Universidad de Valladolid

ESCUELA DE INGENIERÍAS INDUSTRIALES

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

TESIS DOCTORAL:

Tratamiento de aguas residuales de la industria agroalimentaria mediante consorcios de microalgas-bacterias. Valorización de la biomasa algal mediante la obtención de monosacáridos, lípidos y biogás.

Presentada por David Hernández González para optar al grado de doctor por la Universidad de Valladolid

Dirigida por:

María Cruz García González
Mónica Coca Sanz

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Memoria para optar al grado de Doctor,
presentada por el Biotecnólogo:
David Hernández González

Dirigida por:
María Cruz García González y
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La presente tesis doctoral queda registrada en el folio número_______ del correspondiente libro de registro número ____________________

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DAVID HERNÁNDEZ GONZÁLEZ ha realizado bajo su dirección el trabajo "*Tratamiento de aguas residuales de la industria agroalimentaria mediante consorcios de microalgas-bacterias. Valorización de la biomasa algal mediante la obtención de monosacáridos, lípidos y biogas*", en el Instituto Tecnológico Agrario de Castilla y León, y en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, a ______ de_______________ de 2015

Fdo. María Cruz García González   Fdo. Mónica Coca Sanz

Reunido el tribunal que ha juzgado la Tesis Doctoral titulada "*Tratamiento de aguas residuales de la industria agroalimentaria mediante consorcios de microalgas-bacterias. Valorización de la biomasa algal mediante la obtención de monosacáridos, lípidos y biogas*" presentada por el Biotecnólogo David Hernández González y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por_______________ la calificación de _______________.

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PRESIDENTE SECRETARIO

1er Vocal 2º Vocal 3er Vocal

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Resumen

Durante las últimas décadas se ha producido un incremento exponencial en la generación de aguas residuales agroalimentarias como consecuencia del incremento de la producción industrial. Por ello, en la actualidad se pueden encontrar aguas residuales de elevada carga orgánica y una mayor concentración de nutrientes y productos tóxicos, por lo que deben ser tratadas previamente a su vertido para no incurrir en daños medioambientales graves.

En este contexto, a día de hoy existen diversas tecnologías que se emplean para tratar las aguas residuales agroalimentarias, como son los tratamientos físico-químicos y los procesos biológicos (aerobios, anóxicos, anaerobios y sus combinaciones). Estas tecnologías conllevan, en muchos casos, costes energéticos y de operación elevados y no permiten valorizar los nutrientes presentes en el agua residual. Una alternativa a los tratamientos biológicos más convencionales es el uso de consorcios de microalgas y bacterias en simbiosis, donde las bacterias oxidan la materia orgánica, liberando CO_2, amonio, nitratos, nitritos y fósforo soluble y mediante la fotosíntesis oxigénica las microalgas asimilan estos compuestos en forma de biomasa, liberando O_2. Una vez llevado a cabo el tratamiento del agua, las microalgas producidas pueden ser valorizadas en forma de biocombustibles (biodiesel, bioetanol, biogás, etc.) y otros compuestos de alto valor añadido, mejorando la viabilidad económica del proceso global.

En esta tesis se ha estudiado el tratamiento de cuatro aguas residuales agroalimentarias diferentes, tanto en origen como en composición, mediante consorcios de microalgas y bacterias y el aprovechamiento de la biomasa resultante para obtener distintos biocombustibles.

En el **Capítulo 3** se llevó a cabo el pretratamiento físico, químico e hidrólisis enzimática de las microalgas *Chlorella sorokiniana*, *Nannochloropsis gaditana* y *Scenedesmus almeriensis* a fin de identificar aquellos pretratamientos más eficientes y si la combinación de los mismos provoca un incremento en la liberación de azúcares fermentables. Los pretratamientos que conducen a una mayor liberación de monosacáridos fueron aquellos en los que se realizó un pretratamiento ácido en primer lugar y una posterior hidrólisis enzimática.

En el **Capítulo 4** se realizó un estudio comparativo para identificar aquellos procesos de extracción de lípidos más eficientes en las microalgas *Isochrysis* T-ISO, *Nannochloropsis gaditana*, *Scenedesmus almeriensis* y *Tetraselmis* sp. El proceso de extracción que permitió alcanzar los mejores rendimientos fue el MSE, que consiste en el tratamiento con microondas y posterior extracción con CO_2 supercrítico, independientemente del tipo de microalga empleada. En este proceso la biomasa se somete inicialmente a un pretratamiento con microondas y posteriormente a una etapa de extracción con CO_2 supercrítico. De esta forma, el rendimiento de recuperación de lípidos se incrementa en un 15-25%. Con el

objetivo de determinar la influencia del proceso de extracción de lípidos sobre la producción de metano a partir de una misma biomasa, se llevó a cabo su digestión anaerobia con y sin extracción MSE. Los resultados demuestran un considerable incremento de la producción de metano (15-60%) en aquellas muestras sin lípidos como consecuencia de una mayor biodegradabilidad provocada por la aplicación previa del proceso MSE, por lo que la rentabilidad económica del proceso puede verse aumentada en un 30%.

Posteriormente, en los **Capítulos 5** y **6** se llevó a cabo el tratamiento de aguas residuales procedentes de: i) la industria de fritura de patatas, ii) el tratamiento biológico de purines, iii) un matadero industrial de cerdos y iv) una industria de procesado de pescado mediante consorcios de microalgas y bacterias. Durante la operación de los fotobiorreactores se observó que al disminuir el tiempo de retención hidráulico se alcanzaban mejores rendimientos de eliminación de materia orgánica y nutrientes y mayores productividades de biomasa. De los resultados presentados en estos capítulos se desprende que el tratamiento biológico con consorcios microalgas-bacterias es eficaz para reducir el contenido de materia orgánica y nutrientes de las aguas residuales agro-industriales. En todos los ensayos realizados se obtuvieron eficacias de eliminación de DQO del 62-85%. La eliminación de nitrógeno en todos los casos fue del 80-95% mientras que la eliminación de fósforo total osciló entre el 58-90%. En el **Capítulo 5** se optimizó la relación substrato/inóculo para optimizar la producción de metano, de forma que éste se incrementó en más de un 40%.

Las algas producidas durante la operación de los fotobiorreactores alimentados con aguas de matadero (**Capítulo 6)** fueron valorizadas mediante extracción de lípidos empleando el proceso MSE y posterior digestión anaerobia de la biomasa resultante. Estos ensayos confirmaron los resultados obtenidos en el **Capítulo 4** donde se evidenció un incremento de la biodegradabilidad de las algas sometidas a una extracción previa de lípidos, con el consecuente incremento de la producción de metano. Con ello se demuestra que la producción de biocombustibles a partir de microalgas sería más rentable cuando se plantea la obtención de varios bioproductos de manera conjunta, en el marco de una biorrefinería.

Abstract

In recent decades, the increase of the industrial production has caused an exponential augment in the production of agroindustrial wastewater; therefore, agroindustrial wastewater with high organic matter, high nutrient concentration and high toxic compounds can be found. These wastewaters must be treated in order to avoid serious environmental damage.

In this context, different technologies are used to treat agroindustrial wastewater, like physico-chemical and biological processes (aerobic, anoxic, anaerobic and their combinations). However, these technologies have some disadvantages as their high energetic operating costs, as well as the inability to valorise the nutrients present in the wastewater. The use of symbiotic microalgae-bacteria consortia may be an alternative to conventional biological treatments. In these treatments, bacteria oxidize the organic matter, releasing CO_2, ammonia, nitrates, nitrites and soluble phosphorous, while microalgae assimilate these compounds in the form of biomass, and releasing O_2 by oxygenic photosynthesis. The produced microalgae may be further valorised in the form of biofuels (biodiesel, bioethanol, biogas, etc.) and other high-added value compounds, increasing the economic viability of the overall process.

In this thesis, the efficiency of microalgae-bacteria consortium to treat four agroindustrial wastewaters has been evaluated. These wastewaters were different in origin and composition, and the use of the resulting biomass to produce biofuels was studied.

In **Chapter 3** physical, chemical and enzymatic hydrolysis of the microalgae *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus almeriensis* has been carried out. These pretreatments were performed in order to identify which are the most efficient saccharification methods, and to determine if the combination of them could enhance the release of fermentable sugars. The highest release of monosaccharides was obtained using acid pretreatment followed by enzymatic hydrolysis.

In **Chapter 4** a comparative study of different lipid extraction methods was carried out using the microalgae *Isochrysis* T-ISO, *Nannochloropsis gaditana*, *Scenedesmus almeriensis* and *Tetraselmis* sp. The highest yields were achieved using the MSE process, consisting on a microwave pretreatment and subsequently supercritical extraction with CO_2, regardless of the type of microalgae used. During this process, the biomass was initially pretreated with microwave and then subjected to a supercritical CO_2 extraction. In this manner, the lipid extraction yield increased by 15-25%. In order to determine the influence of the lipid extraction on the methane production, the anaerobic digestion of the microalgae with and without MSE extraction was performed. The results showed a considerable increase in the methane production (15-60%)

in those samples without lipids as a consequence of a higher biodegradability caused by the MSE process. Thus, the profitability of the process may be increased by 30%.

In **Chapters 5** and **6** the treatment of agroindustrial wastewater from i) the potato processing industry, ii) the treated liquid fraction of pig manure, iii) an industrial piggery slaughterhouse and iv) a fish processing industry (**Appendix I**)was carried out using microalgae-bacteria consortium. During the experimental set up it was evidenced that the decrease in hydraulic retention time resulted in higher biomass productivity and higher removal efficiencies of organic matter and nutrients. The results presented in **Chapters 5** and **6** demonstrated that the biological treatment with microalgae-bacteria consortia is effective to reduce the content of organic matter and nutrients from agroindustrial wastewaters. In all experimental runs performed, COD removal efficiencies of 62-85% were obtained. Nitrogen removal in all cases was 80-95% while the total phosphorus removal ranged from 58-90%. In **Chapter 5** the substrate/inoculum ratio was optimized to maximize methane production, increasing by more than 40%.

The microalgal biomass produced during the slaughterhouse wastewater treatment (**Chapter 6**) was valorised through lipid extraction using MSE method and subsequently anaerobic digestion of the resulting biomass. These experiments confirmed the results obtained in **Chapter 4**, in which microalgal biomass subjected to lipid extraction increased its biodegradability; thus, an increase in

methane production was obtained. These results demonstrated that the production of biofuels from microalgae is more profitable when different bioproducts are obtained together, in the context of a biorefinery.

Lista de publicaciones

Paper I. Hernández, D., Riaño, B., Coca, M., & García-González, M. C. (2015). Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. *Chemical engineering Journal*, *262*, 939-945.

Paper II. Hernández, D., Solana, M., Riaño, B., García-González, M. C., & Bertucco, A. (2014). Biofuels from microalgae: Lipid extraction and methane production from the residual biomass in a biorefinery approach. *Bioresource technology*, *170*, 370-378.

