

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
RAD: 10-134253- -15		FECHA: 2015-12-17 14:04:23	
DEP: 2020 DIRECCION DE NUEVAS CREACIONES		EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I	
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II		FOLIOS: 5	
ACT: 519 TRASLADOINFORME			

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ALVARO JOSE CORREA ORDOÑEZ

Asunto: Radicación: 10-134253- -15
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 15155
 Folios: 5

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2009/040123				
Fecha de Presentación Internacional	09/04/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado N° 10- 134253 -00000-0000	28/Octubre/2010
Reivindicaciones:	Radicado N° 10- 134253 -00000-0000	28/Octubre/2010
	Radicado N° 10- 134253 -00009-0000	22/Agosto/ 2013
	Radicado N° 10- 134253 -00012-0000	31/Marzo/2014
Prioridad:	US 60/043,620	09/Abril/2008
	US 61/074,610	20/Junio/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas (Art 34 D 486 y Art 19, 22 y 34 PCT)

Se aceptan las modificaciones presentadas comoquiera que se ajustan a las prescripciones contenidas en el reglamento del PCT.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 10 del capítulo reivindicatorio del Radicado N° 10-134253-00012-0000.

Título de la solicitud: “UN MÉTODO PARA MODIFICAR QUÍMICAMENTE UN LÍPIDO DE MICROALGA DEL GÉNERO PROTHOTHECA O CHLORELLA, UN MÉTODO PARA ELABORAR UN JABÓN Y UNA COMPOSICIÓN DE LÍPIDOS DE MICROALGA SAPONIFICADOS” (Modificado en la Resolución N° 10030 del 21 de febrero de 2014)

El problema planteado en esta solicitud consiste en la necesidad de proporcionar, métodos más económicos y eficientes para extraer biomoléculas derivadas de lípidos producidos por microorganismos

Para resolver este problema técnico la solicitud en estudio proporciona un método para la modificación química de lípidos presentes en la biomasa microbiana, un método de saponificación para fabricar un jabón y una composición que comprende una fase ligera con alquilésteres de ácido graso y por lo menos una fase más pesada que contiene biomasa microbiana.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12P 7/64 // 1/04.

5. De la solicitud de patente

Título

El título no corresponde con el objeto reclamado en la solicitud porque este corresponde a la modificación de lípidos de la especie *Prototheca moriformis*. Se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

1- La reivindicación 1 no es clara ni concisa porque:

- Las expresiones “a) cultivar una población de microalgas de la especie *Prototheca moriformis* bajo condiciones heterotróficas para producir biomasa... y; “b) someter el lípido a una reacción química que modifica covalentemente el lípido...” son ambiguas puesto que se refieren a un resultado a alcanzar y no definen las características esenciales del método. Se le sugiere a la solicitante caracterizar el método de modificación química de lípidos de microalgas con las etapas técnicas esenciales que lleven a la ejecución de dicho método, esto se logra de otra manera mencionando en la etapa a) las condiciones específicas de cultivo; el medio de cultivo con sus componentes y proporciones, las temperaturas y días de cultivo, los lípidos modificados y para la etapa b) donde se somete el lípido a una reacción química se debe especificar el tipo de reacción química y condiciones de

dicha etapa y mencionar cuales reactivos son los apropiados para cada una de las reacciones propuestas.

- El término "*condiciones heterotróficas*" es muy general y ambiguo, puesto que no limita los alcances del método reclamado. Se sugiere definir estas condiciones de forma concreta.
- Se caracteriza el método reclamado por el contenido de impurezas que generan color, específicamente carotenoides y/o clorofila, el cual no es una característica técnica de la solicitud puesto que corresponde a un resultado a alcanzar. Se sugiere eliminar la referencia a los resultados a alcanzar del capítulo reivindicatorio.
- Se emplea el término "y/o" generando una variedad de opciones, se sugiere eliminar dicho termino.

2- Las reivindicaciones 1, 5-7 y 10 no son claras porque los términos "*no más*" y "*al menos*", son imprecisos, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

3- Las reivindicaciones 2-4 no son claras ni concisas puesto que hace referencia a un método donde el lípido de microalgas es sometido a la reacción química, lo cual abarca una cantidad de posibilidades aun no exploradas por la solicitante y no define el método. Se le sugiere a la solicitante mencionar las condiciones específicas de la reacción química a las cuales es sometido el lípido de microalgas.

4- La reivindicación 5 no es clara porque:

- Las expresiones "*proporcionar lípido de microalga que comprende no más de 500ppm de impurezas que generan color, en donde el lípido de microalgas se obtiene de microalgas de la especie *Prototheca moriformis**" y "*saponificar el lípido de microalgas para producir un jabón*" no son claras, por cuanto se consideran un resultado a alcanzar y no define las características esenciales del método. Se le sugiere a la solicitante caracterizar el método para fabricar jabón con las etapas técnicas esenciales que lleven a la ejecución de dicho método, esto se logra de otra manera mencionando en la etapa a) las características estructurales de los lípidos empleados en dicho método y para la etapa b) las condiciones de la reacción de saponificación del lípido, recordando que estas se deben encontrar debidamente sustentadas en el capítulo descriptivo.
- No se establecen todas las características técnicas del proceso que se reclama, toda vez que la fabricación de un jabón requiere pasos adicionales a la reacción de saponificación de un lípido. Se sugiere incluir las etapas adicionales de acuerdo a lo enseñado en el capítulo descriptivo (Pág. 169-170).
- Se caracteriza el precursor empleado por su origen y su contenido de impurezas que generan color, específicamente carotenoides y/o clorofila, los cuales no son una característica técnica de un producto químico. Se sugiere caracterizar los lípidos empleados en función de su estructura química o nombre IUPAC.
- Se emplea el término "y/o" generando una variedad de opciones, se sugiere eliminar dicho termino.

5- La reivindicación 6 no es concisa puesto que menciona un método para hacer jabón, teniendo el mismo objeto que la reivindicación 5. Se sugiere a la solicitante eliminar dicha reivindicación y mencionar todas las características técnicas esenciales en la reivindicación 5.

6- Las reivindicaciones 7, 8 y 10 no son claras ni concisas puesto que hace referencia a "*lípido de microalga saponificado*", "*un aceite saponificado*" lo cual es considerado un resultado a alcanzar y no define la composición. Se le sugiere a la solicitante mencionar la composición por medio de los componentes activos, ingredientes y proporciones de los mismos que se encuentre debidamente sustentados en el capítulo descriptivo.

7- La reivindicación 9 no es clara porque hace referencia a la concentración de clorofila en el producto de partida, el cual es considerado como un resultado a alcanzar y no una característica técnica de la composición reclamada. Se le sugiere a la solicitante mencionar la composición por medio de los componentes activos, ingredientes y proporciones de los mismos que se encuentre debidamente sustentados en el capítulo descriptivo.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 09 de abril de 2008, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Xu, et al., 2006. <i>Journal of Biotechnology</i> . Vol. 124. Pages: 499-507.	“High quality biodiesel production from a microalga <i>Chlorella protothecoides</i> by heterotrophic growth in fermenters”	Diciembre/2006
D2	Running J.A., et al. <i>Journal of Experimental Botany</i> . Vol. 54, No. 389, pp. 1841-1849	“The pathway of L-ascorbic acid biosynthesis in the colourless microalga <i>Prototheca moriformis</i> ”	Agosto/2003
D3	González, MJ Ibáñez, et al. <i>Journal of the American Oil Chemists Society</i> Vol. 75.No. 12. Pág. 1735-1740.	“Optimization of Fatty Acid Extraction from <i>Phaeodactylum tricornutum</i> UTEX 640 Biomass”	1998

El documento D1¹ divulga la producción de biodiesel de alta calidad a partir de un cultivo de la microalga *Chlorella protothecoids* por medio de una reacción de transesterificación (Pág. 499, Resumen).

El documento D2² divulga un estudio de las rutas sintéticas encontradas en una cepa mutante de *Prototheca moriformis*, particularmente la ruta relacionada con la producción de ácido L-ascórbico (Pág. 1841, Resumen).

El documento D3³ divulga el aislamiento de ácidos grasos de la microalga *Phaeodactylum tricornutum*, utilizando un método de tres pasos que comprende la extracción de sales de potasio de los ácidos grasos crudos obtenidos por saponificación directa con KOH/Etanil, separación de los lípidos no saponificables por extracción con hexano y acidificación de la solución etanólica de las sales de potasio (Pág. 1735, Resumen).

7. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “Método para modificar químicamente lípidos de microalgas que comprende: (a) cultivar una población de microalgas de la especie *Prototheca moriformis* (...)” (Radicado N° 10-134253-00012-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
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1<http://www.sciencedirect.com/science/article/pii/S0168165606003853>

2<http://www.ncbi.nlm.nih.gov/pubmed/12869519>

3<http://link.springer.com/article/10.1007/s11746-998-0325-z>

Método para modificar químicamente lípidos de microalgas que comprende:	Un método para obtener lípidos de microalgas:	Microalgas del genero <i>Prototheca</i> (Pág. 1841, Col. 2)
(a) Cultivar una población de microalgas de la especie <i>Prototheca moriformis</i> bajo condiciones heterotróficas para producir biomasa que comprende lípido de microalgas y no más de 500 ppm de impurezas que generan color, en donde las impurezas que generan color son carotenoides y/o clorofila, y	Cultivo heterotrófico de <i>C. protothecoides</i> (Pág. 500, Col. 2, numeral 2.3) pulverizar las células en un mortero y extraer el aceite con n-hexano (Pág. 501, Col. 1, numeral 2.4)	Que no tienen pigmentos fotosintéticos
(b) someter el lípido a una reacción química que modifica covalentemente el lípido, donde la reacción química comprende transesterificación, interesterificación, hidroxilación, ciclopropanación, epoxidación, hidriprocesamiento, desoxigenación, isomerización, hidrogenación, saponificación o hidrólisis.	transesterificación acidica por reacción del extracto con metanol en relación molar (56:1) (Pág. 501, Col. 1, numeral 2.4	No se menciona

El documento D1 se considera el estado de la técnica más cercano a la solicitud definida en la reivindicación 1, porque divulga la obtención de lípidos a partir de un cultivo de *C. protothecoides* por medio de una reacción de transesterificación acídica (Pág. 501, Col. 1, numeral 2.4). El documento D1 también menciona reacciones de saponificación entre los lípidos extraídos del cultivo de microalgas y KOH (Pág. 501, Col. 2, numeral 2.5) y la disminución en el contenido de clorofila en las microalgas cultivadas en condiciones heterotróficas (Pág. 503, Col. 1, numeral 3.3).

La diferencia entre el método reclamado en la reivindicación 1 y aquel descrito en el documento D1, radica en la especie de microalga empleada, puesto que en el método reclamado se trata de *Prototheca moriformis* mientras que en el documento D1 es *C. protothecoides*.

El efecto técnico asociado a esta diferencia es la obtención de un aceite con un menor contenido de pigmentos, tal como se observa en la figura 19 de la solicitud.

Por lo tanto, el problema técnico objetivo que pretende resolver la solicitud se puede formular así: cómo modificar el procedimiento divulgado en D1 para disminuir el contenido de pigmentos en el aceite obtenido.

Sin embargo, el documento D2 ya sugería la relación entre las microalgas del género *Prototheca* y *Chlorella*, especialmente con *C. protothecoides*, mencionando que dicho género ha sido llamado “*Chlorella* sin color” y que se diferencia de las especies de *Chlorella* al no presentar pigmentos fotosintéticos (Pág. 1841, Col. 2, segundo párrafo).

Por lo tanto, la persona del oficio normalmente versada en la materia, sustituiría microalgas del género *Prototheca* de acuerdo a lo enseñado en el documento D2, en el proceso divulgado en el documento D1, para llegar así al objeto de la reivindicación 1 de la solicitud, por lo cual se considera obvio.

En consecuencia, la reivindicación 1 de acuerdo a lo divulgado en los documentos D1 y D2, carece de Nivel Inventivo.

Puesto que las reivindicaciones dependientes 2 a 4 no mencionan alguna característica

adicional que haga inventivo el método de la reivindicación 1, tampoco tienen nivel inventivo.

Conclusión:

Las reivindicaciones 1 a 4 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 5 consiste en “Método fabricar jabón que comprende: (a) proporcionar lípido de microalga que comprende (...)” (Radicado N° 10-134253-00012-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D3	D1
Método para fabricar jabón que comprende:	Saponificación directa de la biomasa de microalga: (Pág. 1736, col. 1, segundo párrafo)	Caracterización del contenido de lípidos obtenidos de <i>C. protothecoides</i> , que comprende: (Pág. 501, col. 1, numeral 2.5)
(a) Proporcionar lípido de microalga de la especie <i>Prototheca moriformis</i> que comprende no más de 500ppm que generan color, y	La biomasa de <i>P. tricornutum</i> es tratada con	Extraer los lípidos a partir de un cultivo de <i>C. protothecoides</i> (Pág. 501, Col. 1, numeral 2.5)
(b) Saponificar el lípido de microalga para producir jabón.	Una solución de KOH/Etanol, la saponificación se lleva a cabo a 60°C por una hora	Reacción de saponificación del aceite de microalga (Pág. 501, Col. 2, numeral 2.4)

El documento D3 se considera el estado de la técnica más cercano a la solicitud definida en la reivindicación 5, porque divulga la obtención de jabones a partir de cultivos de microalgas por medio de la saponificación de biomasa de las mismas (Pág. 1736, col. 1, segundo párrafo).

La diferencia entre el método reclamado en la reivindicación 5 y aquel descrito en el documento D3, radica en la especie de microalga empleada como fuente de lípidos para la reacción de saponificación, puesto que en el método reclamado se trata de *Prototheca moriformis* mientras que en el documento D3 es *P. tricornutum*.

No hay evidencia de un efecto técnico asociado a esta diferencia en el proceso de producción de jabones por medio de una reacción de saponificación.

Por lo tanto, el problema técnico objetivo que pretende resolver la solicitud se puede formular así: cómo producir modificar el proceso para producir jabón del documento D3 a partir de cultivos de microalgas como *Prototheca moriformis*.

Sin embargo, el documento D1 ya divulgaba reacciones de saponificación a partir de lípidos de microalgas de la familia *Chlorellaceae*, este documento menciona además una disminución en el contenido de clorofila en estas microalgas cultivadas en condiciones heterotróficas (Pág. 503, Col. 1, numeral 3.3).

