 / 19:20 / 21:30
LOS PITUFOS 2 DIG V-D 11:20 / 15:50 L-V 11:20
SON COMO NIÑOS 2 DIG ESP V-D 18:20 / 20:40 / 22:50 / L-V 15:50 / 18:20 / 20:40 / 22:50

JACARANDAS
SON COMO NIÑOS 2 DIG ESP 11:10 / 13:20 / 15:20 / 17:20 / 19:20 / 21:50
LOS ILUSIONISTAS DIG ESP 18:05 / 22:30
SON COMO NIÑOS 2 DIG ESP 11:50 / 14:00 / 16:05 / 20:20
ONE DIRECTION DIG 3D ASI SOMOS 11:00 / 13:00 / 15:00 / 17:00 / 19:00
JURASSIC PARK 3D ESP 21:00
CAZADORES DE SOMBRAS DIG ESP 11:00 / 13:30 / 16:00 / 18:30 / 21:20
CAZADORES DE SOMBRAS DIG ESP 12:15 / 14:45 / 17:15 / 19:45 / 22:40
EL CONJURO DIG ESP 11:00 / 13:15 / 15:30 / 17:45 / 20:00 / 22:15
EL CONJURO DIG ESP 12:00 / 14:20 / 16:30 / 18:45 / 21:30
ARMADAS Y PELIGROSAS DIG 11:15 / 13:35 / 15:55 / 18:15 / 20:35 / 22:55
ARMADAS Y PELIGROSAS DIG 12:40 / 15:15 / 17:35 / 19:55 / 22:20
NO SE SI CORTARME LAS VENAS DIG 22:50 / 18:35 / 20:45 / 22:50
DINOSAURIOS DIG 11:40 / 13:25 / 15:05 / 16:45

CINEMEX CUAUTLA
ARMADAS Y PELIGROSAS DIG 11:40 / 14:15 / 16:50 / 19:20 / 21:40
SON COMO NIÑOS 2 DIG ESP 11:20 / 13:40 / 15:50 / 18:10 / 20:30 / 22:45
ARMADAS Y PELIGROSAS DIG 12:40 / 15:10 / 17:40 / 20:15 / 22:30
JURASSIC PARK 3D ESP 22:10
ONE DIRECTION DIG 3D ASI SOMOS 12:00 / 14:00 / 16:00 / 18:00 / 20:00
EL CONJURO DIG ESP 11:05 / 13:20 / 15:40 / 18:05 / 20:20 / 22:40
EL CONJURO DIG ESP 12:05 / 14:20 / 16:40 / 19:05 / 21:20
CAZADORES DE SOMBRAS DIG ESP 11:10 / 13:50 / 16:30 / 19:10 / 21:50
CAZADORES DE SOMBRAS DIG ESP 12:10 / 14:50 / 17:30 / 20:10 / 22:50
LOS PITUFOS 2 DIG 12:25 / 14:30 / 16:45
SON COMO NIÑOS 2 DIG ESP 18:50 / 21:10 / 23:20
NO SE SI CORTARME LAS VENAS DIG 11:50 / 14:10 / 16:20 / 18:30 / 20:50 / 23:10
ONE DIRECTION DIG ASI SOMOS 11:00 / 13:00 / 15:00 / 17:00 / 19:00 / 21:00 / 23:00
LOS ILUSIONISTAS DIG ESP 12:20 / 14:40 / 17:10 / 19:30 / 22:00