Paper III. Hernández, D., Riaño, B., Coca, M., & García-González, M. C. (2013). Treatment of agro-industrial wastewater using microalgae–bacteria consortium combined with anaerobic digestion of the produced biomass. *Bioresource technology*, *135*, 598-603.

Paper IV. Hernández, D., Riaño, B., Coca, M., & García-González, M. C. (2015). Microalgal cultivation in high rate algal ponds using slaughterhouse wastewater for biofuel applications. *Chemical engineering journal* (Aceptado).

Paper V (Anexo I). Riaño, B., Hernández, D., & García-González, M. C. (2012). Microalgal-based systems for wastewater treatment:

Effect of applied organic and nutrient loading rate on biomass composition. *Ecological engineering*, *49*, 112-117.

Contribución a los artículos incluidos en la tesis

Paper I. En este artículo he sido el responsable del diseño experimental, de llevar a cabo la experimentación relativa a los pretratamientos y, en colaboración con la Dra. Mónica Coca Sanz y Jesús Rodríguez, de poner a punto el método de análisis de monosacáridos e inhibidores presentes en los hidrolizados de microalgas mediante HPLC. Asimismo, he llevado a cabo el análisis e interpretación de los resultados y la redacción del artículo bajo la supervisión de la Dra. María Cruz García, la Dra. Mónica Coca y la Dra. Berta Riaño.

Paper II. En este artículo he sido el encargado del diseño experimental, de llevar a cabo la preparación de las muestras y, en colaboración con Miriam Solana, de realizar la optimización del tratamiento de extracción de los lípidos presentes en las microalgas mediante fluidos supercríticos. Asimismo, he llevado a cabo la digestión anaerobia de las muestras de microalgas, analizando y evaluando los resultados obtenidos. La redacción del artículo se llevó a cabo bajo la supervisión de la Dra. María Cruz García, la Dra. Mónica Coca, la Dra. Berta Riaño y el Profesor Alberto Bertucco.

Paper III. En este artículo he sido el responsable del diseño experimental en colaboración con la Dra. Berta Riaño. Me he encargado de poner a punto el sistema experimental de

fotobiorreactores, de su puesta en marcha y del control diario de la operación. Las muestras las he analizado en colaboración con Janett Fortes. La digestión anaerobia de la biomasa se realizó en colaboración con la Dra. Berta Riaño. Asimismo, he realizado el análisis y la evaluación de los resultados obtenidos. La redacción del artículo se realizó bajo la supervisión de la Dra. María Cruz García, la Dra. Berta Riaño y la Dra. Mónica Coca.

Paper IV. En este artículo he sido el encargado del diseño experimental, arranque de los fotobiorreactores, control diario del proceso y del análisis de las muestras. En colaboración con Miriam Solana se puso a punto el sistema experimental de extracción de lípidos de algas mediante extracción con fluidos supercríticos y se llevaron a cabo los ensayos bajo unas condiciones previamente fijadas. Llevé a cabo la caracterización de la biomasa y el análisis de azúcares hidrolizados mediante HPLC en colaboración con la Dra. Mónica Coca Sanz y Jesús Rodríguez. He realizado la digestión anaerobia de las muestras. Asimismo, he realizado el análisis y la evaluación de los resultados obtenidos. La redacción del artículo se realizó bajo la supervisión de la Dra. María Cruz García, la Dra. Berta Riaño y la Dra. Mónica Coca.

Paper V (Anexo I). Este artículo corresponde al primer trabajo experimental desarrollado en relación con la tesis doctoral. En este artículo colaboré en el control y supervisión de los fotobiorreactores y en la digestión anaerobia de la biomasa resultante. Toda esta labor experimental se realizó junto con la Dra. Berta Riaño.

CAPÍTULO 1

INTRODUCCIÓN

1.1. Problemática de las aguas residuales agroalimentarias

1.1.1. Antecedentes

Desde comienzos del siglo XX, la producción de aguas residuales agroalimentarias (ARAs) ha crecido exponencialmente fruto de una mayor industrialización y de un mayor consumo de productos alimentarios elaborados. Estos cambios en la sociedad han propiciado una mejora de la calidad de vida pero, al mismo tiempo, han provocado un incremento de la producción de ARAs. Estas aguas tienen una mayor carga orgánica y una mayor concentración de productos químicos y compuestos tóxicos que en el pasado (principalmente metales pesados, pesticidas, compuestos policlorados, etc. (Gogate y Pandit, 2004a)), por lo que deben ser tratadas previamente a su vertido para no incurrir en daños medioambientales graves. En respuesta a esta problemática, las autoridades ambientales de la mayoría de los países industrializados han endurecido la normativa de vertido, obligando a las industrias agroalimentarias contaminantes a tratar sus aguas residuales como paso previo a su vertido. Según la carga del agua residual y su contenido en nutrientes (nitrógeno y fósforo, principalmente) los tratamientos que se realizan pueden variar considerablemente, siendo los más comunes los tratamientos físico-químicos y los procesos biológicos (anaerobios, aerobios, anóxicos y sus combinaciones).

Los tratamientos convencionales de ARAs generalmente emplean un primer tratamiento físico-químico que permite eliminar una parte

importante de la carga orgánica y de los sólidos, seguido de un segundo tratamiento biológico para eliminar nutrientes y materia orgánica. La combinación de unos u otros tratamientos dependerá de las características del agua (Gogate y Pandit, 2004a; Gogate y Pandit, 2004b), pero cabe destacar que estas tecnologías en sí mismas no permiten recuperar buena parte del nitrógeno y el fósforo (Ruíz-Martínez y col., 2012). Además, estos tratamientos precisan de una importante inversión y tienen elevados costes operacionales (principalmente debidos a la necesidad de aireación).

El uso de consorcios de microalgas y bacterias para tratar ARAs puede ser una alternativa a los sistemas convencionales. La aplicación de algas para tratar aguas residuales comenzó a desarrollarse en los años 50 (Oswald, 1957) aunque requiere todavía de investigación para optimizar el proceso. Su uso permite reducir el coste del tratamiento en un 90% aproximadamente (Gómez-Serrano y col., 2015) en comparación con los sistemas aerobios de fangos activos ya que no precisa aireación, permitiendo la eliminación de carbono orgánico y la recuperación de los nutrientes presentes en el agua (Jiménez-Pérez y col., 2004; De Godos y col., 2009a; Park y col., 2011) gracias a la simbiosis algas-bacterias (**Capítulo 5** y **Capítulo 6**).

Existen diversos estudios que han demostrado la viabilidad del tratamiento de ARAs mediante microalgas (De Godos y col., 2009a; Molinuevo-Salces y col., 2010; Boelee y col., 2011). Sin embargo, para obtener productividades de biomasa elevadas es necesario

optimizar las condiciones de operación y seleccionar las cepas idóneas para tratar el agua residual, entre otros parámetros. Según la composición macromolecular de la biomasa algal generada, ésta podrá ser destinada a la producción de compuestos de valor añadido, como biocombustibles, fertilizantes, componentes de interés para alimentación humana y animal, entre otras aplicaciones. A continuación, se describen las características de las principales ARAs y de aquellas que han sido empleadas en la presente tesis, junto con las diferentes alternativas de valorización de la biomasa algal para la producción de biocombustibles y su uso aplicando el concepto de biorrefinería.

1.1.2. Características de las ARAs y sistemas de tratamiento

La industria agroalimentaria es uno de los pilares que sustentan la economía española, ocupando uno de los primeros puestos en generación de empleo. En la actualidad, el 18,2% de las ventas de nuestro país corresponden a productos agroalimentarios, generando una riqueza equivalente al 20% del PIB español (MAGRAMA, 2014). A diferencia de otras industrias, no sólo es un sector estratégico, sino que tiene una presencia significativa en todas las Comunidades Autónomas y está presente en casi todos los núcleos urbanos. En Castilla y León se trata de una industria clave, la cual ingresa anualmente más de 4.800 millones de euros y emplea más de 35.000 trabajadores (MAGRAMA, 2014). Además esta industria es considerada como uno de los principales motores de la economía

española debido a su gran volumen de exportación (principalmente vino, aceite y embutidos).

Sin embargo, se trata de una industria que genera un volumen muy importante de aguas residuales que requieren tratamiento, llegando a superar los 35 millones de m^3 anuales (INE, 2008) sin tener en cuenta el sector ganadero que puede generar 74,2 millones de m^3 al año (MARM, 2010). Las ARAs son producidas durante la actividad industrial por lo que su caudal y características pueden variar considerablemente no solo a nivel estacional, sino incluso durante el mismo día, en función del proceso productivo que se esté llevando a cabo. Puesto que existen industrias agroalimentarias de todo tipo (producción, elaboración, transformación, preparación y conservación de alimentos) y de diversos subsectores agroalimentarios (conservas vegetales, zumos, pescado, productos del mar, leche y sus derivados, mataderos e industrias cárnicas, azucareras, cerveceras, etc.) se pueden encontrar aguas residuales con características muy variables en cuanto a la presencia de carga orgánica, nutrientes, color y compuestos tóxicos. Las aguas de procesado y de limpieza son las más importantes en cuanto a volumen y carga, y suelen caracterizarse por su alto contenido en materia orgánica y sólidos en suspensión, pudiendo incluir aceites, grasas, sales, ácidos, fosfatos, entre otros contaminantes. Con el objetivo de poder comparar la composición de las aguas residuales de las principales agroindustrias, en la **Tabla 1** se muestran los principales parámetros de las mismas.

Tabla 1. Características de las aguas residuales agroalimentarias (Adaptado de Babot y col., 2004 y de Pascual, 2008).

Tipo de industria	SST	DQO	DBO_5	N_T	F_T
			mg/L		
Vegetales, conservas y fruta.	700	5.000	3.000	150	30
Procesado de patata	700	10.000	3.000	150	200
Zumos de frutas	--	5.500	2.500	26,5	21
Procesado de pescado	200-3.000	500-4.500	400-4.000	1-20	5-90
Matadero	220-5.000	750-35.000	500-5.350	48-750	10-90
Purín fresco	46.000-76.000	52.000-73.900	35.000-61.000	3.500-5.400	3.200-6.200

*Abreviaturas: sólidos suspendidos totales (SST), demanda química de oxígeno (DQO), demanda biológica de oxígeno transcurridos 5 días (DBO_5), nitrógeno total (N_T), fósforo total (F_T).