Por lo tanto, la persona del oficio normalmente versada en la materia, sustituiría la biomasa de microalgas de la especie *P. tricornutum* del proceso divulgado en el documento D3, por la biomasa de otras microalgas de la familia *Chlorellaceae* de acuerdo a lo enseñado en el documento D1. Más aun cuando se conoce la relación entre *C. protothecoides* y microalgas del genero *Prototheca*⁴. Por lo cual el proceso reclamado se considera obvio.

En consecuencia, la reivindicación 5 de acuerdo a lo divulgado en los documentos D1 y D3,

⁴Running J.A., et al. *Journal of Experimental Botany*. Vol. 54, No. 389, pp. 1841-1849. The pathway of L-ascorbic acid biosynthesis in the colourless microalga *Prototheca moriformis* Dicponible en: <http://www.ncbi.nlm.nih.gov/pubmed/12869519>

carece de Nivel Inventivo.

Puesto que la reivindicación dependiente 6 no menciona alguna característica adicional que haga inventivo el método de la reivindicación 5, tampoco tiene nivel inventivo.

En conclusión, las reivindicaciones 5 y 6 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 7 consiste en “Una composición que comprende sales de ácidos grasos de lípido de microalga saponificado obtenido de microalgas de la especie *Prototheca moriformis* y que comprende no más de 500ppm de impurezas (...)” (Radicado N° 10-134253-00012-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D3	D1
Composición que comprende:	Solución de jabón obtenida por; (Pág. 1736, col. 1, tercer párrafo)	Caracterización de los lípidos obtenidos a partir de <i>C. protothecoides</i> , que comprende: (Pág. 501, col. 1, numeral 2.5)
Sales de ácidos grasos de lípido de microalga de la especie <i>Prototheca moriformis</i> que comprende no más de 500ppm que generan color, y	Reacción de saponificación de la biomasa de <i>P. tricornutum</i> (Pág. 1736, col. 1, segundo párrafo)	Reacción de saponificación del aceite de microalga (Pág. 501, Col. 2, numeral 2.4)

El documento D3 se considera el estado de la técnica más cercano a la solicitud definida en la reivindicación 7, porque divulga soluciones que comprenden jabones obtenidos a partir de biomasa de microalgas por medio de reacciones de saponificación (Pág. 1736, col. 1, segundo y tercer párrafo).

La diferencia entre la composición reclamada en la reivindicación 7 y la solución descrita en el documento D3, radica en la especie de microalga empleada como fuente de lípidos para la reacción de saponificación, puesto que en el método reclamado se trata de *Prototheca moriformis* mientras que en el documento D3 es *P. tricornutum*.

El efecto técnico asociado a esta diferencia es el contenido de pigmentos en la composición, puesto que el documento D3 establece que la solución obtenida muestra un porcentaje de alrededor de 1% de clorofila en la biomasa seca de *P. tricornutum*, mientras que el contenido de clorofila presente en el aceite de *Prototheca moriformis* de acuerdo a la solicitud es menor al 0.01 mg/Kg (Pág. 174-175).

Por lo tanto, el problema técnico objetivo que pretende resolver la solicitud se puede formular así: cómo producir composiciones que comprenden sales de ácidos grasos obtenidos a partir de cultivos de microalgas, que presenten un menor contenido de pigmentos.

Sin embargo, el documento D1 ya divulgaba reacciones de saponificación y por lo tanto la obtención de jabones, a partir de lípidos de microalgas de la familia *Chlorellaceae*, donde se menciona una disminución en el contenido de clorofila en estas microalgas cultivadas en condiciones heterotróficas (Pág. 503, Col. 1, numeral 3.3).

Por lo tanto, la persona del oficio normalmente versada en la materia, sustituiría la biomasa de microalgas de la especie *P. tricornutum* como fuente de lípidos para la obtención de sales de ácidos grasos del documento D3, por la biomasa de otras microalgas, de acuerdo a lo enseñado en D1, más aun cuando se conoce la relación entre las especies las microalgas

del género *Chlorella*, especialmente con *C. protothecoides*. Por lo cual el objeto reclamado reclamado se considera obvio.

En consecuencia, la reivindicación 7 de acuerdo a lo divulgado en los documentos D1 y D3, carece de Nivel Inventivo.

Puesto que las reivindicaciones dependientes 8 - 10 no mencionan alguna característica adicional que haga inventivo el método de la reivindicación 1, tampoco tienen nivel inventivo.

En conclusión, las reivindicaciones 7 a 10 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Xu, et al., 2006. <i>Journal of Biotechnology</i> . Vol. 124. Pages: 499-507.	1-10	Nivel inventivo
D2	Running J.A., et al. <i>Journal of Experimental Botany</i> . Vol. 54, No. 389, pp. 1841-1849	1-4	Nivel inventivo
D3	González, MJ Ibáñez, et al. <i>Journal of the American Oil Chemists Society</i> Vol. 75.No. 12. Pág. 1735-1740.	5-10	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-12-22

Elaboró: Ruth Andrea Rodriguez

Revisó: Dayron Alexander Ramirez Reyes

INFORME DE BÚSQUEDA DE PATENTES PARA EI RADICADO 10-134253-00012-0000						
CRITERIOS PARA LA BÚSQUEDA						
Palabras clave utilizadas		Prototheca AND oil Chrorella AND Prototheca				
CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA		A61F6/14				
BASES DE DATOS CONSULTADAS.						
Google patents		Espacenet			Science direct	
INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS						
Oficina	Fecha del examen o de reporte de búsqueda de documentos	Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)	Reiv. afectadas	RELEVANCIA (requisito que afecta)	FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA	
					SI	NO
EUROPEA	30/12/2009	US5354878				X
		US2007048848				X
		US2006225341				X
		US2008038804				X
		US5091116				X
		US5888947				X
		Xu, Han et al. High Quality Biodiesel Production from a Microalga Chlorella protothecoides by Heterotrophic Growth in Fermenters. <i>Journal of Biotechnology</i> 126. Elsevier 2006. pp. 499-507	1-10	Nivel Inventivo	X	
		Turmel, M, Otis, C, Mercier, J-P, Gutell, and Lemieux R R, C. Prototheca wickerhamii large subunit ribosomal RNA (rrnL) gene, partial sequence; chloroplast gene for chloroplast product.				X
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USPTO	03/01/2012	Xu, Han et al. High Quality Biodiesel Production from a Microalga Chlorella protothecoides by Heterotrophic Growth in Fermenters. <i>Journal of Biotechnology</i> 126. Elsevier 2006. pp. 499-507	1-10	Nivel Inventivo	X	
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BÚSQUEDA DE SECUENCIAS						
Número de depósito / patente y N° de secuencia % de identidad						
SEQ ID NO (de la solicitud)	NCBI	The Lens	EMBL-EBI	DDJB	Genome Quest	
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High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters

Han Xu, Xiaoling Miao, Qingyu Wu*

Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, PR China

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Abstract

The aim of the study was to obtain high quality biodiesel production from a microalga *Chlorella protothecoides* through the technology of transesterification. The technique of metabolic controlling through heterotrophic growth of *C. protothecoides* was applied, and the heterotrophic *C. protothecoides* contained the crude lipid content of 55.2%. To increase the biomass and reduce the cost of alga, corn powder hydrolysate instead of glucose was used as organic carbon source in heterotrophic culture medium in fermenters. The result showed that cell density significantly increased under the heterotrophic condition, and the highest cell concentration reached 15.5 g L^{-1} . Large amount of microalgal oil was efficiently extracted from the heterotrophic cells by using *n*-hexane, and then transmuted into biodiesel by acidic transesterification. The biodiesel was characterized by a high heating value of 41 MJ kg^{-1} , a density of 0.864 kg L^{-1} , and a viscosity of $5.2 \times 10^{-4} \text{ Pa s}$ (at 40°C). The method has great potential in the industrial production of liquid fuel from microalga.

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Keywords: Biodiesel; Heterotrophic culture; *Chlorella protothecoides*; Fermenter; Corn powder hydrolysate

1. Introduction

As a biodegradable, renewable, and non-toxic fuel, biodiesel fuel has received considerable attention in recent years. It also contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than conventional diesel fuel (Lang et al., 2001; Antolin et al., 2002; Vicente et al., 2004). Biodiesel

fuel, which consists of the simple alkyl esters of fatty acids, is presently making the transition from a research topic and demonstration fuel to a marketed commodity. Annual US production in 2001 has been estimated at 57–76 million liters, with European production more than 10 times that size (Jon Van Gerpen, 2005). However, the economic aspect of biodiesel production limits its development and large-scale use. Biodiesel usually costs over $\text{US\$ } 0.5 \text{ L}^{-1}$, compared to $\text{US\$ } 0.35 \text{ L}^{-1}$ for conventional diesel fuel (Zhang et al., 2003).

Chlorella protothecoides is a microalga that can grow photoautotrophically or heterotrophically under

* Corresponding author. Tel.: +86 10 62781825; fax: +86 10 62781825.

E-mail address: qingyu@tsinghua.edu.cn (Q. Wu).

different culture conditions. Heterotrophic growth of *C. protothecoides* supplied with acetate, glucose, or other organic compounds as carbon source, results in high biomass and high content of lipid in cells (Endo et al., 1977; Wu et al., 1994). With the addition of the organic carbon source (glucose) to the medium and the decrease of the inorganic nitrogen source in the medium, the heterotrophic *C. protothecoides* was cultivated with the crude lipid content up to 55.2%, which was about four times that in photoautotrophic *C. protothecoides* (Miao and Wu, 2004a). Therefore, *C. protothecoides* has not only become an important source of many products, such as aquaculture feeds, human food supplements, and pharmaceuticals (Kyle, 1992; Running et al., 1994; Borowitzka, 1995; Chen, 1996), but also been suggested as a very good candidate for fuel production (Wu et al., 1992; Wen et al., 2002; Miao and Wu, 2004a).

To increase the biomass and reduce the cost of alga, corn powder hydrolysate (CPH) as substrate in heterotrophic growth of *C. protothecoides* was used. *Chlorella protothecoides* was heterotrophically cultured in a 5 L stirred tank fermenter with CPH feeding, which gave significant improvement in cell density (15.5 g L^{-1}) and productivity. High quality biodiesel was obtained from heterotrophic microalgal oil by acidic transesterification. It was characterized by a high heating value of 41 MJ kg^{-1} , a density of 0.864 kg L^{-1} , and a viscosity of $5.2 \times 10^{-4} \text{ Pa s}$ (at 40°C).

2. Materials and methods

2.1. Microalga and medium

Chlorella protothecoides was provided by the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The culture medium and method were as described as Wu et al. (1992). The alga was grown autotrophically and axenically in batch cultures under $28 \pm 1^\circ \text{C}$ with continuous illumination at intensities of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. Aeration was provided by bubbling air at regular pressure. For the heterotrophic growth of *C. protothecoides*, 10 g L^{-1} glucose was added to the basal medium and the glycine was reduced to 0.1 g L^{-1} . The details of the culture of heterotrophic cells were reported in our previous research (Wu et al., 1994).

Table 1
Design of 2^3 factorial experiments

Serial number	A	B	C
1	–	–	–
2	–	+	–
3	+	–	–
4	+	+	–
5	–	–	+
6	–	+	+
7	+	–	+
8	+	+	+

The three factors were alpha-amylase dosage (A), glucoamylase dosage (B), and reaction time (C). Levels were 0.005 g (–), 0.010 g (+) for A, 0.100 g (–), 0.200 g (+) for B and 1 h (–), 2 h (+) for C.

2.2. Production of CPH

The 2^3 factorial experiments were chosen to examine the optimum condition of corn powder hydrolyzed by alpha-amylase and glucoamylase (Table 1). The three factors were alpha-amylase dosage (A), glucoamylase dosage (B), and reaction time (C). Levels were 0.005 g (–), 0.010 g (+) for A; 0.100 g (–), 0.200 g (+) for B and 1 h (–), 2 h (+) for C.

Buffer solution was prepared with 0.2N of dipotassium hydrogen phosphate (103.00 mL) and 0.1N of citric acid (97.00 mL). Accurately 5.000 g corn powder, 10.00 mL buffer solution and distilled water were mixed, and the corn powder was hydrolyzed by alpha-amylase and glucoamylase at 60°C and pH 6.0.

2.3. Cultivation

Chlorella protothecoides in exponentially period was inoculated (10%, v/v) in a liquid medium. Heterotrophic cultivation of *C. protothecoides* was initially carried out in a 500-mL Erlenmeyer flask containing 300 mL medium at $28 \pm 1^\circ \text{C}$ under continuous shaking (180 rpm) and air flowing in the dark (Miao and Wu, 2004a). Further heterotrophic cultivation was performed in a 5-L fermenter (Biostat Q, B.BRAUN, Germany) containing 3.0 L medium, in which concentrated glucose solution was batch-fed. Dissolved oxygen concentration was controlled by increasing agitation speed and airflow. Aeration rate and the agitation speed were variable and initially set at 0.5 vvm and 300 rpm. Temperature was controlled $28 \pm 1^\circ \text{C}$.

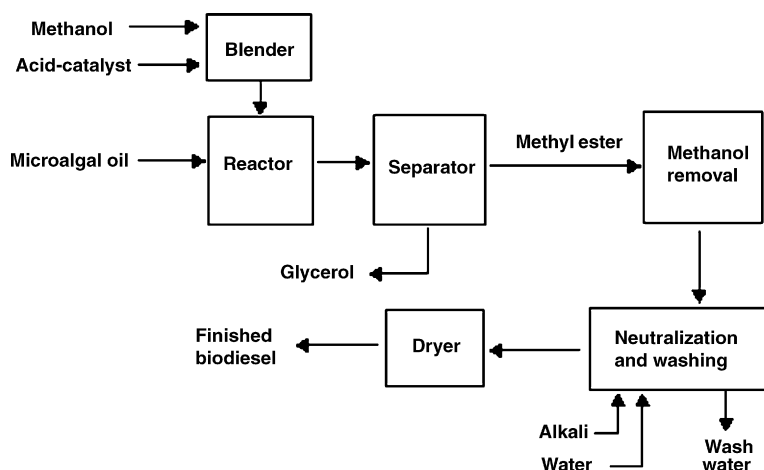


Fig. 1. Process flow schematic for biodiesel production.

2.4. Biodiesel preparation

Cells were harvested by centrifugation, washed with distilled water, and then dried by a freeze dryer. The main chemical components of heterotrophic *C. protothecoides* were measured as previous study (Miao et al., 2004b). Microalgal oil was prepared by pulverization of heterotrophic cell powder in a mortar and extraction with *n*-hexane.