Los principales sistemas de tratamiento de ARAs están orientados al tratamiento integral del influente, por lo que generalmente constan de dos etapas. En la primera etapa se realizan tratamientos físicos y químicos de manera combinada para eliminar los sólidos presentes en el agua mediante sedimentación, separación mecánica, deshidratación y adición de reactivos orgánicos o inorgánicos. Posteriormente se lleva a cabo el tratamiento de la fase soluble (Metcalf y Eddy, 2003) mediante tratamientos biológicos aerobios, anaerobios y/o anóxicos según las características de la fase soluble y la mayor o menor presencia de nutrientes (nitrógeno y fósforo). Los tratamientos aerobios con fangos activos están ampliamente extendidos, especialmente cuando las cargas no son muy elevadas, pero requieren de una gran cantidad de oxígeno que debe ser suministrado, por lo que se incurren en importantes costes de aireación (De Godos y col., 2009a). En los sistemas aerobios de fangos activos el fósforo soluble generalmente se elimina mediante adición de sales de aluminio, cloruro férrico o hidróxido de calcio, formando precipitados que se recogen con el fango mientras que el

nitrógeno se elimina mediante procesos de nitrificación/desnitrificación (Metcalf y Eddy, 2003). Este proceso consiste en la oxidación del NH_4^+ a NO_2^- por medio de las bacterias *Nitrosomonas* y *Nitrosoccocus* y posteriormente el NO_2^- se oxida a NO_3^- por medio de las bacterias *Nitrobacter* (Rittman y McCarty, 2001). Finalmente se produce la reducción del NO_3^- dando lugar a N_2 gas –que pasa a la atmósfera– mediante bacterias heterótrofas en condiciones de anoxia. El tratamiento mediante digestión anaerobia se emplea cuando la carga orgánica es muy elevada, eliminando el carbono orgánico que se transforma en CH_4 y CO_2, pero dejando los nutrientes en el medio (Speece, 2008).

El tratamiento de ARAs con consorcios de microalgas-bacterias es especialmente interesante no solo debido a la posibilidad de tratar las aguas, sino debido a que puede permitir reutilizar el agua residual tratada (R.D. 1620/2007) y a que la biomasa algal puede ser valorizada (**Figura 1**) incrementando la rentabilidad del proceso.

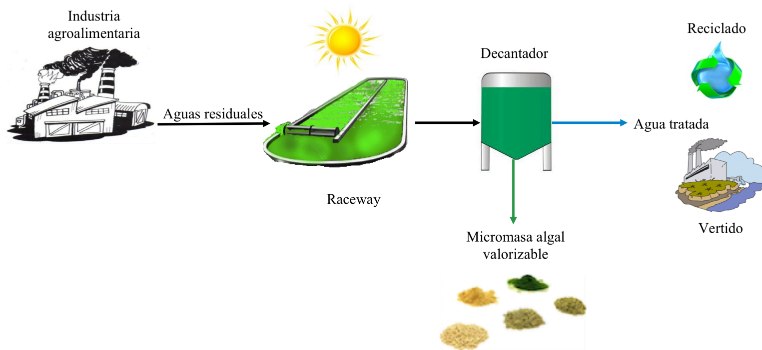

Figura 1. Esquema del proceso de tratamiento de ARAs con consorcios de microalgas-bacterias y posterior aprovechamiento de la biomasa residual y del agua ya tratada.

En el **Capítulo 5** de la tesis, se ha abordado el tratamiento de las aguas generadas en una planta de procesado de patatas situada en Valladolid (España), mediante la aplicación de consorcios de microalgas y bacterias. En esta industria se generan aguas residuales que contienen aceites de fritura y detergentes procedentes del lavado de las freidoras. Además se trata de aguas con un cierto color amarillento y que contienen materia orgánica soluble fácilmente biodegradable (Pascual, 2008). Las aguas generadas durante la actividad industrial pueden ser divididas en dos grupos: i) aguas procedentes del lavado de las patatas: contienen una menor concentración de materia orgánica, no contienen productos químicos y su concentración de DQO, ST, SV y nutrientes son bajas; y ii) aguas procedentes del lavado de las freidoras: poseen una carga orgánica elevada, mayor concentración de nutrientes, detergentes y grasas.

Para el desarrollo de esta tesis se han utilizado aguas provenientes de la limpieza de las freidoras que contenían una carga orgánica y de nutrientes elevada. Por ello, en el **Capítulo 5** se trató de determinar si el uso de consorcios de microalgas-bacterias puede suponer una alternativa viable de tratamiento de dichas aguas.

Así mismo, en el **Capítulo 5** de la tesis se ha empleado el efluente de una planta de tratamiento de purines para realizar un tratamiento con consorcios de microalgas y bacterias. Se ha seleccionado este agua residual debido a la importancia del sector porcino en España, que representa más del 30% de la cría de ganado, con un volumen

de facturación superior a los 4000 millones de euros. En la actualidad más de 25 millones de cerdos son criados y sacrificados anualmente en España, de los cuales más de 3 millones se producen en Castilla y León (MAGRAMA, 2014; García-González y col., 2015). Como resultado de la elevada producción de ganado porcino intensivo en Castilla y León se genera un total de 6,3 millones de toneladas de deyecciones al año que deben ser correctamente gestionadas para evitar problemas medioambientales (García-González y col., 2015). Este elevado volumen de purines puede ser aplicado en campo como fertilizante siempre y cuando se cumplan unas determinadas condiciones contempladas en el R.D. 261/1996 sobre protección de las aguas contra la contaminación producida por los nitratos procedentes de actividades agrarias; y a nivel autonómico regulado por el Decreto 40/2009, por el que se designan las zonas vulnerables a la contaminación de las aguas por nitratos procedentes de fuentes de origen agrícola y ganadero. Sin embargo, en aquellas zonas consideradas vulnerables es necesario tratarlo previamente para eliminar las altas cargas de materia orgánica y nutrientes (principalmente nitratos) (Flotats y col., 2009; De Godos y col., 2009a). En la actualidad se emplean múltiples tecnologías para tratar los purines de cerdo, siendo una de las técnicas más utilizadas la separación de la fase líquida de la sólida mediante coagulación-floculación (Riaño y García-González, 2014). La fracción líquida del purín (altamente cargada) es tratada biológicamente para oxidar la materia orgánica mediante bacterias aerobias mientras que la fracción sólida se utiliza directamente como abono o bien se composta. Una vez eliminada la mayor parte

de la materia orgánica y de los nutrientes presentes en la fracción líquida del purín mediante tecnologías convencionales, se produce un efluente terciario que contiene materia orgánica recalcitrante y una baja concentración de nutrientes que son difícilmente eliminables mediante tratamientos convencionales.

Con el objetivo de valorizar los nutrientes y eliminar la materia orgánica recalcitrante que aún queda en el agua residual tratada, en el **Capítulo 5** de esta tesis doctoral, se ha trabajado con el efluente de un tratamiento biológico aerobio de purines. Se ha seleccionado esta tecnología, ya que debido a las complejas interacciones entre los consorcios de microalgas-bacterias, es posible eliminar el carbono orgánico residual recalcitrante y los nutrientes restantes, puesto que la fotosíntesis oxigénica realizada *in situ* permite a las bacterias su eliminación (Muñoz y Guieysse, 2006). Además, este método permite valorizar los nutrientes en forma de biomasa microalgal (González-Fernández y col., 2010) en posteriores aplicaciones.

En el **Capítulo 6**, se ha llevado a cabo el tratamiento de aguas residuales procedentes de un matadero de cerdos. Se trata de aguas residuales que contienen principalmente sangre, por lo que son de un color rojo intenso, con materia orgánica fácilmente biodegradable (BREF, 2005) y una concentración de nutrientes disueltos muy escasa (Pascual, 2008). La selección de este efluente para su estudio en esta tesis se justifica por la gran importancia del sector cárnico (mataderos) tanto a nivel regional como nacional. El

volumen de ventas que genera este mercado solo en Castilla y León es superior a los 1.600 M€ anuales con una producción de carne cercana a las 600.000 toneladas al año (Estudio del sector cárnico en Castilla y León, 2006). Fruto del sacrificio y la elaboración de la carne, se generan aguas residuales altamente cargadas (**Tabla 1**) que potencialmente pueden emitir olores y que en caso de vertido tendrían unas consecuencias medioambientales muy severas en el ecosistema. Los residuos generados en estas industrias son tratados convencionalmente mediante secado, procesado de la sangre, incineración y tratamientos aerobios (BREF, 2005). Como consecuencia del sacrificio y la elaboración de la carne, en Castilla y León se generan 2,97 Mm^3 anuales (BREF, 2005) por lo que cualquier mejora realizada en los sistemas de tratamiento tendrá un impacto directo en la economía del sector. En el caso de los mataderos, el uso de consorcios de microalgas-bacterias para tratar ARAs puede suponer una importante mejora frente a los sistemas convencionales debido a que (Gómez-Serrano y col., 2015):

- Se trata de una tecnología más económica que los sistemas aerobios.
- El agua residual tratada puede ser reutilizada de acuerdo al R.D. 1620/2007.
- La biomasa resultante puede ser posteriormente empleada para generar electricidad y calor (uno de los principales gastos de los mataderos) mediante combustión directa o digestión anaerobia y posterior combustión del biogás.

1.1.3. Tratamiento de aguas residuales mediante consorcios de microalgas-bacterias

En general, las microalgas son un grupo heterogéneo de organismos microscópicos (2-200 μm), unicelulares, con capacidad de realizar procesos fotosintéticos y que no presentan ningún tipo de diferenciación (tallo-raíz-hoja). Se trata de un grupo muy diverso de microorganismos con más de 29.000 especies (Brock y col., 2009). Dentro de las microalgas se engloban las cianobacterias (microorganismos fotosintéticos oxigénicos) y organismos procariotas fotosintéticos (Mata y col., 2010). Estos microorganismos pueden ser autótrofos, heterótrofos o mixotróficos dependiendo de las condiciones de cultivo, y en su mayoría suelen encontrarse en ambientes acuáticos (agua dulce o salada). Se estima que se conocen unas 350.000 especies distintas pudiéndose clasificar en procariotas (Cyanophita y Prochlorophyta) y eucariotas (Glaucophyta, Rodophyta, Heterokontophyta, Hatophyta, Cryptophyta, Dinophyta, Euglenophyta, Chorarachniophyta y Chlorophyta). La gran diversidad de especies de microalgas permite encontrarlas en hábitats muy diferentes con perfiles bioquímicos muy distintos, por lo que sus usos potenciales son muy diversos.

Debido a la gran simplicidad de estos microorganismos y a su reproducción asexual, los ciclos de división celular son significativamente más rápidos que en organismos superiores, pudiéndose alcanzar producciones 10 veces superiores a las de las plantas. Por lo tanto, la producción anual de biomasa producida en

cada hectárea de superficie es considerablemente más elevada en microalgas (alcanzando hasta 150 toneladas/ha (Pittman y col., 2011)) que en cultivos convencionales. Así mismo, las microalgas son organismos especialmente eficientes a la hora de captar nutrientes como el nitrógeno y el fósforo del medio en que se encuentran y asimilarlos en forma de biomasa (Jiménez-Pérez y col., 2004). Como se ha indicado previamente, debido a que las microalgas no tienen estructuras diferenciadas, la composición de toda la biomasa producida es igual (Brock y col., 2009) y se puede aprovechar toda la biomasa generada para el fin preestablecido, mientras que en otras biomasas como por ejemplo en cultivos energéticos (maíz, remolacha, chopo, etc.) solo se aprovecha el fruto o la leña y el resto se desecha (hojas y raíces).

1.1.3.1. Potencial de los consorcios microalgas-bacterias en simbiosis para tratar ARAs

Los primeros tratamientos de aguas residuales con microalgas en reactores abiertos fueron realizados por Oswald (1957). En este primer estudio se demostró la posibilidad de tratar aguas residuales poco cargadas con microalgas, recuperando así los nutrientes en forma de biomasa microalgal que posteriormente pudiera ser valorizada. El uso de aguas residuales como fuente de nutrientes para producir microalgas supone un ahorro económico considerable tanto en el tratamiento del agua residual como en la producción de las mismas (Gómez-Serrano y col., 2015), además de una mejora medioambiental.

El tratamiento de aguas residuales mediante esta tecnología consiste en el establecimiento de un consorcio de bacterias aerobias y microalgas que actúan de manera simbiótica. El agua residual a tratar contiene una parte de los nutrientes ya mineralizados, por lo que están fácilmente disponibles para las algas (amonio, nitrato, fósforo soluble, minerales disueltos. etc.) y otra parte se encuentra en la materia orgánica presente en los sólidos suspendidos del agua. Estos sólidos suspendidos son degradados aeróbicamente por las bacterias (con consumo de oxígeno), liberando al medio CO_2 y nutrientes, los cuales quedan disponibles para las algas (amonio, nitrato, fósforo, etc.). Mediante la fotosíntesis oxigénica, el dióxido de carbono liberado por las bacterias es empleado por las microalgas para su desarrollo, liberando oxígeno que es empleado por las bacterias aerobias para su respiración (**Figura 2**). En este sentido, la formación de 1 kg de biomasa algal permite fijar 1,8 kg de CO_2 que no es emitido a la atmósfera, por lo que este tratamiento reduce la emisión de gases de efecto invernadero a la atmósfera (Molinuevo-Salces y col., 2010). El proceso de fotosíntesis oxigénica, permite a las microalgas transformar compuestos inorgánicos en orgánicos mediante procesos de oxidación-reducción, donde el CO_2 y el H_2O son transformados en carbohidratos mediante la energía aportada por la luz, liberando oxígeno. Para que se lleve a cabo este proceso, es necesaria la presencia de macronutrientes (principalmente nitrato y fosfato) y micronutrientes (principalmente metales) que son empleados como cofactores (Brock y col., 2009). Durante la fotosíntesis oxigénica, la concentración de O_2 disuelto en el agua y el pH se elevan

considerablemente provocando la desactivación de patógenos y permitiendo la eliminación de metales pesados (Muñoz y Guieysse, 2006).

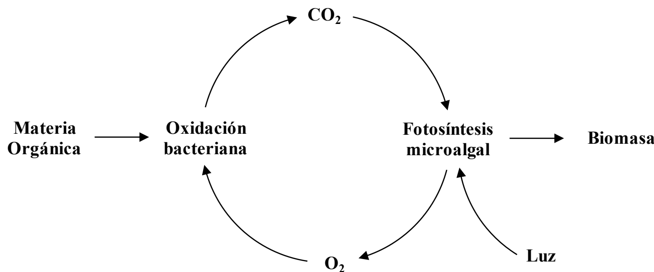

Figura 2. Principio de oxigenación fotosintética en el proceso de eliminación de materia orgánica (DBO) en sistemas de tratamiento de aguas residuales con microalgas (adaptado de Muñoz y Guieysse, 2006).

Una de las principales ventajas de la fotosíntesis oxigénica en comparación con otros sistemas de tratamiento de ARAs, es la elevada capacidad de las microalgas para asimilar el nitrógeno (NH_4^+ y NO_3^-) y el fósforo soluble (PO_4^{-3}) en forma de biomasa algal, debido principalmente a que las microalgas suelen contener entre un 45-60% de proteína (en peso seco), junto con importantes cantidades de fosfolípidos y ácidos nucleicos por lo que las necesidades de nutrientes son elevadas (Muñoz y Guieysse, 2006).

A pesar de que el tratamiento de ARAs con consorcios microalgas-bacterias ha mostrado ser, en muchas ocasiones, más interesante desde un punto de vista técnico-económico (Gómez-Serrano y col., 2015), en la actualidad su uso está menos extendido que los procesos de fangos activos, en parte debido a que los sistemas de

depuración con algas requieren de una superficie considerablemente superior a la necesaria en los sistemas de fangos activos (Craggs y col., 2011). En la **Tabla 2** se comparan las principales características los sistemas aerobios convencionales y de los que emplean consorcios de microalgas-bacterias.

Tabla 2. Comparativa de dos sistemas de tratamiento de aguas residuales. Sistema Aerobio (SA) y Sistema Basado en Microalgas (SBM) (Adaptado de Borowitzka, 2005; Park y col., 2011; Craggs y col., 2011).

Características	SA	SBM
Costes de construcción	Medios	Medios-Bajos
Capacidad de carga tratada (kg DBO_5/(ha d))	5000-7500	100-150
Costes operacionales	Medios	Bajos
Tolerancia a cargas elevadas	Si	No
Necesidad de aireación	Si	No
Ahorro energético	No	80-90%
Emisiones de CO_2	Si	No
Valor de la biomasa generada	Bajo	Medio-Alto
Estado de la técnica	Desarrollado	Poco desarrollado

En la actualidad el uso de consorcios de microalgas-bacterias está encaminado al tratamiento eficiente de ARAs y a la recuperación de los nutrientes presentes (valorización en forma de biomasa) (Ahluwalia y Goyal 2007; De Godos y col., 2009a, Posadas y col., 2014) tal como se muestra en el **Capítulo 5** y en el **Capítulo 6**. En la **Tabla 3** se recogen los resultados de diversos tratamientos realizados con consorcios de microalgas-bacterias empleando distintos tipos de aguas residuales. Los resultados muestran una gran variabilidad tanto en las microalgas empleadas como inóculo, como en el tipo de agua tratada, productividad o superficie empleada. Por lo tanto, existen importantes diferencias en los resultados de fitodepuración (Termini y col., 2011) entre unas y

Tabla 3. Tratamientos de aguas residuales con consorcios de microalgas-bacterias

Microalgas inoculadas	Agua residual empleada	Productividad de la biomasa	Superficie del reactor (m^2)	Eliminación de carbono (%)	Eliminación de nitrógeno (%)	Eliminación de fósforo (%)	Referencia
Chlorella sorokiniana	Purín de cerdo	---	0,025	75	72	---	González y col., 2008
Chlorella sorokiniana	Industria de procesado de patata	0,19 g/(L·d)	0,051	85	>95	81	**Capítulo 5**
Chlorella sorokiniana	Tratamiento secundario de purines	0,26 g/(L·d)	0,051	62	83	58	**Capítulo 5**
Chlorella sorokiniana	Tratamiento secundario de purines	0,26 g/(L·d)	0,051	62	83	58	**Capítulo 5**
Microalgas agua dulce	Industria de procesado de café	---	0,0231	56	80	---	Posadas y col., 2014
Microalgas agua dulce	Matadero de pescado	0,11 g/(L·d)	0,046	70	68	42	Riaño y col., 2011
Microalgas agua dulce	Purín tratado anaeróbicamente	0,33 g/(L·d)	0,046	67	>95	84	Molinuevo y col., 2010
Microalgas agua dulce	Digestato de purín de cerdo	6,8 g/(m^2·d)	0,01	---	94	56	Chen y col., 2012
Microalgas agua dulce	Sintética/ doméstica	7,7 g/(m^2·d)	0,02	---	83	85	Boelee y col., 2011
Microalgas agua dulce	Matadero de cerdo	10,7 g/(m^2·d)	0,43	86	83	91	**Capítulo 6**
Microalgas agua dulce	Purín de cerdo	27,7 g/(m^2·d)	1,8	76	88	93	De Godos y col., 2009[a]
Microalgas agua dulce	Aguas de fábrica alfombras/Doméstica primaria	28,1 g/(m^2·d)	5,5	---	>95	>95	Chinnasamy y col., 2010
Microalgas agua dulce	Doméstica primaria	35 g/(m^2·d)	0,5	91	70	85	Posadas y col., 2013

otras aguas residuales. Sin embargo, a partir de los resultados se desprende que los consorcios de microalgas-bacterias son capaces de fitodepurar aguas residuales urbanas, agrarias e industriales de manera eficiente.

El tratamiento de ARAs mediante consorcios de microalgas-bacterias conduce a porcentajes de eliminación de carbono orgánico y nutrientes superiores al 50% en aguas domésticas (Boelee y col., 2011; Posadas y col., 2013), en aguas ganaderas (De Godos y col., 2009b; Molinuevo-Salces y col., 2010; Chen y col., 2012) y en aguas procedentes de industrias alimentarias (Wilkie y Mulbry, 2002). Se han observado productividades de biomasa y porcentajes de eliminación muy diferentes entre las referencias consultadas, pudiendo ser atribuidas a diversos factores entre los que cabe destacar (Acién-Fernández y col., 2001; Kuei-Ling y Chang, 2010):

- Las ARAs suelen tener color, lo que puede ocasionar que llegue menos luz a las microalgas y disminuya por tanto la captación de nutrientes.
- En el tratamiento de ARAs, la proporción C/N/P viene determinada por el influente, por lo que las microalgas deben adaptarse a los nutrientes presentes, habiendo por regla general un déficit de algún nutriente esencial.
- La concentración de nutrientes y materia orgánica en las ARAs puede variar significativamente en función de las características del proceso productivo.

- En muchas ocasiones, las ARAs provienen de tratamientos secundarios, por lo que la materia orgánica presente en el agua es recalcitrante, pudiéndose producir un déficit de CO_2.
- Si se produce un aumento importante de la carga orgánica disponible, las bacterias se ven beneficiadas debido a su mayor tasa de reproducción, provocando un desbalance en los microorganismos y empeorando la eficiencia del tratamiento.
- Las microalgas presentes en el tratamiento de ARAs no se encuentran en monocultivo, por lo que existe una competencia inter-específica, que puede provocar una disminución de la productividad.
- Posibilidad de aparición puntual de sustancias tóxicas.
- Imposibilidad de controlar las poblaciones de algas que crecen en el ARA.

Una vez establecido el consorcio de microalgas-bacterias, se alcanza un punto de equilibrio en la biomasa producida, donde aproximadamente el 90% en masa son microalgas y el 10% bacterias (Morales-Amaral y col., 2015). La composición de carbohidratos, lípidos y proteínas de la biomasa puede variar en gran medida (Sialve y col., 2009) según las condiciones de cultivo. Por ello, es necesario hacer una caracterización de la biomasa para determinar la mejor forma de valorización de la misma.