Biodiesel was obtained from heterotrophic microalgal oil by acidic transesterification (Fig. 1). The optimum process combination was 100% catalyst quantity (based on oil weight) with 56:1 molar ratio of methanol to oil at temperature of 30 °C, which reduced product specific gravity from an initial value of 0.912 to a final value of 0.864 in about 4 h of reaction time.

2.5. Analytical methods

Cell growth was measured by means of the absorbance of the suspension at 540 nm as Becker (1994) showed. This value was transformed to biomass concentration, by a regression equation as

$$y = 0.2821x \quad (R^2 = 0.996, P < 0.05)$$

where y (g L⁻¹) is the cell concentration and x is the absorbance of the suspension at 540 nm.

Lipid in the algal cells was extracted according to Zhu et al. (2002). Glucose was analyzed with the method of Miller (1959).

The saponification (189.3 mg KOH g⁻¹) and acid value (8.97 mg KOH g⁻¹) of the microalgal oil were determined according to the method of Vicente et al. (2004). The molecular weight of the oil was calculated from saponification and acid value as

$$M = \frac{168300}{SV - AV},$$

where M is the molecular weight of the oil, SV the saponification value, and AV is the acid value. The average molecular weight of the oil was 933.

The properties of biodiesel such as density, viscosity, flash point, cold filter plugging point, solidifying point, and heating value were measured. The elemental compositions of biodiesel were determined by a CE-440 elemental analyzer (Peng et al., 2001).

The composition of the biodiesel was derivatized and analyzed by gas chromatographic–mass spectrometric analysis. Gas chromatography was performed on a 0.25 mm (i.d.) × 30 m fused silica column lined with a 0.25 μm film of polyethylene glycol (VF-5ms, from VARIAN, America). Samples (0.2 μL) were injected in split mode (split/column flow ratio 30:1). The column head pressure of the carrier gas (helium) was 3 kPa at the initial oven temperature, and its flow rate 1.0 mL min⁻¹. The injection temperature was 290 °C; the oven temperature was 100 °C for 2 min, rose to 300 °C over 20 min and was held at this temperature for 20 min (total run time 42 min). The GC–MS

Table 2
Influence of two enzymes and reaction time on DE value of hydrolysate

Alpha-amylase/ glucoamylase	Volume of solution (mL)	1 h				2 h			
		Absorbency	Glucose concentration ($\mu\text{g mg}^{-1}$)	Total glucose (g)	Dextrose equivalent (%)	Absorbency	Glucose concentration ($\mu\text{g mg}^{-1}$)	Total glucose (g)	Dextrose equivalent (%)
0.005/0.100	164	0.926	192.1	3.15	63.0	1.096	218.7	3.59	71.8
0.005/0.200	169	0.905	188.8	3.19	63.8	0.949	195.7	3.31	66.2
0.010/0.100	179	0.851	183.5	3.28	65.6	0.801	172.5	3.09	61.8
0.010/0.200	187	0.811	174.0	3.25	65.0	0.613	143.0	2.67	53.4

Temperature = 60 °C, pH 6.0.

apparatus was linked to a PC running software for data acquisition and processing.

3. Results and discussion

3.1. Hydrolysate from corn powder

Corn powder was hydrolyzed under 2^3 factorial experiments. Comparing the dextrose equivalents (DE) after 1 h, when the dosage of alpha-amylase fixed, the dosage of glucoamylase did not play a decisive role (Table 2). This meant that, starch was hydrolyzed but not saccharified with the reaction time of 1 h. After 2 h, all the reactions were determinately excessive under the conditions, except alpha-amylase and glucoamylase were 0.005 and 0.100 g. Because the reducing sugar produced in the excessive reactions was disproportioned. The optimum condition of the reaction in laboratory was alpha-amylase 0.005 g and glucoamylase 0.100 g per 5.000 g corn powder, at 60 °C and pH 6.0. After 2 h, the DE value reached 71.8%.

3.2. Cultivation of heterotrophic *Chlorella* in flasks

As shown in Fig. 2, the cell growth reached maximum value (3.92 g L^{-1}) after 144 h culture with the substrate of CPH, while the maximum value was 3.74 g L^{-1} with the substrate of glucose. It indicated that, it was feasible to use CPH as organic carbon

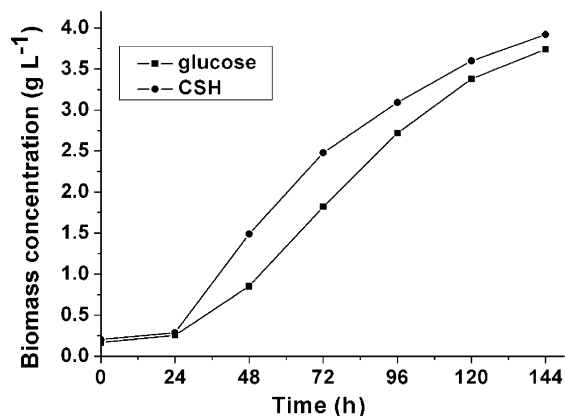


Fig. 2. Growth curve comparison of heterotrophic *Chlorella* between glucose and CPH medium in flasks.

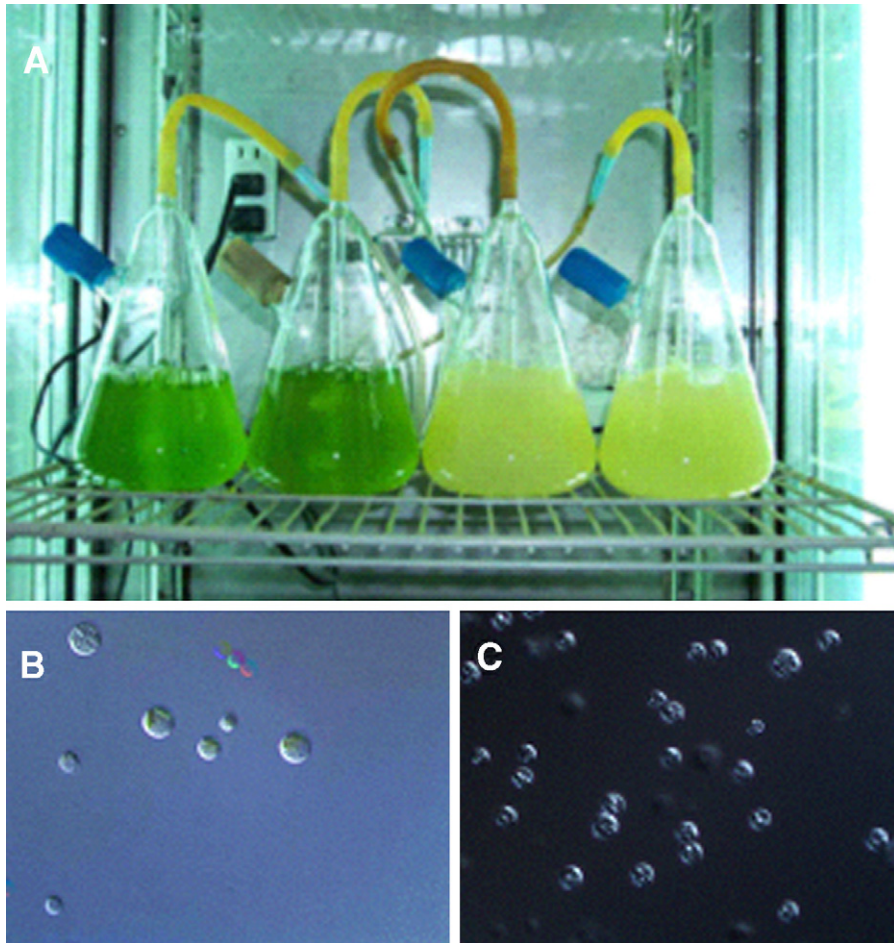


Fig. 3. (A) Growth of the cells of *C. protothecoides* under autotrophic (left, green) and heterotrophic (right, yellow) culture conditions. (B and C) Cells of autotrophic and heterotrophic *C. protothecoides* under differential interference microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

to cultivate *Chlorella*. During most of the cultivation time, CPH resulted superior to glucose solution, which due to CPH contained some beneficial components to *Chlorella*. Lipid content in the algal cells was 54.7% with glucose feeding, and 55.3% with CPH feeding, which was not significantly different.

3.3. The main chemical components of heterotrophic *Chlorella* cells

As shown in Fig. 3, heterotrophic growth of *Chlorella protothecoides* results in not only the disappearance of chlorophyll in cells (Fig. 3A) but also accumulation of high lipid content in cells. Lipid con-

tent in heterotrophic cells reached as high as 55.2%, which was about four times that in autotrophic cells (Table 3). The heterotrophic cells were full of lipid vesicles, which can be easily observed under differ-

Table 3

Contents of the main chemical components of autotrophic (AC) and heterotrophic (HC) *Chlorella protothecoides* cells

Component (%)	AC	HC
Protein	52.64 ± 0.26	10.28 ± 0.10
Lipid	14.57 ± 0.16	55.20 ± 0.28
Carbohydrate	10.62 ± 0.14	15.43 ± 0.17
Ash	6.36 ± 0.05	5.93 ± 0.04
Moisture	5.39 ± 0.04	1.96 ± 0.02
Others	10.42 ± 0.65	11.20 ± 0.61

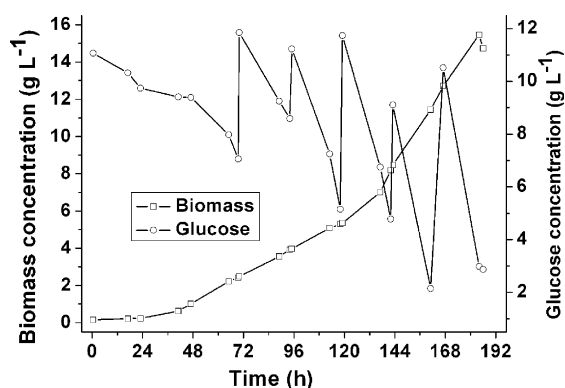


Fig. 4. Growth and glucose consumption curve of heterotrophic *Chlorella* in 5 L fermenter.

ential interference microscopy (Fig. 3C). The lipid-soluble compounds from the autotrophic cells appeared in a blackish green with chlorophyll and carotenoid as the major components, whereas the lipid-soluble compounds from the heterotrophic cells appeared in a state of light yellow grease, which were mainly lipid compounds (referred as oil). The fatty acid composition of the oil has been demonstrated to be mainly composed of oleic acid, linoleic acid, cetane acid, etc. by hydro-sis, esterification, and gas chromatographical analysis as reported in our previous work (Wu et al., 1992).

3.4. Cultivation of heterotrophic *Chlorella* in the fermenter

As shown in Fig. 4, the cell growth reached 15.5 g L^{-1} after 184 h culture in 5 L bioreactor, then decreased to 14.3 g L^{-1} in the subsequent 2 h culture. A high density heterotrophic culture of *C. protothe-*

coides with CPH feeding was established in the 5 L stirred tank fermenter. Lipid content in the algal cells cultivated in the fermenter was 46.1%, which was a little lower than that in the Erlenmeyer flasks.

3.5. Biodiesel produced from heterotrophic *Chlorella*

To assess the potential of biodiesel as a substitute of diesel fuel, the properties of biodiesel such as density, viscosity, flash point, cold filter plugging point, solidifying point, and heating value were determined. A comparison of these properties of diesel fuel (Ma and Hanna, 1999; Lang et al., 2001; Al-Widyan and Al-Shyukh, 2002; Antolin et al., 2002; Vicente et al., 2004), biodiesel from microalgal oil and ASTM biodiesel standard is shown in Table 4. Most of these parameters comply with the limits established by ASTM related to biodiesel quality (Antolin et al., 2002).

The physical and fuel properties of biodiesel from microalgal oil in general were comparable to those of diesel fuel. The biodiesel from microalgal oil showed much lower cold filter plugging point of -11°C in comparison with the diesel fuel (Table 4).

The gas chromatograph of biodiesel is shown in Fig. 5. The fatty acid methyl esters (FAMES) of the biodiesel are presented in Table 5. There were nine FAMES derivatized in the biodiesel, and the most abundant composition was oleic acid methyl ester with the content of 60.84%. Oleic acid methyl ester, octadecadienoic acid methyl ester, and octadecanoic acid methyl ester are 18 carbon acid methyl esters, and the total content of these three FAMES was over 80%. This resulted in the high quality of the biodiesel.

Table 4

Comparison of properties of biodiesel from microalgal oil and diesel fuel and ASTM biodiesel standard

Properties	Biodiesel from microalgal oil	Diesel fuel ^a	ASTM biodiesel standard
Density (kg L^{-1})	0.864	0.838	0.86–0.90
Viscosity ($\text{mm}^2 \text{ s}^{-1}$, cSt at 40°C)	5.2	1.9–4.1	3.5–5.0
Flash point ($^\circ\text{C}$)	115	75	Min 100
Solidifying point ($^\circ\text{C}$)	–12	–50–10	–
Cold filter plugging point ($^\circ\text{C}$)	–11	–3.0 (max –6.7)	Summer max 0 Winter max < –15
Acid value (mg KOH g^{-1})	0.374	Max 0.5	Max 0.5
Heating value (MJ kg^{-1})	41	40–45	–
H/C ratio	1.81	1.81	–

^a The data about diesel fuel was taken from published literature as indicated in the text.