A pesar de que existen numerosos trabajos en los que se demuestran las ventajas del uso de consorcios de microalgas-bacterias para tratar ARAs en comparación con los sistemas convencionales, todavía existen diversos cuellos de botella que deben ser solventados para poder implementar esta tecnología a gran escala, como son:

i) mejora de los rendimientos de eliminación-recuperación completa de los nutrientes del agua residual (tratado en los **Capítulos 5** y **6**) a fin de cumplir los límites de descarga marcados por la autoridad competente;
ii) desarrollo de estudios a media-gran escala (abordado en el **Capítulo 6**);
iii) mejora de la viabilidad económica del proceso mediante la valorización de la biomasa producida en forma de biocombustibles y productos de alto valor añadido (analizado en los **Capítulos 3**, **4**, **5** y **6**);
iv) realización de estudio económico preliminar del proceso (estudiado en los **Capítulos 4** y **6**).

1.1.3.2. Fotobiorreactores utilizados en el tratamiento de ARAs

Los sistemas de depuración de ARAS que emplean microalgas se pueden clasificar en sistemas abiertos o cerrados. Las principales diferencias entre ambos se recogen en la **Tabla 4**.

Tabla 4. Comparación de sistemas de tratamiento de aguas residuales con microalgas (adaptado de: Demirbas y Demirbas, 2010; Acién y col., 2012; Muñoz y col., 2015).

Reactores	Sistemas cerrados	Sistemas abiertos
Inversión inicial (€/m^2)	500-3.000	2-20
Productividad (t/(ha año))	92,9	38,5
Eficiencia fotosintética (%)	4-6	2
Coste energético (W/m^3)	50-1.000	0,1-10
Coste medio (€/t)	12.600	227
Ratio de eficiencia energética	4,33	7,01
Eficiencia del mezclado	Elevada	Escasa
Riesgo contaminación	Media	Alta
Control de especies	Alta	Baja
Volumen de agua tratada	Elevada	Media
Control de parámetros	Mayor	Menor
Necesidad de limpieza	Mayor	Menor
Inhibición por O_2	Mayor	Menor
Pérdidas de agua (evaporación)	Menor	Mayor

Ambos sistemas presentan ventajas y desventajas. Normalmente, los sistemas cerrados se emplean cuando el producto tiene un alto valor añadido y el cultivo debe ser puro, por lo que se debe cultivar en condiciones de esterilidad. Sin embargo, en el tratamiento de ARAs, es más interesante usar fotobiorreactores abiertos ya que el coste de la depuración por cada m^3 de agua residual tratado es mucho menor y estos sistemas son más sencillos de operar (Park y col., 2011). Tal y como se muestra en la tabla anterior, existen diferencias muy marcadas en los costes iniciales y los costes medios por tonelada de biomasa producida entre ambos sistemas. Estas diferencias hacen inviable el uso de los sistemas cerrados para tratar aguas residuales en la actualidad salvo unas pocas excepciones (Richmond, 1999), por lo que alrededor del 99% de los tratamientos de aguas residuales con consorcios de microalgas-bacterias se realizan en sistemas abiertos (Benemann, 2013).

Habitualmente se emplean fotobiorreactores de tratamiento de alta productividad (HRAP, High Rate Algal Ponds, por sus siglas en inglés), que están especialmente diseñados y operados para optimizar la captación de luz y CO_2, maximizando el tratamiento de aguas residuales y la productividad. Los reactores abiertos HRAP tipo raceway consisten en estanques de una profundidad de 10-40 cm divididos mediante un panel central en 2 o en 4 y que se encuentran en constante agitación mediante paletas a una velocidad promedio de unos 31 cm/s, homogenizando así el cultivo y permitiendo el acceso a los nutrientes y a la luz a todos los microorganismos (Tredici, 2004). Estos fotobiorreactores están especialmente indicados para el tratamiento de ARAs debido a que sus costes de construcción y operación son considerablemente menores que en otros fotobiorreactores y a su fácil escalabilidad (Benemann, 2013). Para maximizar el caudal de ARAs tratado por unidad de superficie, es necesario que la actividad fotosintética de las algas sea máxima, por lo que una cuestión clave es aumentar la relación superficie/volumen, ya que ello provoca un incremento de la cantidad de luz que llega al cultivo y se incrementa la asimilación de nutrientes del medio en forma de biomasa. Este aumento de la actividad fotosintética provoca una mayor liberación de oxígeno, favoreciendo la mezcla, disminuyendo el número de unidades formadoras de colonias de cultivo y por lo tanto, mejorando la calidad del agua residual tratada (Tredici, 2004). Por todo ello, en el **Capítulo 5** se han empleado reactores abiertos de 5 L simulando un lagunaje, mientras que en el **Capítulo 6** se han empleado reactores HRAP tipo Raceway de 75 L.

1.1.3.3. Microalgas empleadas durante la presente tesis

Respecto al tratamiento de aguas residuales con consorcios de microalgas y bacterias, la bibliografía consultada apunta a una gran variedad de especies utilizadas. Los géneros más empleados son *Anabaena, Chlamydomonas*, *Chlorella, Phormidium, Pseudoanabaena*, *Scenedesmus*, *Ulothrix*, (Wilkie y Mulbry, 2002; Cho y col., 2011; Posadas y col., 2014), ya que son capaces de tolerar altas concentraciones de materia orgánica y nutrientes. En ocasiones, también se emplean inóculos que han crecido en el propio agua residual estancada que se va a tratar, o bien procedentes de un lagunaje. Las microalgas identificadas en mayor medida en lagunajes son: *Chlamydomonas*, *Chlorella*, *Chlorococcal*, *Microspora*, *Navicula*, *Nitzschia*, *Phormidium*, *Pseudoanabaena*, *Scenedesmus*, *Stigeoclonium* y *Teilingia*.

Durante el desarrollo de esta tesis se han empleado tanto cultivos puros, microalgas cultivadas en medios enriquecidos bajo condiciones de esterilidad, como microalgas obtenidas durante el tratamiento de ARAs. Dentro del primer grupo se encuentran *Chlorella sorokiniana*, *Isochrysis* T-ISO, *Nannochloropsis gaditana*, *Scenedesmus almeriensis* y *Tetraselmis* sp. (**Figura 3**) que fueron seleccionadas por su perfil rico en ácidos grasos ω-3 y ω-6.

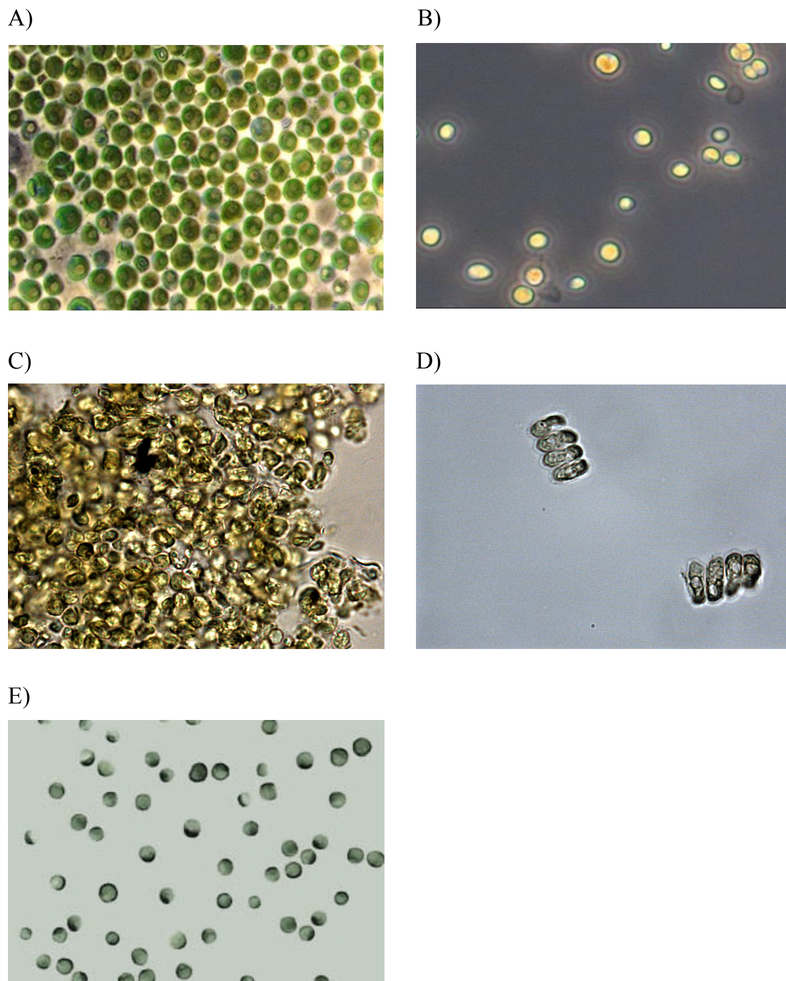

Figura 3. Cultivos puros de microalgas cultivadas en medios minerales. A) *C. sorokiniana* (50X), B) *N. gaditana* (100X), C) *Isochrysis* T-ISO (100X), D) *S. almeriensis* (100X), E) *Tetraselmis* sp. (100X).

Las microalgas *C. sorokiniana* y *S. almeriensis* fueron seleccionadas debido a su capacidad para crecer en aguas residuales con alta carga orgánica y alta concentración de nutrientes (Mendez y col., 2014). *C. sorokiniana* fue empleada en el **Capítulo 5** como inóculo para realizar el tratamiento de las aguas residuales de la

industria de procesado de patata y purines. Durante el periodo experimental la población inicialmente inoculada varió, detectándose la presencia de otras especies como *Nitzschia* y *Chlorococcal* aunque *Chlorella* continuó siendo la especie mayoritaria (**Figura 4 a, b**). Por otra parte, en el **Capítulo 6** se emplearon microalgas procedentes de un lagunaje como inóculo, el cual estaba mayoritariamente formado por especies como *Chlamydomonas subcaudata* y *Anabaena* sp., cambiado durante la operación del fotobiorreactor hacia especies de microalgas filamentosas donde predominaba mayoritariamente *Phormidium tergestinum* y en menor medida *Anabaena* sp. (**Figura 4 c, d**).

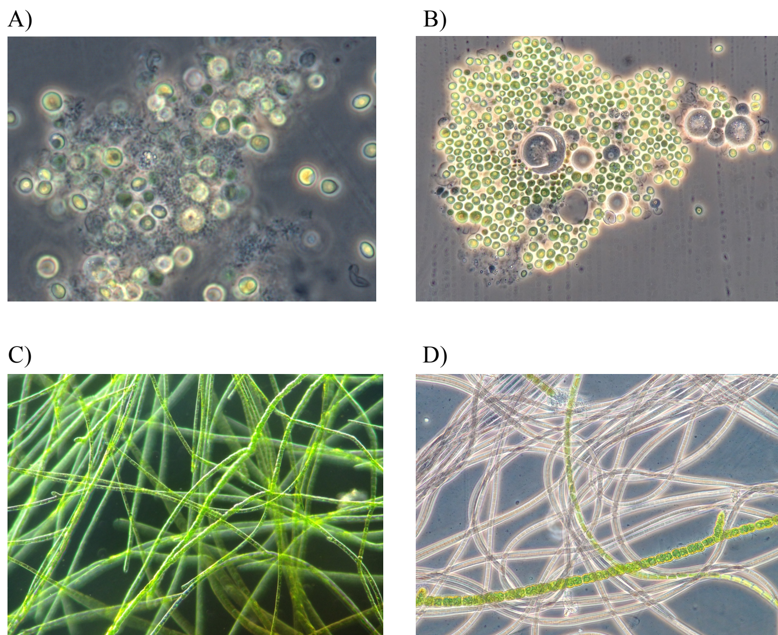

Figura 4. Microalgas obtenidas durante tratamiento de A) aguas de efluente secundario de purines (50X), B) aguas del procesado de patatas (20X), aguas residuales de matadero con C) luz ambiental (20X) y D) con luz artificial (20X).

1.2. Microalgas como fuente de biocombustibles

En la actualidad, las microalgas se emplean principalmente como suplemento alimenticio animal y humano (Lewis y col., 2000), como fuente de ácidos grasos poliinsaturados (PUFAs) (Spolaore y col., 2006; Cerón-García y col., 2013), β -caroteno, carotenoides, astaxantina (Spolaore y col., 2006), elaboración de compuestos farmacéuticos (Mata y col., 2010), uso en la industria agrícola como biofertilizante (Mulbry y col., 2005) y por sus componentes antioxidantes y antiinflamatorios (Chisti, 2007; Guedes y col. 2011) entre otras aplicaciones. Su impacto en la economía todavía es limitado, ya que los sistemas de producción y extracción de compuestos aún no están suficientemente desarrollados y optimizados (Acién y col., 2012; Halim y col., 2012; Chisti, 2013); sin embargo, no se debe subestimar su posible influencia en la economía a medio plazo. Las algas son microorganismos especialmente interesantes debido a que pueden ser cultivados en condiciones muy diversas (Williams y Laurens, 2010), son capaces de depurar aguas residuales (Chisti, 2013; Posadas y col., 2013) y se puede inducir la acumulación de compuestos de interés (lípidos, carbohidratos, astaxantina, carotenoides, etc.) bajo determinadas condiciones de operación (Dragone y col., 2011).

Debido a su gran variabilidad bioquímica y a su elevada productividad, las microalgas se han constituido como una interesante alternativa a los combustibles fósiles. Aunque se han publicado resultados prometedores sobre la viabilidad del uso de

estos biocombustibles (Stephens y col., 2010), es necesario ahondar en la producción de algas ricas en carbohidratos y lípidos como fuente potencial de biocombustibles. La gran variabilidad bioquímica de las microalgas atiende principalmente a dos motivos: i) a la especie empleada y ii) a la modificación del metabolismo celular como consecuencia de situaciones que generan estrés en el alga. Según la especie empleada se puede observar como unas especies son más propensas a acumular carbohidratos (*Spyrogyra* sp., *Porphyridium cruentum*, etc.), lípidos (*Nannochloropsis* sp., *Chlorella vulgaris* , etc.), proteínas (*Arthrospira maxima*, *Spirulina platensis*, etc.) o compuestos de alto valor añadido (*Haematoccocus pluvialis, Dunaliella salina, Isochrysis* sp.*, Tetraselmis* sp.) (Becker y col., 2004; Sialve y col., 2009).

A continuación se describe la situación actual de los combustibles fósiles y el uso de biocombustibles producidos a partir de microalgas.

1.2.1. Problemática actual de los combustibles fósiles

Los combustibles fósiles son aquellos procedentes de biomasas acumuladas durante millones de años y que se han ido transformando en sustancias de gran contenido energético. Dentro de estos combustibles se engloba el petróleo y sus derivados, el carbón, gas natural, etc. El consumo de estos combustibles fósiles se ha incrementado un 50% en las últimas dos décadas fruto de la rápida industrialización de las economías emergentes que

representan el 80% del aumento global del consumo de energía (Rühl, 2014). Según el informe de la BP *Statistical Review of World Energy* de Junio de 2014 (Rühl, 2014), del total de la energía producida a nivel mundial durante el año 2013, el 83% procedía de combustibles fósiles (petróleo 32%, gas natural 24% y carbón 30%), mientras que un 4% tenía su origen en energía nuclear, un 7% en energía hidráulica, un 2% en energías renovables (fotovoltaica y eólica) y un 1% biocombustibles. Según el informe "*Key World Energy Statistics*" publicado por la Agencia Internacional de la Energía (IEA, 2007), el consumo energético mundial ha pasado de 5.800 millones de toneladas equivalentes de petróleo (Mtoe) a 13.800 Mtoe en 40 años (**Figura 5**).

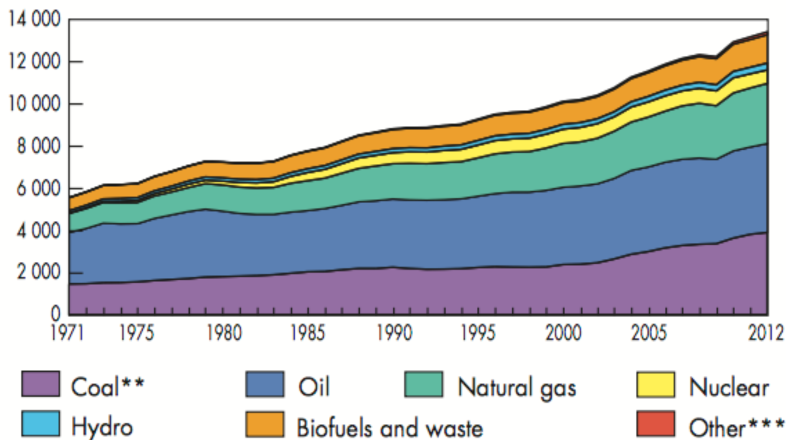

Figura 5. Consumo total de energía según el tipo de combustible (Mtoe) entre 1971 y 2012 (Rühl, 2014).

El uso de combustibles fósiles como principal fuente de energía supone no sólo su agotamiento, sino también la emisión a la atmósfera de gran cantidad de compuestos contaminantes tanto

primarios (óxidos de azufre, CO, CO_2, NO_X, metales pesados,, etc.) como secundarios (oxidantes fotoquímicos, ozono, H_2SO_4, HNO_3, etc), lo que contribuye al deterioro de la calidad del aire, el calentamiento global, la desertización y la erosión del medio (Den Elzen y Schaeffer, 2002). Si las perspectivas ambientales de la OCDE para el año 2050 se cumplen, la contaminación del aire se convertirá en la principal causa ambiental de mortandad prematura. Por todo ello, es necesario encontrar una alternativa que permita reducir la dependencia del uso de combustibles fósiles.

1.2.2. Biocombustibles: una alternativa a los combustibles fósiles

Biocombustible es el término empleado para denominar a los combustibles sólidos, líquidos o gaseosos producidos de forma directa o indirecta a partir de biomasa, por lo tanto se trata de una fuente renovable de energía (FAO, 2012). Los biocombustibles son alcoholes, éteres, ésteres y otros compuestos químicos obtenidos a partir de biomasa (normalmente cultivos vegetales o residuos derivados de estos). Su producción está suscitando un gran interés a nivel internacional, ya que son muchos los biocombustibles que pueden ser obtenidos de la biomasa, destacando entre ellos el biodiesel, bioetanol y biogás. El uso de biocombustibles juega un papel crucial a la hora de evitar una dependencia excesiva de los combustibles fósiles, permitiendo asegurar el abastecimiento de combustibles de una forma sostenida en el tiempo. Además, los biocombustibles promueven una mayor sostenibilidad, ya que todo

el CO_2 emitido a la atmósfera como consecuencia de su combustión ha sido previamente capturado en forma de biomasa.

Según el origen de la biomasa, los biocombustibles pueden ser clasificados como de primera, segunda, tercera o cuarta generación (Naik y col., 2010) (**Tabla 5**). Una de las principales ventajas del uso de microalgas para la producción de biocombustibles de tercera generación en comparación con los biocombustibles de primera y segunda generación es que no requieren tierra cultivable, por lo que se pueden emplear terrenos no aptos para usos agrícolas para emplazar en ellos grandes fotobiorreactores donde crecer algas, evitando una competencia directa por el suelo con los cultivos convencionales (Dragone y col., 2011).

Tabla 5. Clasificación de los biocombustibles en base al sistema de producción (Naik y col., 2010).

Tipo de biocombustible	Materia prima	Tipo de biocombustible
Primera generación	Azúcar, almidón, aceites vegetales, grasas animales, etc.	Bio-alcoholes, aceites vegetales, biodiesel, syngas, biogás.
Segunda generación	Cultivos no alimenticios, paja de cereales, madera, residuos sólidos, cultivos energéticos.	Bio-alcoholes, biodiesel, bio-hidrógeno, diesel procedente de maderas, biogás.
Tercera generación	Algas, árboles bajos en lignina,	Bio-alcoholes, biodiesel, biogás, biohidrógeno.
Cuarta generación	Algas y bacterias modificadas genéticamente.	Bio-alcoholes, biodiesel

Esta tesis doctoral se centra en los biocombustibles de tercera generación producidos a partir de microalgas. Los biocombustibles de tercera generación, también conocidos como biocarburantes avanzados, al igual que los de segunda generación proceden de biomasas no alimentarias, pero se diferencian de éstos en que

emplean tierras no aprovechables para cultivos convencionales. Debido a su gran complejidad técnica, requieren de mano de obra altamente cualificada para su obtención (Richmond, 2008).

1.2.2.1. ¿Por qué utilizar algas para producir biocombustibles?

Las microalgas pueden constituir una alternativa viable a los combustibles fósiles, ya que son especialmente adecuadas por ser un sumidero de CO_2, por sus altos rendimientos productivos y por la posibilidad de acumular, potencialmente, altas concentraciones de carbohidratos y lípidos (Chisti, 2007; Kuei-Ling y col., 2010; Chen y col., 2011) mediante la generación de situaciones de estrés abiótico (privación de determinados nutrientes, estrés lumínico, cambios de temperatura, etc.). Por todas estas características, Campbell (1997) auguró que en un futuro a medio plazo las algas serán una de las principales fuentes de biocombustibles del planeta.