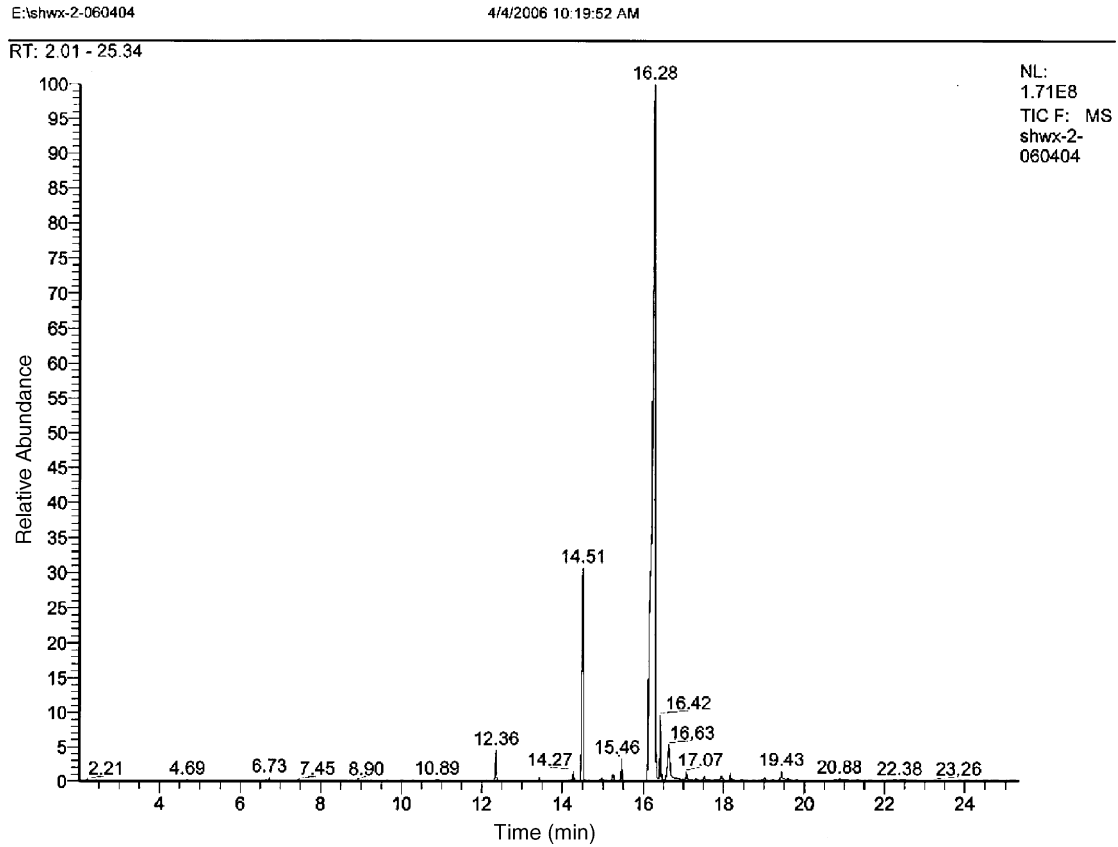


Fig. 5. Gas chromatograph of the fatty acid methyl ester in biodiesel.

Table 5

Fatty acid methyl esters in the biodiesel

No.	Molecular formula	Relative molecular mass	Fatty acid methyl ester	Relative content (%)
1	C ₁₅ H ₃₀ O ₂	242	Methyl tetradecanoate	1.31
2	C ₁₇ H ₃₄ O ₂	270	Hexadecanoic acid methyl ester	12.94
3	C ₁₈ H ₃₆ O ₂	284	Heptadecanoic acid methyl ester	0.89
4	C ₁₉ H ₃₄ O ₂	294	9,12-Octadecadienoic acid methyl ester	17.28
5	C ₁₉ H ₃₆ O ₂	296	9-Octadecenoic acid methyl ester	60.84
6	C ₁₉ H ₃₈ O ₂	298	Octadecanoic acid methyl ester	2.76
7	C ₂₀ H ₃₈ O ₂	310	10-Nonadecenoic acid methyl ester	0.36
8	C ₂₁ H ₄₀ O ₂	324	11-Eicosenoic acid methyl ester	0.42
9	C ₂₁ H ₄₂ O ₂	326	Eicosanoic acid methyl ester	0.35

The results suggest that the new process, which combines bioengineering and transesterification, was a feasible and effective method for the production of high quality biodiesel from heterotrophic microalgal oil. The biodiesel from heterotrophic microalgal oil could be a competitive alternative to conventional diesel fuel.

4. Conclusion

The research of liquid fuel produced from microalga was begun at middle 1980s in 20 centuries. Transesterification and catalytic cracking were usually adopted to convert fat in the cell of microalga as gasoline and

diesel at that time. This kind of method was limited by low temperature, and the outcome function was highly influenced by the fat constitute. What was more, the fat content in the microalga was required to be very high, otherwise the economic performance would be hard to acquire.

Because it was hard to obtain microalga with high content of fat, scientists turned to develop a new method pyrogenation. The method of pyrolyzing microalga to produce liquid fuel was put forward by Doctor Bayer in Germany in 1986. In 1993, high quality oil of low nitrogen and low sulphur was got successfully by Professor Ben-Zion Ginzburg in Israel, using *Dunaliella salina* as material of pyrogenation. Liquefaction of *Botryococcus braunii* was performed with sodium carbonate as a catalyst for conversion into liquid fuel and recovery of hydrocarbons at high pressure (10 MPa N₂ press) under 300 °C. The income liquid oil reached 57–64 wt%, and the quality was comparable to petroleum (Dote et al., 1994). *Dunaliella tertiolecta* with a moisture content of 78.4 wt% was converted directly into oil by thermochemical liquefaction at 340 °C in 60 min holding time. The oil yield was about 37% on an organic basis, and had a viscosity of 150–330 mm² s⁻¹ and a calorific value of 36 MJ kg⁻¹ (Minowa et al., 1995).

To increase the fat content in microalga, the technique of metabolic controlling through heterotrophic growth of *C. protothecoides* was applied, which resulted in the crude lipid content of 55.2% (Wu et al., 1994). The microalgal oil was efficiently extracted from the heterotrophic cells, and then transmuted into biodiesel by acidic transesterification.

The present study introduced an integrated method for the production of biodiesel from heterotrophic *C. protothecoides*. CPH was got from corn powder by the co-hydrolyzation of alpha-amylase and glucoamylase with the dosages of 0.005 and 0.100 g per 5.000 g corn powder, at 60 °C and pH 6.0 after 2 h reaction. The DE value reached 71.8%. CPH was used as the substrate of heterotrophic growth of *C. protothecoides* which was cultivated in Erlenmeyer flasks and 5 L fermenter. In Erlenmeyer flasks, the cell had the concentration of 3.92 g L⁻¹ and the lipid content of 55.3% after 144 h culture with CPH feeding, which was superior to glucose feeding. In 5 L fermenter, the cell growth reached 15.5 g L⁻¹ after 184 h culture, and then microalgal oil was efficiently extracted from the heterotrophic cells. Biodiesel which was obtained from heterotrophic

microalgal oil by acidic transesterification was characterized by a high heating value of 41 MJ kg⁻¹, a density of 0.864 kg L⁻¹, and a viscosity of 5.2 × 10⁻⁴ Pa s (at 40 °C). The results suggest that the new process was a low-cost, feasible, and effective method for the production of high quality biodiesel from microalga.

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The pathway of L-ascorbic acid biosynthesis in the colourless microalga *Prototheca moriformis*

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RESEARCH PAPER

The pathway of L-ascorbic acid biosynthesis in the colourless microalga *Prototheca moriformis*

Jeffrey Allan Running*, Richard Paul Burlingame† and Alan Berry‡

Bio-Technical Resources, 1035 South 7th Street, Manitowoc, WI 54220, USA

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Abstract

When mutant strain UV77–247 of *Prototheca moriformis* Kruger was fed D-[1-¹³C]Glc, it synthesized L-ascorbic acid (AA) with approximately three-quarters of the label at the C-1 position and the remaining label at the C-6 position, showing that AA is made by a non-inversion (retention) pathway, i.e. C-1 of Glc becomes C-1 of AA. The label present at C-6 is consistent with the glycolytic conversion of Glc to 3-carbon intermediates and subsequent gluconeogenesis. Compounds suggested as intermediates in inversion-type pathways were not converted to AA. Most strains converted Man to AA at a rate greater than they did Glc. Enzyme activities leading from Fru-6-P to the formation of GDP-Man were identified in all strains, but none of these activities correlated with the mutants' abilities to accumulate AA. However, there was a strong correlation between GDP-Man-3,5-epimerase activity and AA accumulation. Wild-type *P. moriformis* ATCC 75669 and mutant strains of varying AA-synthesizing abilities rapidly converted L-Gal or L-galactono-1,4-lactone to AA. Based on this data, a biosynthetic pathway from Glc to AA is proposed in which the epimerase is the rate-limiting activity in AA synthesis.

Key words: GDP-Mannose-3,5-epimerase, L-ascorbic acid, microalga, *Prototheca*.

Introduction

L-ascorbic acid (AA; vitamin C) is essential for the growth and nutrition of humans and other animals. Currently, AA

is produced commercially by either the Reichstein process, which entails one bacterial fermentation and several chemical synthesis steps (Jaffe, 1984; Reichstein and Grussner, 1934) or by fermentation to produce 2-keto-L-gulonic acid, followed by a chemical conversion to AA (Hancock and Viola, 2001). A one-step fermentation process has been described for the production of AA using the heterotrophic green microalga *Chlorella pyrenoidosa* (Running *et al.*, 1994). That work resulted in increases in intracellular AA content of more than 70-fold, and in cells containing more than 2% of their dry weight as AA (Doncheck *et al.*, 1996). Virtually none of the AA produced was extracellular. It has also been shown that *C. protothecoides* and members of all *Prototheca* species synthesize AA from Glc (Running *et al.*, 2002).

The genus *Prototheca* is a member of the microalgal Class Chlorophyceae and is related to the genus *Chlorella* (Huss and Sogin, 1990; Kessler, 1982; Pore, 1985), especially *C. protothecoides* (Huss *et al.*, 1999). *Prototheca* has been called a 'colourless *Chlorella*' (Casselton and Stacey, 1969; Walker *et al.*, 1975), but its members have characteristics which distinguish them from members of most *Chlorella* species: (1) they do not have photosynthetic pigments (Pore, 1993); (2) they cannot use nitrate as a sole nitrogen source (Pore, 1985, 1993); (3) they are all thiamine auxotrophs (Pore, 1993); (4) they are acidophilic (Huss *et al.*, 1988); and (5) they can use a variety of hydrocarbons as sole carbon sources (Koenig and Ward, 1983; Walker *et al.*, 1975; Walker and Pore, 1978).

Strain-improvement efforts using *Prototheca* generated many mutant strains with increased abilities to accumulate AA (Running *et al.*, 2002). A number of mutants accumulated less AA than the wild-type (wt) strain; AA

* To whom correspondence should be sent. Fax: +1 920 684 5519. E-mail: jeff@biotechresources.com

† Present address: Dyadic International Inc., 140 Intracoastal Pointe Drive, Suite 404, Jupiter, FL 33477-5094, USA.

‡ Present address: Biotechnology Department, Research and Development, Roche Vitamins AG, CH-4070 Basel, Switzerland.

Abbreviations: AA, L-ascorbic acid; GMP, GDP-Mannose pyrophosphorylase; L-GalL, L-galactono-1,4-lactone; L-GalDH, L-galactose dehydrogenase; PMI, phosphomannose isomerase; PMM, phosphomannomutase.

was undetectable in some. These mutants were used to aid in identifying a pathway for AA biosynthesis that involves Man-containing intermediates. This pathway, identified in *P. moriformis* earlier (Berry *et al.*, 1999), is similar to that recently described in higher plants (Wheeler *et al.*, 1998). Both proposals incorporate the key observation that the carbon chain of metabolized Glc is not inverted in the synthesized AA (see Results and discussion), unlike the pathways described for animals and some algae (Grün and Loewus, 1984; Helsper *et al.*, 1982; Isherwood *et al.*, 1954b; Shigeoka *et al.*, 1979; see Results and discussion). Two recent reviews provide detailed descriptions of the AA synthesis pathways in various organisms (Loewus, 1999; Smirnov *et al.*, 2001).

Materials and methods

Strains, growth media and culture conditions

Wild-type strains were obtained from the American Type Culture Collection, Manassas, VA (ATCC), the Culture Collection of Algae and Protozoa at the University of Texas at Austin (UTEX) or from Professor R Scott Pore (West Virginia University Medical School, Charleston, WV). Strains were grown in a Glc minimal medium containing (l^{-1}): 1.3 g KH_2PO_4 , 3.8 g K_2HPO_4 , 7.7 g Na_3 -citrate, 3.7 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 1.5 mg $FeSO_4 \cdot 7H_2O$, 3–6 mg thiamine HCl, 30–60 g Glc, and 2 ml of trace metals solution. The trace metal solution contained (l^{-1}): 20 ml HCl, 156 mg $CoCl_2 \cdot 6H_2O$, 914 mg H_3BO_3 , 1.76 g $ZnSO_4 \cdot 7H_2O$, 1.23 g $MnSO_4 \cdot H_2O$, 48 mg $Na_2MoO_4 \cdot 2H_2O$, 11.4 g $CaCl_2 \cdot 2H_2O$, 80 mg $VOSO_4 \cdot 2H_2O$, 40 mg $Ni(NO_3)_2 \cdot 6H_2O$, and 40 mg Na_2SeO_3 . For Mg-limiting growth, the $MgSO_4 \cdot 7H_2O$ was reduced to 20 mg l^{-1} . The standard growth temperature was 35 °C.

AA analysis

For colorimetric analysis of AA, a modification of the dipyriddy method (Omaye *et al.*, 1979) was used. HPLC analysis was based on that of Grün and Loewus (1983). Supernates were chromatographed on a Bio-Rad HPX-87H organic acid column (Bio-Rad Laboratories, Richmond, CA) with 13 mM nitric acid as solvent, at a flow rate of 0.7 ml min^{-1} at room temperature. Detection was at 254 nm. This system can distinguish between AA and D-erythorbic acid, the 5-epimer.

[^{13}C]labelling

Shake flask cultures were grown to moderate cell density under Mg-limiting conditions, with D-[^{13}C]labelled Glc (Cambridge Isotope Laboratories, Andover, MA) as 10% of the total Glc (40 g l^{-1}). The combined culture supernates (90 ml) were clarified by the addition of four parts ethanol and overnight storage at –20 °C, with precipitated solids removed by centrifugation. The supernate was evaporated to approximately 350 ml, 500 ml of water was then added, and the resulting solution was again evaporated to 350 ml. The pH of 80 ml aliquots was brought to 2.0 with HCl, 5 g of AG501-X8 mixed bed resin (Bio-Rad Laboratories, Richmond, CA) was added, and the pH of the mixture was kept between 2.0 and 3.0 by periodic additions of HCl until the pH, AA content (as measured with test strips; EM Science, Gibbstown, NJ), and conductivity had stabilized. The resin was removed by filtration and the samples were lyophilized. Samples were subjected to [^{13}C]nuclear magnetic resonance analysis using a Varian XL-400 spectrometer. Isotopic enhancements were determined by integrating relevant peaks, subtracting the contribution from natural abundance ^{13}C and dividing by the latter.

Whole cell conversions of potential AA precursors

Shake flask-grown cells were washed and resuspended in buffer (5 mM citrate/20 mM phosphate, pH 4.5) or fresh growth medium. Cell suspensions were supplemented with carbon sources, as indicated in the results. Supplemented cell suspensions were incubated for 24–30 h at 35 °C with shaking. The entire suspension was extracted with 5% trichloroacetic acid and assayed for AA.

Phosphomannose isomerase (PMI) and phosphomannomutase (PMM) assays

Magnesium-limited cells, washed and resuspended in 50 mM TRIS/10 mM $MgCl_2$, pH 7.5, were broken in a French press and centrifuged at 30 000 g for 30 min. The resulting supernates were desalted using Pharmacia PD-10 columns (Pharmacia Biotech, Uppsala, Sweden). Activities of PMI and PMM were determined according to the methods of Sa-Correia *et al.* (1987). Protein in the original extracts was determined by the method of Bradford (1976). All biochemicals were purchased from Sigma Chemical Co. (St Louis, MO).

GDP-Man pyrophosphorylase (GMP) assay

Cells in 50 mM phosphate buffer, pH 7.0, containing 20% (v:v) glycerol and 0.1 M NaCl (3 ml buffer g^{-1} wet cells) were broken in a French press. The crude extracts were centrifuged at 15 000 g for 15 min. The reactions were carried out in the forward direction by adding various volumes of extracts to solutions of 50 mM phosphate/4 mM $MgCl_2$ buffer, pH 7.0, containing 1 mM GTP. The reactions were initiated by addition of 1 mM (final) Man-1-P. The final reaction volume was 0.1 ml. The reaction mixtures were incubated at 30 °C for 10 min, filtered through 0.45 μm PVDF syringe filters and analysed for GDP-Man by HPLC. A Supelcosil SAX1 column (4.6 $\times$ 250 mm; Supelco, Bellefonte, PA) was used with solvent mixtures (flow rate 1 ml min^{-1}) of (A) 6 mM potassium phosphate, pH 3.6 and (B) 500 mM potassium phosphate, pH 4.5. The step gradient was: 0–3 min, 100% (A); 3–10 min, 79% (A); 10–15 min, 29% (A). Column temperature was 30 °C.

GDP-Man-3,5-epimerase assay

Cells were harvested after 43–48 h growth under Mg-limiting conditions, washed and resuspended in 50 mM MOPS/5 mM EDTA, pH 7.2. Cells were disrupted in a French press and centrifuged at 20 000 g for 20 min. Fifty μl aliquots of dilutions of the resulting extracts were added to 10 μl of 6.3 mM Na-[U- ^{14}C]GDP-Man (NEN Life Science Products, Boston, MA). This substrate had an activity of 16 μCi ml^{-1} before dilution into the reaction mixture. The reactions were stopped after 10 min by transferring 20 μl of each mixture into a microfuge tube containing 20 μl of 250 mM trifluoroacetic acid (TFA) and 1.0 g l^{-1} each Man and Gal. These tubes were sealed and boiled for 10 min, cooled, spun at 10 000 g for 60 s, and 5 μl of each hydrolysate was spotted on plastic-backed EM Science (Gibbstown, NJ) silica gel 60 thin-layer chromatography plates, with 1 cm lanes created by scoring with a blunt stylus. After drying, plates were twice chromatographed for 2.5 h in ethyl acetate:isopropanol:water, 65:22.3:12.7 (plates were dried between runs). Spots of free sugars were visualized with a *p*-anisaldehyde spray (Krebs *et al.*, 1969), followed by heating at 105 °C for about 15 min. Spots corresponding to Gal and Man were cut from the plates and counted in a scintillation counter (Model LS2800, Beckman Instruments, Inc, Irvine, CA). For time-zero control counts, 16.7 μl of each extract dilution was added to 23.3 μl of the labelled substrate above, which had been diluted 1 to 7 with the TFA/Man/Gal solution.

Biochemicals

D-Glucosone was prepared from Glc using pyranose-2-oxidase prepared from *Polyporus obtusus* by the method of Koths and Halenbeck (1986). L-sorbose and L-idonic acid were gifts from Frank Loewus, Washington State University, Pullman, WA. All other chemicals were of the highest grade commercially available.

Results and discussion

¹³C-NMR

When cultures of strain UV77-247 were grown on D-[1-¹³C]Glc and the AA isolated from those cultures was analysed by quantitative [¹³C]NMR, about three-quarters of the label was found at the C-1 position, and the remaining label was found at C-6 (Table 1). Further analyses suggested that the label at C-6 was due to catabolism of the Glc to 3-carbon intermediates and subsequent gluconeogenesis, similar to that found in carrots by Krook *et al.* (1998, 2000). Isotopic enhancements in the 'mirror image' positions were seen in AA recovered from cultures grown with D-[2-¹³C]Glc or D-[3-¹³C]Glc; about one-fifth of the labels were found at C-5 and C-4, respectively.

The small, but significant, amount of enhancement observed in other, 'non-mirror image', positions is consistent with flux through the pentose phosphate pathway, the overall balanced equation for which is:



Based on the known biochemistry, it would be expected that carbon flux through this pathway would result in AA with isotopic enhancement at positions (1) and (3) when cells were grown on D-[2-¹³C]Glc and enhancement at position (2) when cells were grown on D-[3-¹³C]Glc. This was indeed observed. That there is twice as much enhancement at C-1 as there is at C-3 after growth on D-[2-¹³C]Glc is also predicted.

Conversion of putative pathway intermediates by strain UV77-247

When resting cells of strain UV77-247 were incubated with various compounds, some of which have been proposed as intermediates in the AA biosynthetic pathways in animals, plants and other algae, AA accumulated to various extents, depending on the substrate (Table 2). L-Gal and L-galactono-1,4-lactone (L-GalL) were converted to AA more rapidly than was Glc, suggesting that these compounds are intermediates in the biosynthetic pathway from Glc, with the rate-limiting steps occurring upstream of those compounds.

Saito *et al.* (1990) proposed a pathway in bean and spinach in which Glc is converted to AA through D-glucosone and L-sorbose. Strain UV77-247 converted D-glucosone to AA better than it did L-sorbose. In the absence of transport considerations, this is the opposite of

Table 1. Labelling pattern in AA after cells were fed D-[1-¹³C], [2-¹³C] or [3-¹³C]Glc

Cells of strain UV77-247 were grown to moderate density in Mg-limiting medium with D-[1-¹³C], [2-¹³C] or [3-¹³C]Glc as 10% of the total Glc (40 g l⁻¹). The clarified, deionized culture broth was analysed by nmr to determine the labelling pattern in synthesized AA.

Carbon position in AA	% of label in AA carbons when cells were fed Glc labelled at:		
	C-1	C-2	C-3
1	73.4	7.3	2.8
2	0	73.0	6.3
3	0	3.6	69.7
4	0	0	19.7
5	0	16.1	1.4
6	26.6	0	0

Table 2. AA concentrations in resting-cell suspensions of *P. moriformis* strain UV77-247 incubated with potential AA precursors

Cells from Glc-depleted flask cultures were washed, resuspended in complete medium with Glc replaced by one of the listed compounds (50 mM), incubated for 24–30 h with shaking at 35 °C and whole broths were assayed for AA.

Substrate	AA (mg l ⁻¹), less no-substrate control	
	Trial 1	Trial 2
L-Gal	623	430
L-GalL	476	393
Man		174
Fru	248	120
D-glucosone	247	174
Glc	242	116
Gal	200	73
D-glucono-1,5-lactone	40	
D-gulono-1,4-lactone	24	
GlcUA	22	
L-sorbose	0	
2-keto-D-gluconic acid	-1	
D-glucurono-6,3-lactone	-13	
D-gluconic acid	-33	
D-GalUA	-45	
L-idonate	-46	

what one might expect if the D-glucosone pathway is correct, since L-sorbose is downstream from D-glucosone in that pathway. The importance of this pathway has also been questioned by Pallanca and Smirnoff (1999).

GlcUA and D-glucurono-6,3-lactone, intermediates in the biosynthetic pathway in rats (Isherwood *et al.*, 1954a, b) were not converted to AA. Neither was GalUA, proposed as an intermediate in early descriptions of the AA biosynthetic pathway for plants (Isherwood *et al.*, 1954a) and algae (Shigeoka *et al.*, 1979). Other conceivable precursors not converted to significant levels of AA included D-gluconolactone, D-gulono-1,4-lactone,

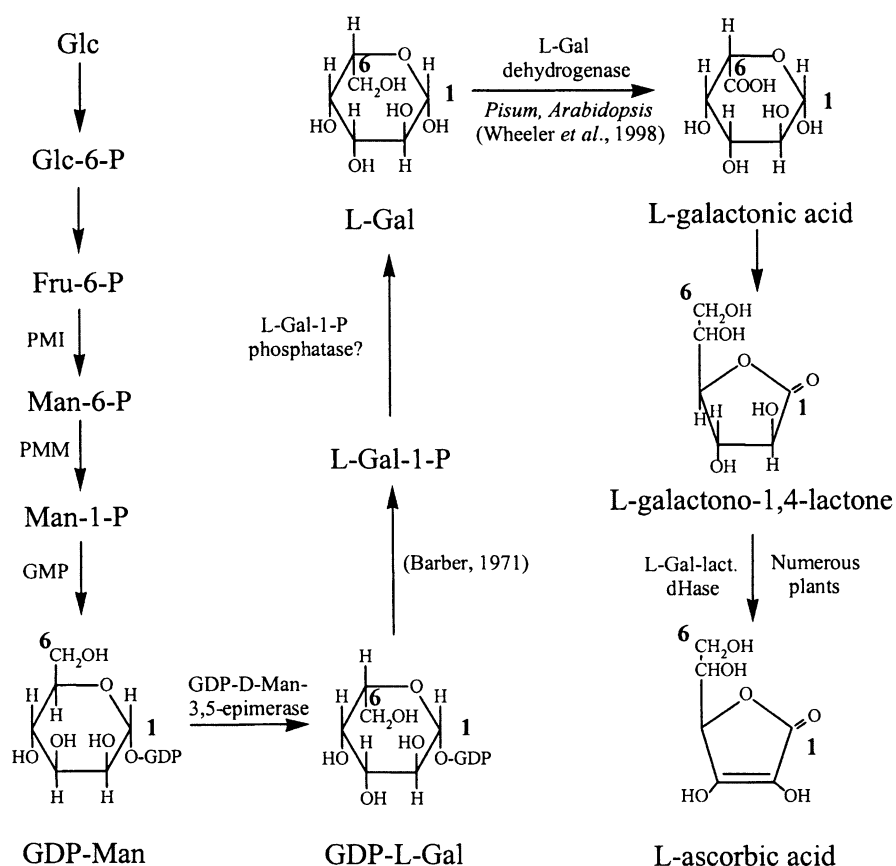


Fig. 1. Proposed pathway from Glc to AA in *P. moriformis*. The activities of PMI, PMM, GMP and GDP-Man-3,5-epimerase were assayed in mutant strains. PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-Man pyrophosphorylase.

2-keto-D-gluconic acid, D-gluconic acid and L-idonic acid. The lack of conversion of the last two is consistent with the results of ^{14}C labelling studies in beans by Saito and Loewus (1989). In a second trial, UV77-247 converted Man and D-glucosone to AA at greater rates than it did Glc. Again, L-Gal and L-GalL were rapidly converted. These data are consistent with the pathway shown in Fig. 1.

Conversion of potential AA biosynthesis intermediates by mutant *P. moriformis* strains

Mutants with varying abilities to accumulate AA were tested for their abilities to convert Glc, Man, L-Gal and L-GalL into AA (Table 3). Three strains (designated type I) that overproduce AA in Glc-fed culture and are typified by ATCC PTA-111 converted Man to AA as well or better than they did Glc, and converted L-Gal and L-GalL to AA at higher rates than they converted either D-sugar. Five other strains (type II), exemplified by the immediate descendant of ATCC PTA-111, UV244-1, did not convert either Glc or Man to AA, yet readily converted L-Gal and L-GalL, strongly suggesting blocks in the pathway upstream from L-Gal. Strain UV244-15 (type III) converted Glc and Man to AA, but did not convert either L-Gal or L-

GalL. This strain is a moderately good producer of AA, and this result suggests an inability to transport these two exogenously supplied compounds into the cell. Strain UV244-16 (type IV) converted Glc to AA and converted the other three compounds less well. This strain may well have a lesion that reduces the efficiency of the last enzymatic step in the pathway and this would explain its overall weak AA productivity from Glc.

Enzyme activities in mutants

Magnesium-limited cells of the wt and 14 mutant isolates ultimately derived from it were assayed for four key enzyme activities shown in Fig. 1. Table 4 summarizes the results of these assays. All of the strains that produced measurable amounts of AA had measurable epimerase activity. The converse was not true. Four of the strains that synthesized trace amounts or no AA had significant epimerase activities. Since these strains can synthesize AA from L-Gal and L-GalL, their genetic lesions are likely to lie in genes that affect the conversion of GDP-L-Gal to L-Gal. As shown in Fig. 2, there is a strong correlation between AA accumulation and GDP-Man-3,5-epimerase activity. This argues strongly for GDP-Man-3,5-epimerase

Table 3. AA concentrations in resting cell suspensions of strain ATCC PTA-111 and three immediate descendants of various AA-synthesizing abilities after incubation with potential AA precursors

Strain	Specific AA formation (mg l ⁻¹ culture A ₆₂₀ ⁻¹)	Substrate			
		Glc	Man	L-Gal	L-GalL
		AA, mg l ⁻¹ , less no-substrate control			
Type I ATCC PTA-111	73.7	44	80	138	120
Type II UV244-1	0.0	0	0	148	146
Type III UV244-15	24.5	51	68	4	0
Type IV UV244-16	5.0	46	25	25	25

Table 4. Specific enzyme activities (mU)^a of selected mutant *Prototheca* strains

Strain	Specific AA formation (mg l ⁻¹ culture A ₆₂₀ ⁻¹)	PMI ^b	PMM ^b	GMP ^b	Epimerase ^b
UV164-6	78.4				0.79
ATCC PTA-111	73.7	10.8	69.6	2.6	0.78
UV140-1	69.9				0.78
ATCC 209681	61.4				0.58
UV77-247	44.4				0.52
ATCC PTA-112	40.1	11.1	45.8	4.3	0.39
UV244-15	24.5	14.3	41.5	3.1	0.42
NA21-14	23.6	12.1	60.3	2.4	0.27
ATCC 75669 ^c	21.9				0.28
UV244-16	5.0	16.5	85.6	4.3	
ATCC PTA-113	2.0	17.7	47.0	2.0	0.03
UV218-1	0.4	15.9	72.1	2.7	0.83
UV213-1	0.1	19.7	47.7	3.2	0.60
UV82-21	0.0	14.6	70.6	4.1	0.15
UV244-1	0.0	18.2	51.1	5.5	0.15

^a Units: PMI and PMM, nmoles NADP reduced min⁻¹ mg⁻¹ protein; GMP, nmoles GDP-Man formed min⁻¹ mg⁻¹ protein; epimerase, nmoles GDP-L-Gal formed min⁻¹ mg⁻¹ protein.