Sin embargo, el uso de microalgas para producir biocombustibles también tiene una serie de desventajas que dificultan su escalabilidad:

i) Son organismos relativamente poco conocidos y con un comportamiento muy variable según la especie empleada (Tomaselli, 2008).
ii) Su producción requiere de formación cualificada y conocimientos técnicos complejos (Richmond, 2008).

iii) Generalmente es necesario recoger, concentrar y secar la biomasa, por lo que se incurre en fuertes costes (Acién y col., 2012).

iv) El proceso completo de producción de biocombustibles a partir de microalgas conlleva importantes costes (en el año 2008 se estimó que el precio de 1 litro de biodiesel de microalgas tenía un coste 14,5 veces superior a 1 litro de petrodiesel (Kovacevic y Wesseler, 2010)).

No obstante, se espera que el desarrollo de esta tecnología conlleve una disminución de los costes de producción de 10-15 veces en los próximos 20 años (Chisti, 2008). En la actualidad, la mayor parte de la investigación está encaminada a maximizar la producción de algas (Ho y col., 2012; Sforza y col., 2012; Bennet y col., 2014) y a acumular determinados carbohidratos y lípidos para producir biocombustibles (Dragone y col., 2011; Praveenkumar y col., 2012), así como a la minimización de los costes de producción y extracción (Acién y col., 2012).

A partir de las microalgas se pueden obtener diversos biocombustibles entre los que cabe destacar biodiesel, bioetanol, biometanol, biohidrógeno y biogás (Melis, 2002; Gravilescu y Chisti, 2005; Kapdan y Kargi, 2006; Spolaore y col., 2006). Una de las principales características de las microalgas es su gran variabilidad bioquímica (contenido en proteínas, carbohidratos y lípidos) no solo a nivel de especie, sino en función de las

condiciones de cultivo. Existen especies que tienen concentraciones de carbohidratos menores del 20% (*Spirulina platensis*), mientras que otras superan el 60% (*Spirogyra* sp.). Del mismo modo ocurre referente a la concentración de lípidos, pudiéndose encontrar especies como *Chlorella pyrenoidosa* que contiene un 3% de lípidos y otras como *Chlorella vulgaris* que pueden superar el 55% (**Tabla 6**).

Tabla 6. Composición general de las principales microalgas (% peso seco).

Especie	Proteínas	Carbohidratos	Lípidos	Referencia
Anabaena cilindrica	43-56	25-30	4-7	Becker, 2007
Aphanizomenon flos-aquae	62	23	3	Becker, 2007
Arthrospira maxima	61-71	13-16	6-7	Becker, 2007
Chlamidomonas reinhardtii	48	17	21	Becker, 2007
Chlorella pyrenoidosa	57	26	2	Becker, 2007
Chlorella sorokiniana	45-55	18-38	15-35	Sialve y col., 2009
Chlorella vulgaris	51-58	12	14-56	Becker, 2007
Dunaliella salina	57	32	6	Becker, 2007
Euglena gracilis	39-61	14-18	14-20	Sialve y col., 2009
Isochrysis sp.	41-58	18-21	25-33	Becker, 2007
Isochrysis T-ISO	55	24	19	**Tesis doctoral***
Nannochloropsis gaditana	60	21	19	**Tesis doctoral***
Porphyridium cruentum	28-39	40-57	9-14	Becker, 2007
Scenedesmus almeriensis	50	22	24	**Tesis doctoral***
Scenedesmus obliquus	50-56	10-17	12-14	Sialve y col., 2009
Spirogyra sp.	6-20	33-64	11-21	Becker, 2007
Spirulina platensis	46-63	8-14	4-9	Sialve y col., 2009
Tetraselmis sp.	56	18	15-23	**Tesis doctoral***

* **Tesis doctoral:** Datos que no aparecen en los capítulos 3, 4, 5 y 6 pero que han sido determinados experimentalmente durante el transcurso de esta tesis.

Cuando las microalgas son cultivadas en condiciones sub o supra-óptimas (condiciones de estrés) reaccionan mediante cambios metabólicos que atienden a una estrategia para hacer frente a estas condiciones ambientales adversas (**Figura 6**) (Markou y Nerantzis, 2013). Estos cambios dinámicos en el medio provocan una modificación de la composición tanto a nivel macromolecular (proteínas, carbohidratos y lípidos) como a nivel de producción de

metabolitos secundarios (síntesis de vitaminas, hormonas, sustancias antioxidantes, antibióticos, etc. (Skäjnes y col., 2012)), produciéndose mayoritariamente una acumulación de lípidos (Praveenkumar y col., 2012; Xin y col., 2011) o carbohidratos (Dragone y col., 2011; Ho y col., 2013), que pueden ser empleados por la industria para la producción de biocombustibles.

Especie	Condiciones de estrés	Compuestos acumulados	Referencias
Dunaliella sp. *Haematococcus pluvialis* *Botryococcus braunii*	Salinidad →	β-caroteno Astaxantina Lípidos	Loeblich, 1982 Sarada y col., 2002 Rao y col., 2007
Chlorella sp. *Scenedesmus* sp. *Chlamydomonas reinhardtii*	Déficit de N y P →	Lípidos Carbohidratos	Praveenkumar y col., 2012 Xin y col., 2010 James y col., 2013 Dragone y col., 2011
Dunaliella viridis *Scenedesmus* sp. *Nannochloropsis salina*	Exceso de luz →	Lípidos Carbohidratos	Gordillo y col., 1998 Hodaifa y col., 2009 Sforza y col., 2012
Chlorella vulgaris *Chlorella minutissima* *Nannochloropsis* sp. *Tetraselmis chuti* *Pavlova luthens*	Metales pesados (Fe, Cu, Zn, Mn) →	Lípidos	Liu y col., 2008 Yang y col., 2014 Richards y Mullins, 2013
Parietochloris incisa *Scenedesmus* sp. *Porphyridium cruentum*	Temperatura →	Lípidos Carotenoides Luteína	Solovchenko, 2011 Shi y Chen, 2002 Xin y col., 2011

Figura 6: Acumulación de compuestos de interés tras someter a las microalgas a diferentes condiciones de estrés.

1.2.2.2. Producción de bioetanol a partir de microalgas

El bioetanol es un combustible producido a partir de la fermentación de los azúcares presentes en la biomasa, siguiendo la ecuación (1):

$$C_nH_{2n}O_n \text{ (azúcar)} \rightarrow (n/3)C_2H_5OH + (n/3)CO_2 + \text{Energía} \quad (1)$$

Para la producción de bioetanol, generalmente se emplean biomasas ricas en azúcares, almidón o celulosa que deben ser previamente sacarificadas. Posteriormente el microorganismo transforma los azúcares fermentables en etanol y libera CO_2 (Brennan y Owende, 2010). El bioetanol se separa del medio de fermentación mediante un proceso de destilación, obteniéndose por una parte el biocombustible, y por otra la torta que contiene la biomasa resultante. Este residuo puede ser posteriormente valorizado ya que tiene un contenido elevado en lípidos o proteínas.

Saccharomyces cerevisiae es el organismo más empleado para producir bioetanol a escala industrial debido a su fácil manejo y a que es capaz de metabolizar los principales azúcares de seis carbonos. Sin embargo, una parte importante de los monómeros que se liberan durante la hidrólisis de las algas no pueden ser metabolizados por *S. cerevisiae*, por lo que los rendimientos de producción de etanol pueden ser reducidos. Por esta razón, cada vez se están empleando con mayor frecuencia otros microorganismos capaces de fermentar una mayor variedad de azúcares, donde destacan microorganismos como *Pichia stipitis, Zimomonas mobilis*

o *Saccharomyces bayanus* y con cepas modificadas de *Escherichia coli* (Harun y col., 2010; Kim y col., 2012; Bellido y col., 2014). Estas cepas están empezando a ser cada vez más empleadas (Bajpai, 2013). Se estima que a partir de una hectárea de terreno cultivada con algas se podrían producir entre 46.000 y 140.000 litros de bioetanol al año (Cheryl, 2010). Sin embargo, actualmente existe una importante diferencia entre la producción teórica y la real. En general, bajo condiciones óptimas donde las microalgas crecen de forma exponencial, las microalgas no acumulan grandes concentraciones de carbohidratos (15-20%) salvo ciertas especies como *Spyrogyra* sp. (33-64%), *Scenedesmus dimorphus* (21-52%) o *Porphyridium cruentum* (40-57% en peso seco) (Harun y col., 2010). Como se ha indicado anteriormente, bajo ciertas condiciones de estrés (**Figura 6**), determinadas especies de microalgas sí son capaces de acumular cantidades significativas de carbohidratos. Por lo tanto, previamente a la hidrólisis y fermentación de los carbohidratos, es necesario que las algas hayan sido cultivadas en condiciones que favorezcan la acumulación de carbohidratos. Una de las principales ventajas del uso de microalgas frente a otras materias primas de origen lignocelulósico es la ausencia de lignina, facilitando el pretratamiento (John y col., 2011). En las microalgas, una parte considerable de los carbohidratos se encuentran formando parte de la pared celular (Miranda y col., 2012) mientras que otra parte se encuentra en el interior celular en forma de gránulos de almidón dentro de vacuolas. Para poder acceder al interior celular y fermentar estos carbohidratos se emplean principalmente dos estrategias (Wingren y col., 2003; Harun y col., 2014):

i) Hidrólisis de los carbohidratos y posterior fermentación (Separate Hydrolysis and Fermentation (SHF)).
ii) Fermentación directa de la biomasa añadiendo enzimas hidrolíticas (Simultaneous Saccharification and Fermentation (SSF)).

Debido a que la pared celular de las microalgas es, en general, difícilmente hidrolizable por los microorganismos fermentadores, no es posible realizar una fermentación directa de la biomasa, sino que es necesario llevar a cabo un pretratamiento previo para romper las paredes celulares y sacarificar los carbohidratos (Harun y Danquah, 2011). En el desarrollo de esta tesis (**Capítulo 3**) se han aplicado tratamientos físicos, químicos y enzimáticos a la biomasa algal para favorecer la liberación de monosacáridos. Se consideran azúcares fermentables glucosa, manosa, galactosa, xilosa, arabinosa, ramnosa, fucosa y maltosa.

En la **Tabla 7** se resume el efecto de distintos pretratamientos en la liberación de azúcares a partir de distintos tipos de microalgas junto con la producción potencial y real de bioetanol. La producción potencial fue calculada multiplicando la cantidad de azúcares fermentables hidrolizados por 0,51, siendo 0,51 el rendimiento máximo de conversión de monosacáridos a etanol (Bajpai, 2013).

Como se muestra en la **Tabla 7**, las concentraciones de azúcares hidrolizados varían considerablemente según la especie y el pretratamiento. Debido a la diversidad en la composición,

concentración de carbohidratos (**Tabla 6**) y estructura de la pared celular de las microalgas, los pretratamientos se pueden comportar de manera diferente, por lo que unos pueden ser muy eficientes en unas especies, y no así en otras. Por lo tanto, los resultados de la bibliografía consultada difícilmente pueden ser comparables entre sí. Además hay que tener en cuenta que las condiciones de operación, el medio de cultivo (ya sea medio enriquecido o agua residual), el tipo de reactor en que se han cultivado, el déficit o exceso de nutrientes, etc., son factores que provocan importantes cambios en la composición y estructura de los carbohidratos del alga. Esta falta de resultados comparables sirvió como base del trabajo experimental desarrollado en el **Capítulo 3**, donde se aplicaron tratamientos físicos, químicos y enzimáticos a tres especies distintas, en concreto a *Chlorella sorokiniana*, *Nannochloropsis gaditana* y *Scenedesmus almeriensis*.