^b PMI: phosphomannose isomerase; PMM: phosphomannomutase; GMP: GDP-Man pyrophosphorylase; Epimerase: GDP-Man-3,5-epimerase.

^c Wild-type strain.

activity being the rate-limiting step in the biosynthesis of AA in these cells and supports the AA synthesis pathway depicted in Fig. 1.

Non-inversion nature of AA pathway in *Prototheca*

Relying on labelling studies by Horowitz and colleagues (Horowitz *et al.*, 1952; Horowitz and King, 1953), Isherwood *et al.* (1954b) proposed an AA biosynthetic pathway in animals in which the carbon chain of Glc is inverted (C-1 of Glc becomes C-6 of AA; Fig. 3). They found that cress seedlings can synthesize small amounts of AA from D-GalUA methyl ester (Mapson and Isherwood, 1956), but the poor conversion argued against this compound as a natural substrate. Recent results (Davey *et al.*, 1999) have revived interest in this route as an alternative AA biosynthetic pathway in plants. Loewus' group has shown by tracer studies that cress seedlings and parsley (Loewus, 1963), strawberries (Loewus, 1963;

Loewus *et al.*, 1956, 1958; Loewus and Kelly, 1961), and the green alga *C. pyrenoidosa* (Renstrøm *et al.*, 1982/1983) synthesize AA from Glc without inverting the carbon chain configuration (C-1 of Glc becomes C-1 of AA).

Studies of AA biosynthesis in other algae have indicated an inversion pathway (Fig. 3) that involves GalUA (Grün and Loewus, 1984; Helsper *et al.*, 1982; Shigeoka *et al.*, 1979). One of these, *Euglena gracilis*, is a flagellated, photosynthetic protist. The other two are heterokont algae (Daughbjerg and Anderson, 1997), the flagellated chrysophyte *Ochromonas danica*, and the diatom, *Cyclotella cryptica* (Bacillariophyta). Their possession of the inversion pathway has phylogenetic implications, allying them biochemically with animals and separating them from organisms that use a non-inversion pathway, such as higher plants (Wheeler *et al.*, 1998) and green algae (Renstrøm *et al.*, 1982/1983). Indeed, many diatom genes more

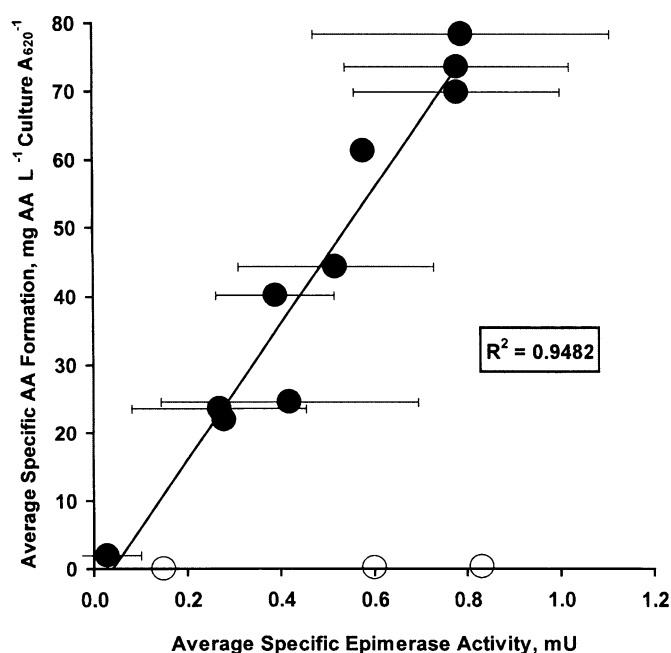


Fig. 2. Correlation of average specific epimerase activities with average whole broth AA specific formations of *P. moriformis* ATCC 75669 and fourteen mutants ultimately derived from it. The AA non-producing strains represented by open symbols probably are affected in steps between GDP-L-Gal and L-Gal.

closely resemble animal genes than those of higher plants (Qiu and Palmer, 1999). Since the colourless genus *Prototheca* fits into the clade encompassing higher plants and chlorophytes (Chesnick *et al.*, 1996), and chlorophytes are the algae most closely related to higher plants (Hicks *et al.*, 2001), it is not surprising that the AA biosynthesis pathway in *Prototheca* (Berry *et al.*, 1999) is similar to the pathway in higher plants (Wheeler *et al.*, 1998).

The [¹³C]labelling data in support of the non-inversion nature of AA synthesis in *Prototheca* are consistent with the inability of this organism to convert into AA compounds which are associated with an inversion pathway (Table 2). This study's [¹³C]labelling results agree with the [¹⁴C]labelling results reported by Loewus *et al.* for higher plants (Loewus, 1963; Loewus *et al.*, 1956, 1958; Loewus and Kelly, 1961) and *Chlorella* (Renström *et al.*, 1982/1983). About three-quarters of the label originating in C-1 of Glc was found in C-1 of AA made by *P. moriformis*. In line with the similar results of Krook *et al.* (1998, 2000), it is believed that the remaining label found at C-6 of AA is due to metabolism of Glc through triose-phosphate intermediates. After the formation of glyceraldehyde-3-P (Gly-3-P) and dihydroxyacetone-P (DHAP), and the conversion of the DHAP into a second molecule of Gly-3-P, the C-1 and C-6 carbons of Glc both become C-3 of Gly-3-P. When C-1-labelled Glc is metabolized to triose phosphates in glycolysis and then re-formed during subsequent gluconeogenesis, the newly

formed Glc would be labelled at either or both C-1 and C-6. If this re-formed Glc were then used to make AA, one would expect the AA to be similarly labelled. Consistent with this type of 'isotopic mixing' is the observation that Suc obtained from D-[1-¹³C]labelled Glc was labelled at positions 1, 6 and 1', and presumably 6' (data not shown), with the C-1 to C-6 ratio consistent with that observed for AA. Similar isotopic enrichment of Suc was also seen by Krook *et al.* (2000).

The non-inversion nature of the pathway is also supported by the C-2 and C-3 labelling data (Table 2). In both cases, about three quarters of the label in Glc remained at that same carbon in AA, and about one-fifth of the label was detected in the 'mirror-image' positions in AA, consistent with glycolytic isotopic mixing. Small amounts of label were also detected in 'non-mirror image' AA carbons, consistent with a small amount of the labelled Glc passing through the pentose phosphate pathway before gluconeogenesis.

Reactions in proposed pathway

Roberts (1971) fed labelled Man to corn root tips and found the label in polysaccharide, specifically in the residues of Man, L-Gal and Fuc. No label was detected in Glc, Gal, Ara, or Xyl. Man is converted to AA by *Prototheca* cells as well or better than Glc. This argues for Man being downstream from Glc in the pathway. The enzymes involved in the conversion of Fru-6-P to GDP-Man (PMI, PMM, GMP) have been well characterized in plants (Feingold and Avigad, 1980; Oesterhelt *et al.*, 1997), yeast (Boles *et al.*, 1994; Hashimoto *et al.*, 1997), filamentous fungi (Smith and Payton, 1994), red algae (Oesterhelt *et al.*, 1996), and bacteria (Koplin *et al.*, 1992; Sa-Correia *et al.*, 1987). These enzyme activities were identified in all mutants of *P. moriformis*, but there was no correlation between any of these activities and their abilities to accumulate AA. Barber (1971, 1975) identified in *C. pyrenoidosa* enzyme activities for the conversion of GDP-Man to GDP-L-Gal (GDP-Man-3,5-epimerase) and conversion of GDP-L-Gal to L-Gal-1-P. The present study found a strong correlation between mutants' GDP-Man-3,5-epimerase activities and AA synthesizing abilities (Fig. 2), implicating this enzyme activity as the rate-limiting step in AA biosynthesis. These reactions logically link Man to the compounds which are rapidly converted to AA in *Prototheca*, namely L-Gal and L-GalL. It is highly probable that the four strains that showed little or no AA accumulation, but measurable epimerase activity (Fig. 2; Table 4), have genetic lesions affecting activities between GDP-L-Gal and L-Gal, since their abilities to convert L-Gal to AA are unimpaired (Table 3, 'Type II').

Based on their discovery of an L-Gal dehydrogenase (L-GalDH), Wheeler *et al.* (1998) have recently proposed a similar pathway in higher plants. The same authors (Gatzek *et al.*, 2002) saw no increase in AA content

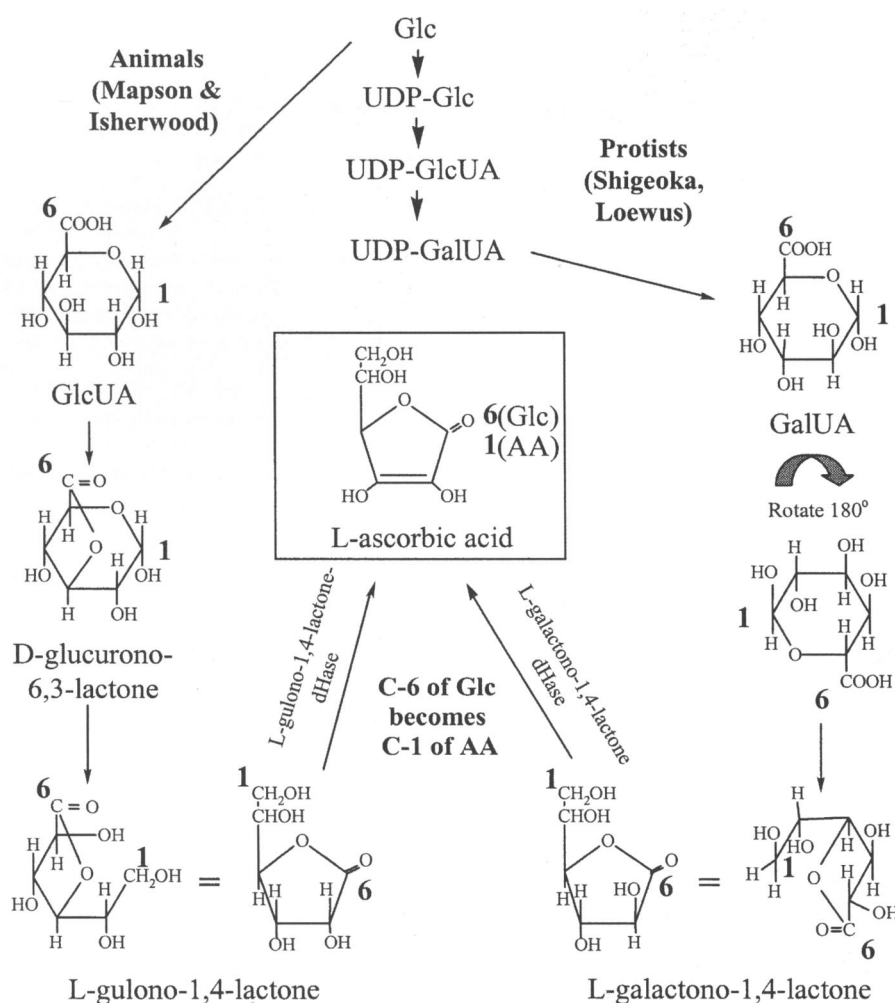


Fig. 3. Proposed biosynthetic pathways from Glc to AA in non-chlorophyte algae and animals. Inversion of the carbon chain was shown in the algae *Cyclotella* (Grün and Loewus, 1984), and *Poteriochromonas* (Helsper *et al.*, 1982). The latter saw AA enhancement with free GalUA, as did Shigeoka *et al.* in *Euglena* (Shigeoka *et al.*, 1979), but it is thought that UDP intermediates are the natural precursors. The inversion route in animals is based on results in rats (Isherwood *et al.*, 1954b).

when that *Arabidopsis* enzyme was overexpressed in tobacco. Although AA pool size increased under high illumination, L-GalDH activity did not. Peltzer *et al.* (1999) saw differences in the total AA and reduced/oxidized AA ratios in illuminated/darkened leaf parts of *Coleus*, and concluded that the L-GalDH may be light-modulated. They saw no difference in L-GalDH activities of light- and dark-harvested leaves, at physiological pHs. If the reaction rates in the *Coleus* AA pathway are similar to those in *Prototheca*, it is difficult to see how L-GalDH is responsible for the 30–50% increase in total AA levels seen under illumination. It is plausible that the difference in AA levels reflect an increase in the activity of another pathway enzyme under illumination. Although one must be wary of translating *in vitro* enzyme reaction rates to *in vivo* conditions, Table 4 shows epimerase rates that are more than an order of magnitude lower than those measured for PMM and PMI, and three or more times

lower than the GMP rates. Figure 2 clearly shows that the increase in productivity in the *Prototheca* mutants was due to increases in epimerase activity, implicating this enzyme as the rate-limiting step. Wolucka *et al.* (2001) have also demonstrated a strong correlation between epimerase activity and AA content of *Arabidopsis* cells. The proposed pathway, first described by Berry *et al.* (1999) and Wheeler *et al.* (1998), clears up decades of speculation as to the main route of AA synthesis in plants. These discoveries bring closer the possibilities of genetically engineering plants for increased AA content, and industrial microbes for the fermentative production of AA at large scale.

Acknowledgement

We thank Gary Juneau for NMR analysis of [^{13}C]-containing samples.

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Optimization of Fatty Acid Extraction from *Phaeodactylum tricornutum* UTEX 640 Biomass

M.J. Ibáñez González^a, A. Robles Medina^{a,*}, E. Molina Grima^a,
A. Giménez Giménez^a, M. Carstens^b, and L. Esteban Cerdán^a

^aDepartamento de Ingeniería Química, Universidad de Almería, E-04071 Almería, Spain, and ^bDepartamento de Ingeniería Química, Universidad de la Patagonia, Comodoro Rivadavia, Argentina

ABSTRACT: Fatty acids in the microalga *Phaeodactylum tricornutum* were isolated using an optimized three-step method: extraction of crude fatty acid potassium salts made by direct saponification of lipids in the microalgal biomass with KOH/ethanol (96%, vol/vol), separation of unsaponifiable lipids by extraction with hexane, and final purification of fatty acids by acidification of the alcoholic solution of potassium soaps followed by extraction of fatty acid into hexane. Direct saponification was carried out in ethanol (96%, vol/vol) using 2.09 mL ethanol (96%) per gram of wet biomass (10 mL/g of dry biomass) mixed with 0.4 g KOH/g of biomass. Under these conditions the fatty acid yield was 87%. The optimal water content of the alcoholic solution for extraction of the unsaponifiables was established as 40%, w/w. Data on equilibrium carotenoid distribution between the alcoholic (40%, w/w water) and hexane phases were determined. These data allow prediction of the carotenoid yields with different volumes of hexane in several extraction steps. The optimal pH of the alcoholic solution before extracting the purified fatty acid was established as pH 6, and the equilibrium fatty acid distribution between the alcoholic and hexane phases was determined. This optimized method permitted a 20% reduction in the production costs of highly purified eicosapentaenoic acid (EPA) in the three-step preparative process (extraction of fatty acid, concentration of polyunsaturated fatty acids by the urea method, and EPA fractionation through preparative high-performance liquid chromatography) previously developed by the authors.

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KEY WORDS: Eicosapentaenoic acid (EPA), fatty acid extraction, fatty acid purification, *Phaeodactylum tricornutum*.

Technology has been evaluated for the potential production of polyunsaturated fatty acids (PUFA) by microorganisms (1). Human diets formulated to contain PUFA reportedly produce a preventive/regulatory effect in the treatment of circulatory system diseases (2). Today's technology has made oils from microalgae competitive with those from fish as a source of these raw materials (3). Under certain growth conditions, the microalga *Phaeodactylum tricornutum* has a high rate of eicosapentaenoic acid (EPA, 20:5n-3) production. EPA is a fatty acid essential for human metabolism and is involved in

blood lipids equilibrium. EPA lowers triglyceride levels in blood serum, reduces the degree of platelet aggregation, is anti-inflammatory (4), and prevents hypertriglyceridemia (5) and various carcinomas (6). Total *Phaeodactylum tricornutum* fatty acids include 20–40%, w/w, EPA and small amounts of other PUFA that are difficult to separate from EPA during the purification process (7,8).

Current processes cannot provide the highly purified products required by the pharmaceutical industry for preclinical and clinical trials (2). Therefore, further development in this area of microalgal biotechnology will require rapid reliable methods of PUFA extraction and purification from the microalgal biomass. At the same time, such treatments must minimize autooxidative degradation and the presence of artifacts. Furthermore, solvents used to extract PUFA for the pharmacological industry must be selected bearing in mind their toxicity, handling, safety, and cost.

A three-step process for obtaining highly purified PUFA from cod liver oil and microalgae has recently been developed. The three steps are, fatty acid extraction by direct saponification of biomass, enrichments of PUFA by urea fractionation, and isolation of PUFA through preparative high-performance liquid chromatography (HPLC). In this way, 96%-pure EPA and 95%-pure docosahexaenoic acid (DHA) from the microalga *Isochrysis galbana* (9), 96%-pure EPA from *P. tricornutum* (8,10), and 94%-pure EPA and 81%-pure arachidonic acid (AA) from *Porphyridium cruentum* (11) have been obtained. A preliminary estimate of the cost of obtaining EPA from wet *P. tricornutum* biomass by this procedure puts the production cost of 96%-pure EPA at US\$188 per gram, extraction by direct saponification of biomass and purification through preparative HPLC being the most expensive steps (67% of the total costs) (10). The objective of this work was to reduce extraction costs by optimizing the first step (the extraction of fatty acid from biomass).

MATERIALS AND METHODS

Microalgal biomass. Wet biomass of the marine microalga *P. tricornutum* UTEX 640 was used as an oil-rich substrate with a high proportion of EPA. Cells were grown in an outdoor tubular photobioreactor, harvested by centrifugation at

*To whom correspondence should be addressed. E-mail: arobles@ualm.es

1800 × g, and then stored at -18°C until use. The wet paste biomass contained $20.9 \pm 1.3\%$ dry biomass. The total fatty acid content in the biomass was $10.9 \pm 0.5\%$ of the dry weight, and the EPA content was 26% of the total fatty acids.

Fatty acid extraction from biomass. Fatty acid extraction was performed by the three-step method shown in Figure 1, that is, direct saponification of wet biomass, followed by extraction of unsaponifiables and extraction of purified fatty acids.

Direct saponification of wet biomass. In a typical experiment 4.8 g of wet biomass (1 g of dry biomass) was treated with 76 mL of ethanol (96%, vol/vol), containing 1.6 g of KOH (85% pure), in a 2.5-L reactor that was jacketed for temperature control. Saponification was carried out at 60°C for 1 h with constant agitation in an argon atmosphere. The mixture obtained was then filtered through a 100–160 μm microporous glass filter, and the biomass residue was washed with 20 mL of ethanol (96%).

Extraction of unsaponifiables. In a typical experiment 20 mL of water was added to 96 mL of the soap solution (11% w/w water and 47 ± 2 mg/L of carotenoids) to obtain a solution with 28% w/w water, and unsaponifiables were extracted in subdued light at 20°C by adding hexane and shaking. The two phases were then separated and two aliquots of each

phase taken for carotenoid determination. To study equilibrium carotenoid distribution between the alcoholic phase and hexane, different volumes of hexane were added to 25 mL of a 40% w/w, water/alcoholic solution (34.0 ± 1.1 mg/L carotenoids). These extractions were carried out at 20 and 40°C . Unsaponifiables were also separated from the soap solution in several extraction steps with different volumes of hexane.

Extraction of purified fatty acids. In a typical experiment the pH of the 40% w/w water/alcoholic solution was adjusted to pH 1.0 using 35% HCl. The fatty acid concentration of this solution was 903 ± 18 mg/L. The equilibrium distribution of fatty acids was determined by adding different volumes of hexane to 25 mL of fatty acid solution. Extractions were performed in subdued light in an argon atmosphere at 20°C with agitation. The phases were separated, and an aliquot of each was taken for fatty acid determination. Extraction of fatty acid was also carried out in several steps with different volumes of hexane (Fig. 1).

Carotenoid determination. Carotenoids were determined as a measure of the unsaponifiable extraction yields using a modified version of the method employed by Whyte (12). To determine carotenoids in the alcoholic solution a known volume of this solution was dried under a N_2 stream. The residue was resuspended in an aqueous solution with 60% KOH, and the carotenoids were extracted with ethyl ether. Then the ethyl ether phase was dried, and carotenoids were resuspended in a known volume of acetone. To determine carotenoids in the hexane phase a known volume of this solution was dried, and the residue was resuspended in a known volume of acetone. The optical densities (OD) of these suspensions were determined at 444 nm using acetone as the reference. Carotenoids concentration (C) was determined by Equation 1:

$$C (\text{mg L}^{-1}) = 4.32 \text{ OD} - 0.0439 \quad [1]$$

This equation was obtained using β -carotene as the solute and acetone as the solvent with a correlation coefficient (r^2) of 0.9998. Equation 1 was used to determine carotenoids in the initial alcoholic solution and in hexane upon reaching equilibrium. Carotenoids in the alcoholic phases were determined by difference between the amount of initial carotenoids and the amount in the hexane phase.

Fatty acid determination. Fatty acids in feedstock, hexane, and alcoholic phases were analyzed by capillary gas chromatography (GC). To determine fatty acids in the hexane phase, a known volume was dried in a N_2 stream, and methylation was by direct transesterification following the method of Lepage and Roy (13). Methylation and methyl ester analysis have been described elsewhere (14). To determine fatty acids in the alcoholic phase, a known volume was mixed with an equal volume of water, and HCl was added to pH 1.0; fatty acids were extracted three times with hexane, and the amount in hexane was then analyzed as indicated above. Fatty acid yields were then calculated (14). The total fatty acid content in biomass was determined by direct transesterification of fatty acid in biomass.

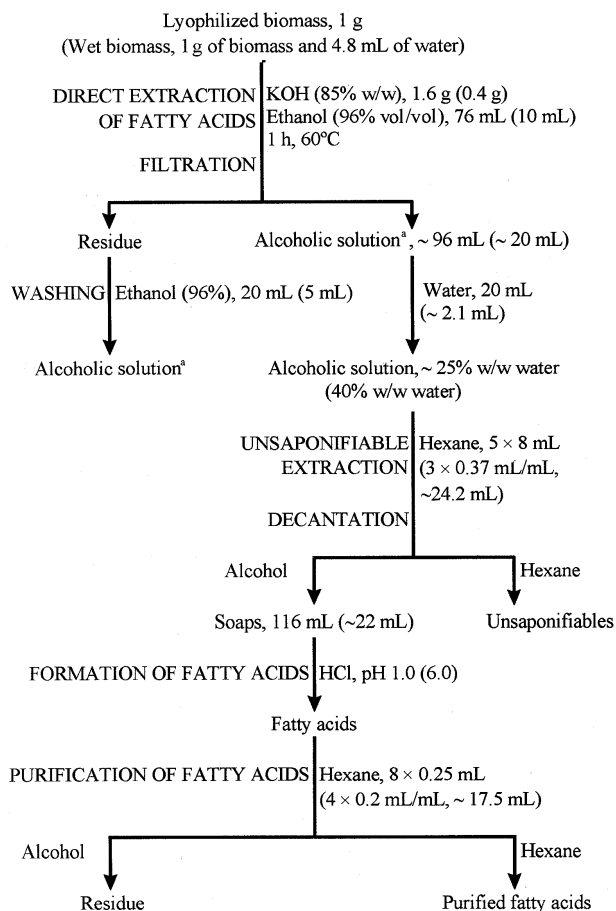


FIG. 1. Original and optimized (in parentheses) process to extract and purify fatty acids from the microalga *Phaeodactylum tricornutum*. (*The two alcoholic solutions are mixed.)

Protein and chlorophyll determination. Proteins were determined in the final alcoholic solution after separating the purified fatty acids, as described by Lowry *et al.* (15). Chlorophylls in the biomass were measured according to the method of Hansmann (16).

RESULTS AND DISCUSSION

Fatty acid extraction from biomass. In earlier work several low-toxicity solvents were evaluated for extracting fatty acids from the microalgae *I. galbana* (14) and *P. tricornutum* (8). The best solvents were ethanol (96% vol/vol) and hexane/ethanol (96%) (1:2.5, vol/vol). However, lyophilized biomass was extracted and large volumes of solvents were used (Fig. 1). Table 1 shows that fatty acid yield decreased about 4% when the ratio of ethanol (96%)/wet biomass decreased from 20 to 1 mL/g, and the ethanol concentration decreased from 92 to 55% (vol/vol), as consequence of water present in the wet biomass. Therefore, an ethanol (96%)/wet biomass ratio of 1.05 mL/g, which is much lower than what was used previously (76 mL/g of lyophilized biomass) (8), seems to be enough. However, 2.09 mL of ethanol (96%) per gram of wet biomass (10 mL/g of dry biomass) was used, because the subsequent filtration took too long with an ethanol (96%)/wet biomass ratio of 1.05 mL/g. The partial yields of EPA were similar to the overall fatty acid yields (Table 1). When the extraction was made from lyophilized biomass a fatty acid yield of 96.2% was obtained (Table 1). The decrease in the fatty acid extraction yield obtained with wet biomass can be compensated by the decrease in cost because the lyophilization is omitted.

Extraction of unsaponifiables. The crude fatty acid extract obtained with ethanol (96%)/KOH (i.e., the initial alcoholic solution in Fig. 1) contains the potassium salts of fatty acids,

pigments such as carotenoids and chlorophylls (the chlorophylls in *P. tricornutum* biomass were about 1% by dry weight), proteins (the protein concentration in the final alcoholic solution was 14.8 g/L) and other lipid and nonlipid contaminants. The unsaponifiable lipids, such as carotenoids, can be extracted by treating the crude extract with apolar solvents such as chloroform or hexane, in which soaps are not soluble. Here hexane was used because it is less toxic than chloroform. The alcoholic solution contained a little water [i.e., only the water added with the wet biomass and ethanol (96% vol/vol)]. An increase in the water content (obtained by adding water to a solution with 11% w/w water and 45 mg/L of unsaponifiables as β -carotene decreased the miscibility of the alcoholic solution and hexane and increased the unsaponifiable extraction yield. At water contents of 11, 30, 40, 50, 60, and 70% w/w, the unsaponifiable extraction yields with respect to the amount of unsaponifiables contained in the alcohol solution were 15.3, 24.6, 37.6, 50.3, 60.1, and 54.6% w/w of β -carotene, respectively (extraction carried out at 20°C and an alcohol solution/hexane ratio 1:1, vol/vol). At water percentages over 60%, emulsions formed, the stability of which increased with water content. Also, when the 50–60% water/alcoholic solutions were later acidified to extract the fatty acids (Fig. 1), emulsions again formed. Emulsions make extraction difficult and decrease the fatty acid recovery. So an optimal water content of 40% was chosen to prevent emulsions formation. In a previous article (8), it was reported that emulsions formed more easily when the biomass residue was not separated from the soap solution by filtration and when this residue was washed with water instead of ethanol. The alcoholic solution contained surfactant agents, such as soaps and proteins, that facilitated and stabilized the alcoholic/hexane emulsion. An increase in the water content increased the amount of hydrating water that stabilized the hydrophilic moieties of the surfactant agents, thus stabilizing the emulsion.

To optimize the hexane/alcoholic solution ratio used for extracting the unsaponifiables, we determined the equilibrium carotenoid distribution between the hexane and alcoholic (40% w/w water) phases and/or the relationship between the extraction yield and the hexane/alcohol ratio (Fig. 2, Table 2). Table 2 shows that the recovery of unsaponifiables with hexane increased with the hexane/alcohol phase ratio. However, a large volume of hexane was required to extract the unsaponifiables quantitatively. The equilibrium distribution constant values (Table 2) and the equilibrium data (Fig. 2) also show that equilibrium was greatly displaced to the alcoholic phase and that when the temperature was increased from 20 to 40°C, the equilibrium was only slightly displaced to the hexane phase. The equilibrium data enable prediction of the unsaponifiable yield when the extraction is done in one or several steps with different volumes of hexane. Table 3 compares the extraction yields obtained experimentally (by a five-step hexane extraction) and those obtained from the equilibrium data. The calculation of the latter is based on a sequence of five batch extraction steps, typical in the chemical process industry, assuming that equilibrium has been attained in each

TABLE 1
Influence of Ethanol (96%)/Wet Biomass ($20.9 \pm 1.3\%$ dry biomass) Ratio on the Fatty Acid Yield Obtained by Direct Saponification of Wet *Phaeodactylum tricornutum* Biomass

Ethanol (96%)/ wet biomass (mL/g)	Ethanol (96%)/ dry biomass (mL/g)	Ethanol ^a (%) (vol/vol)	Fatty acid yield ^b (%)	EPA yield (%)
1.05	5	55	86.0	86.0
2.09	10	70	87.0	90.2
2.93	14	76	86.3	89.7
3.97	19	80	86.9	88.5
7.94	38	87	88.5	89.2
11.91	57	90	90.6	90.9
18.88	76	92	90.4	92.1
19.86	95	92	90.0	92.2
—	76 ^c	96	96.2 ^d	98.3 ^d

^aThe concentration of ethanol diminishes as a result of the water contained in the wet biomass.

^bFatty acids were extracted by four aliquots of 0.2 mL hexane per mL of alcohol solution at 20°C. Percentage of fatty acid extracted with respect to the total amount of fatty acids contained in the initial biomass.

^cEthanol/dry biomass ratio corresponds to the usual solvent/biomass ratio used by the method of Bligh and Dyer (Ref. 18).

^dThese yields were obtained using lyophilized biomass.

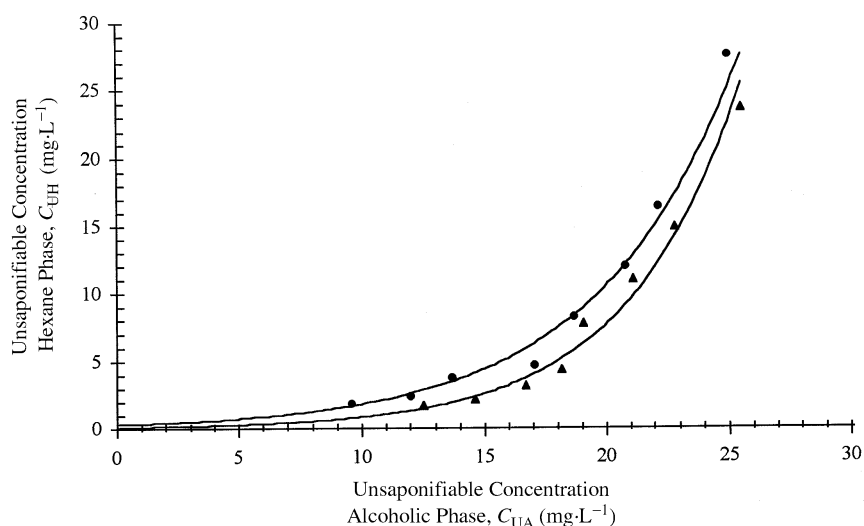


FIG. 2. Equilibrium distribution of unsaponifiables between the alcoholic (40% w/w water) phase and hexane at 20 (▲) and 40°C (●). The experimental data were fit by the equations $C_{UH} = 0.10 \exp(0.217 C_{UA})$ at 20°C and $C_{UH} = 0.32 \exp(0.175 C_{UA})$ at 40°C. The correlation coefficients (r^2) were 0.9757 and 0.9797, respectively. C_{UH} is the concentration of unsaponifiables in the hexane phase ($\text{mg}\cdot\text{L}^{-1}$) and C_{UA} is the concentration of unsaponifiables in the alcoholic phase. The mean deviations between the experimental C_{UH} and the values predicted by the equations are 12.5 and 9.5% of the experimental C_{UH} at 20 and 40°C, respectively.

step and further that the two phases are totally immiscible (17). Good correlation is observed (less than 10% difference). Table 3 shows that maximal unsaponifiable yields of 48.7% were obtained with a total hexane/alcohol solution ratio of 1.87 vol/vol in five extraction steps. Some unsaponifiables not extracted will remain in the final fatty acid extract, although unsaponifiable recovery here (48.7%) is better than in the original procedure (38%, Fig. 1) because the extraction is from an alcoholic phase with a lower water content (approx-

mately 25% w/w water, Fig. 1). Depending on the intended use of the final fatty acid extract, more extractions of unsaponifiables may be necessary.

Extraction of purified fatty acids. In early work (8) the pH of the alcoholic potassium salt solution was adjusted to 1 before extracting the purified fatty acids. However in some experiments carried out using the optimized conditions, emulsions formed when the pH was lowered to this value and hexane was added. At this point, solubility of surfactant agents such as proteins contained in the alcoholic solution may decrease with the change in pH. Even small amounts of these surfactant agents can accumulate in the alcoholic solu-

TABLE 2
Influence of the Hexane/Alcohol Solution Ratio (H/A) on the Unsaponifiable Extraction Yields (R_U) and Equilibrium Distribution Constants (K_U) of Unsaponifiables Between the Hexane and the Alcoholic Solution

H/A (vol/vol)	Extraction temperature 20°C		Extraction temperature 40°C	
	K_U^a	R_U^b (% w/w of β -carotene)	K_U^a	R_U^b (% w/w of β -carotene)
80.0	—	100	—	—
40.0	5.43	99.7	—	98.9
14.0	0.14	71.7	0.20	79.8
10.0	0.15	64.9	0.21	73.0
6.0	0.19	56.1	0.28	66.3
4.0	0.24	50.8	0.27	55.1
2.0	0.41	45.7	0.44	47.7
1.2	0.52	38.9	0.58	40.9
0.8	0.65	33.4	0.74	36.0
0.4	0.93	25.1	1.10	26.6

^a $K_U = C_{UH}/C_{UA}$, where C_{UH} is the concentration of unsaponifiables in the hexane phase and C_{UA} is the concentration of unsaponifiables in the alcoholic phase.

^bPercentage of unsaponifiables extracted with respect to the amount of unsaponifiables contained in the initial alcoholic solution.

TABLE 3
Comparison Between the Yields of Unsaponifiables Obtained by Extraction with Hexane and the Yield Predicted by the Equilibrium Data

Extraction ^a step	Unsaponifiable yield (% w/w of β -carotene)		
	Step ^b	Σ Step ^c	Equilibrium data ^d
1	26.0	26.0	29.1
2	9.0	35.0	40.0
3	6.3	41.3	45.6
4	4.3	45.6	50.0
5	3.1	48.7	53.5

^aExtractions were made at 20°C using a hexane/alcohol solution (40% w/w water) ratio (H/A) of 0.37 vol/vol per step.

^bUnsaponifiable yield obtained experimentally. Percentage of unsaponifiables extracted with respect to the amount of unsaponifiables contained in the alcohol solution.

^cUnsaponifiable yield obtained experimentally. Percentage of unsaponifiables extracted with respect to the amount of unsaponifiables contained in the initial alcohol solution.

^dUnsaponifiable yield obtained from the equilibrium data presented in Figure 2 [$C_{UH} = 0.10 \exp(0.217 C_{UA})$]. For abbreviations see Table 2.

TABLE 4

Influence of the Hexane/Alcohol Solution Ratio on the Fatty Acid Yields and Distribution Constants of Fatty Acids and Eicosapentaenoic Acid^a

H/A (vol/vol)	K_F	K_{EPA}	R_F (%)
14.0	—	—	100
10.0	—	—	99.4
6.0	—	—	98.7
4.0	17.8	18.5	98.3
2.0	16.2	16.9	97.1
1.2	15.0	15.8	97.4
0.8	15.9	16.8	92.6
0.4	18.5	17.8	83.6
0.2	26.1	25.0	81.7

^aExtraction temperature, 20°C. H/A = hexane/alcohol solution ratio. $K_F = C_{FH}/C_{FA}$, where C_{FH} is the fatty acid concentration in the hexane phase and C_{FA} is the fatty acid concentration in the alcohol phase. K_{EPA} = distribution constant for eicosapentaenoic acid (EPA) concentration in the hexane phase/EPA concentration in the alcoholic phase. R_F = percentage of fatty acids extracted with respect to the amount of fatty acids contained in the alcoholic solution.

tion/hexane interface, hence stabilizing the emulsion. Thus, a study of the influence of pH on the fatty acid yield was carried out. Compared to the fatty acid and EPA yields obtained at pH 1, fatty acid and EPA yields did not decrease when pH was adjusted between 3 and 6. At pH 7, low fatty acid and EPA extraction yields were obtained presumably because the fatty acids were still partially in the form of potassium salts.

The equilibrium distribution of fatty acids between the hexane and alcoholic phases was similar to the equilibrium distribution of unsaponifiables. Table 4 shows that the recovery of fatty acids with hexane increases with the hexane/alcohol solution ratio. In this case, a quantitative extraction of fatty acids is possible using relatively low hexane/alcohol

TABLE 5

Comparison Between the Fatty Acid and EPA Yields Obtained by Hexane Extraction and Predicted by the Equilibrium Data

Extraction ^a step	Fatty acid yield (%)			EPA yield (%)		
	Step ^b	Σ Step ^c	Equilibrium data ^d	Step ^b	Σ Step ^c	Equilibrium data ^d
1	78.6	78.6	81.0	79.2	79.2	83.7
2	11.2	89.8	96.7	11.8	91.0	96.8
3	4.7	94.5	99.4	4.7	95.6	99.3
4	1.9	96.4	99.9	1.7	97.4	99.8

^aExtractions were made using a hexane/alcohol solution (40% w/w water) ratio of 0.20 vol/vol per step.

^bFatty acid (or EPA) yield obtained experimentally, expressed as percentage of fatty acids (or EPA) extracted with respect to the amount of fatty acids (or EPA) contained in the alcoholic solution.

^cFatty acid (or EPA) yield obtained experimentally, expressed as percentage of fatty acids (or EPA) extracted with respect to the amount of fatty acids (or EPA) contained in the initial alcohol solution.

^dFatty acid (or EPA) yields obtained from the equilibrium data presented in Figure 3; [$C_{FH} = 261.6 \exp(0.0174 C_{FA})$]. For abbreviations see Tables 2 and 4.

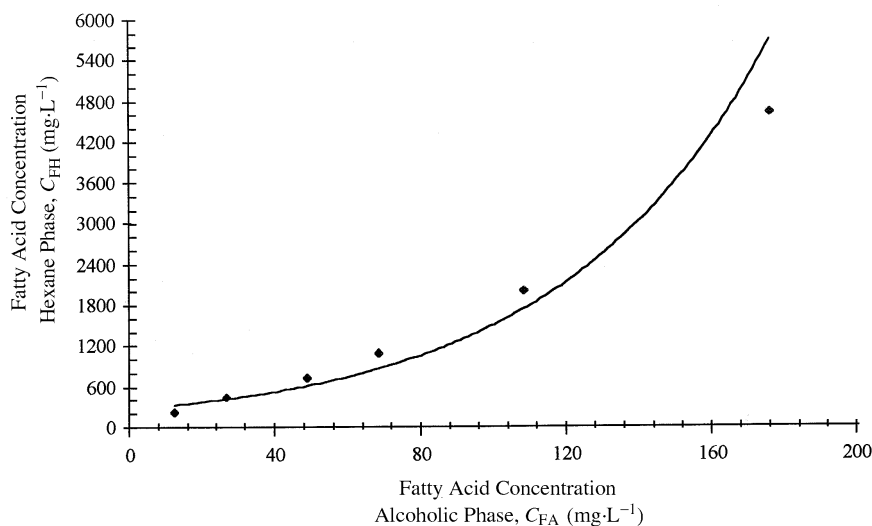


FIG. 3. Equilibrium distribution of fatty acids between the alcoholic (40% w/w water) phase and hexane at 20°C. The experimental data were fit by the equation $C_{FH} = 261.6 \exp(0.0174 C_{FA})$, with a correlation coefficient (r^2) of 0.9501. C_{FH} is the fatty acid concentration in the hexane phase ($\text{mg}\cdot\text{L}^{-1}$) and C_{FA} is the fatty acid concentration in the alcoholic phase; the mean deviation between the experimental C_{FH} and the values predicted by the equation is 20% of the experimental C_{FH} .

phase ratios. The EPA distribution constants are similar to those of the overall fatty acids, so similar EPA yields can be obtained. The equilibrium data represented in Figure 3 enable prediction of the fatty acid yields when the extraction is done in one or several steps with different volumes of hexane. Table 5 compares the extraction yields obtained experimentally (by a four-step hexane extraction) to those based on a sequence of four batch extraction steps, assuming that equilibrium is reached in each step and that the two phases are totally immiscible (17). Good correlation between the two yields may be observed (less than 4% difference).

Comparison of the original and optimized process. The optimized conditions identified in this work were: 10 mL

ethanol (96% vol/vol)/g biomass (2.09 mL/g wet biomass); washing of the biomass residue with 5 mL ethanol (96%)/g biomass; extraction of unsaponifiables by three extractions with 0.37 mL hexane/mL alcoholic solution (40% water w/w) each; and extraction of fatty acids by four extractions with 0.2 mL hexane/mL alcoholic solution (pH 6). The fatty acid yield was 87%, which was lower than the yield obtained in the original process (96.2%) (10). This is mainly because wet biomass was used instead of lyophilized biomass (see the last line in Table 1); however, elimination of the cost of lyophilization justifies this lower fatty acid yield. Figure 1 compares both original and optimized processes (amount in parentheses). As observed above, an important reduction in the amounts of hexane (about 90%) and ethanol (96%) (about 84%) has been achieved. In the earlier process the costs of extraction and purification to obtain highly purified EPA from *P. tricornutum* were estimated at about US\$188 per gram of 96% pure EPA, which included culture, extraction, concentration, and purification (10). By only optimizing the fatty acid extraction step, this cost could be reduced by about 20% (the production cost would be US\$150 per gram of highly purified EPA). The production cost of highly purified EPA from cod liver oil was estimated at about US\$369 per gram (10). After similar optimization, the cost of production of EPA from cod liver oil could only be reduced by about 8%, because only the volume of hexane in the final purification of the fatty acid was reduced. The extraction of fatty acids from cod liver oil is quite different, as the first step is saponification and it is not necessary to extract unsaponifiables (9,14). Although this method has been optimized for the microalga *P. tricornutum*, it can be extended to other microalgae such as *I. galbana* or *P. cruentum* with hardly any modification.

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