Como se puede observar en la **Tabla 7**, los tratamientos más eficientes resultaron ser aquellos que combinan un tratamiento físico-químico, debido a que el tratamiento físico (normalmente térmico en autoclave) produce la ruptura de las paredes celulares permitiendo al compuesto químico (normalmente ácido) acceder al interior celular e hidrolizar los carbohidratos. También se han mostrado muy eficientes en el proceso de sacarificación la combinación de pretratamientos ácidos en autoclave seguidos de hidrólisis enzimática (Lee y col., 2011; Möllers y col., 2014).

Tabla 7. Sacarificación de carbohidratos y producción de etanol de microalgas.

Pretratamiento	Proceso	Especie	Azúcares fermentables hidrolizados (mg azúcares/g alga)	Etanol potencial (mg etanol/g alga)	Etanol real (mg etanol/g alga)	Referencia
Físico	Microondas	*Chlorella sorokiniana*	21	10,7	---	**Capítulo 3**
Físico	Microondas	*Scenedesmus almeriensis*	2	1,0	---	**Capítulo 3**
Físico	Sonicación	*Scenedesmus obliquus*	25	12,75	---	**Tesis doctoral***
Físico	Homogenización	*Scenedesmus obliquus*	16	8,2	---	Miranda y col., 2012
Físico	CO_2 supercrítico	*Chlorococcum* sp.	201	102,5	---	Harun y col., 2014
Físico	CO_2 supercrítico	Microalgas agua dulce	250	127,5	---	**Capítulo 6**
Químico	NaOH	*Chlorella sorokiniana*	8	4,1	---	**Tesis doctoral***
Químico	H_2SO_4	*Chlorella vulgaris*	472	240,7	233	Ho y col., 2013
Químico	H_2SO_4	*Nannochloropsis gaditana*	93	47,4	---	**Capítulo 3**
Químico	HCl	*Scenedesmus obliquus*	130	66,3	---	Miranda y col., 2012
Enzimático	Celulasas	*Scenedesmus almeriensis*	70	35,7	---	**Capítulo 3**
Enzimático	Amilasas	*Chlorella sorokiniana*	101	51,5	---	**Capítulo 3**
Enzimático	Celulasas + amilasas	*Chlorella vulgaris*	510	260,1	178	Ho y col., 2013
Enzimático	Celulasas + xilanasas + amilasas	*Chlorella homosphaera*	48	24,5	---	Rodrigues y col., 2011
Combinado	H_2SO_4 + celulasas + amilasas	*Dunaliella tertiolecta*	450	229,5	140	Lee y col., 2013
Combinado	H_2SO_4 + celulasas	*Gracilaria salicornia*	54	27,5	7,9	Wang y Wang, 2011
Combinado	H_2SO_4 + celulasas	*Nannochloropsis gaditana*	129	65,8	---	**Capítulo 3**

* **Tesis doctoral:** Datos que no aparecen en los capítulos 3, 4, 5 y 6 pero que han sido determinados experimentalmente durante el transcurso de esta tesis.

En los tratamientos enzimáticos se suelen emplear distintos tipos de celulasas y amilasas para provocar la hidrólisis de los carbohidratos en monosacáridos. Las celulasas más empleadas suelen ser i) endoglucanasas, que atacan regiones donde las fibras de celulosa tienen una baja cristalinidad, ii) exoglucanasas, que liberan moléculas de celobiosa de los extremos de los carbohidratos, y iii) β-glucosidasas, que hidrolizan la celobiosa en glucosa. Los tratamientos con α-amilasas catalizan la hidrólisis del enlace α 1-4 del almidón, mientras que las glucoamilasas provocan la ruptura de enlaces α 1-4 y α 1-6 desde los extremos no reductores de las cadenas. Una de las principales ventajas del uso de enzimas frente a agentes químicos es su gran especificidad y que no favorecen la formación de sustancias inhibitorias.

Los tratamientos que emplean ácido diluido y otros agentes químicos agresivos pueden provocar la degradación de la celulosa, hemicelulosa y azúcares simples (Martin y Johnson, 2003) en compuestos inhibidores para los microorganismos fermentadores como furfural, 5-hidroximetilfurfural (HMF), ácido acético, ácido fórmico, ácido láctico, ácido oxálico, glicoaldehídos, polialcoholes, etc. (García-Aparicio y col., 2006), que impiden el crecimiento de los microorganismos y la fermentación de los azúcares presentes. Estas sustancias inhiben el crecimiento a bajas concentraciones (Delgenes y col., 1996; Taherzadeh, 2000; Miranda y col., 2012) por lo que hay que prestar especial atención a su formación durante el pretratamiento. Por otra parte, aquellos pretratamientos que emplean agentes químicos como H_2SO_4, HCl, NaOH, etc., pueden

interferir en el crecimiento de los microorganismos que realizan la fermentación, aunque previamente a la inoculación del microorganismo, el pH haya sido ajustado. Esto es debido a que la conductividad eléctrica del medio aumenta considerablemente y a que la presencia de determinadas sustancias químicas impide el correcto desarrollo del microorganismo, por lo que disminuye o incluso se inhibe completamente la fermentación.

De todos los inhibidores previamente mencionados, existen trabajos en los que se ha detectado la presencia de ácido oxálico, ácido acético, ácido fórmico, ácido láctico, furfural e HMF en hidrolizados de *Chlamydomonas reinhardtii*, *Chlorella sorokiniana, Chlorococcum* sp., *Laminaria japonica*, *Nannochlropsis gaditana*, *Saccharina japonica, Scenedesmus almeriensis*, *Scenedesmus* sp., *Nizimuddinia zamardini*, entre otras especies (Taherzadeh 2000; Harun y col., 2011; Yazdani y col., 2011; Miranda y col., 2012). La concentración de inhibidores es significativamente mayor en aquellos tratamientos que emplean concentraciones elevadas de ácido y altas temperaturas (Miranda y col., 2012), que en aquellos basados en tratamientos enzimáticos (Yazdani y col., 2011) pero en ningún caso fueron tan elevadas como para inhibir la fermentación.

Para evitar que los inhibidores interfieran en la fermentación se puede realizar un lavado de la biomasa pretratada o realizar una detoxificación con carbonato cálcico o carbón activo (Arslan y Eken-Saracoglu, 2010; Kuhad y col., 2010). Ambos procesos se han aplicado sobre material lignocelulósico pero no se han encontrado referencias relativas a microalgas. En cualquier caso, resulta más

interesante emplear pretratamientos menos agresivos y que generen concentraciones menores de inhibidores, o el uso de microorganismos genéticamente modificados capaces de tolerar concentraciones más elevadas de inhibidores o que cuenten con una batería de enzimas capaces de degradar por sí mismos (sin necesidad de pretratamiento) los carbohidratos de la biomasa algal (Harun y col., 2014). Por último, es importante remarcar que los pretratamientos son considerados la parte más costosa del proceso de producción de bioetanol, por lo que una buena optimización de este paso supondrá un considerable ahorro económico (Choi y col., 2010).

1.2.2.3. Producción de biodiesel a partir de microalgas

El biodiesel está formado por ésteres metílicos de ácidos grasos de cadena larga derivados de lípidos provenientes de material vegetal, animal o algal. Este biocombustible proviene de la trans-esterificación de los triglicéridos (lípidos) a temperaturas próximas a 55 ºC en presencia de un catalizador, generalmente NaOH o KOH, y de metanol (**Figura 7**). De esta reacción se obtiene biodiesel y un subproducto (glicerol), que es separado del biocombustible. Los ésteres metílicos son estructuralmente muy diferentes a aquellos que provienen del petro-diesel. Sin embargo, el biodiesel puede ser igualmente empleando en motores diesel sin necesidad de ningún tipo de modificación. Entre las principales propiedades del biodiesel se puede destacar que es miscible con el petro-diesel, es renovable, emite una menor cantidad de gases de efecto invernadero, es

biodegradable, tiene un escaso contenido en sulfuros y compuestos aromáticos y es un lubricante muy eficiente. Una de las principales ventajas del uso de biocombustibles es que son producidos por el mismo país que los consume, lo cual puede suponer una fuente de ingresos para el país, empleo para la población y un posible recurso que se puede exportar a terceros países (Venkata y col., 2014).

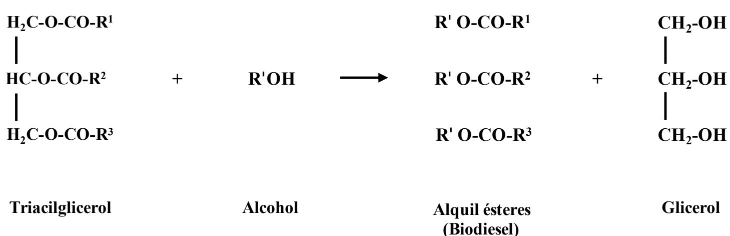

Figura 7. Reacción de trans-esterificación de lípidos (Adaptado de Venkata y col., 2014).

Durante los últimos 25 años se ha estudiado la producción de microalgas para la extracción de lípidos en forma de triacilgliceroles (TAGs) (Nagle y Lemke., 1990; Fajardo y col., 2007; Praveenkumar y col., 2012). Potencialmente, la mayor parte de las algas tienen la capacidad de acumular en torno a un 50% de lípidos (en peso seco) bajo unas determinadas condiciones de operación (Chisti, 2007). La mayor parte de los lípidos neutros presentes en las microalgas son TAGs que pueden ser fácilmente trans-esterificados a ácidos grasos de metil ésteres (FAMEs). Como se ha indicado en el apartado 1.2.2.1., cuando las microalgas son sometidas a ciertas condiciones de estrés la producción de lípidos puede aumentar considerablemente. Esto es debido a que el estrés genera una

reorganización de la membrana celular, liberando a su vez grupos “acilo” que sirven como donantes para la formación de TAGs, favoreciendo la acumulación de lípidos en las microalgas (Moellering y Benning, 2010; Xin y col., 2010; Chen y col., 2011). Dentro de los distintos factores que inducen la acumulación de lípidos se puede destacar el déficit de nutrientes (Praveenkumar y col., 2012), y más concretamente la falta de nitrógeno, que provoca la acumulación de TAGs en los cloroplastos y en el retículo endoplasmático, siendo sintetizados los lípidos más rápidamente (Goodson y col., 2011).

Es importante tener en cuenta que existe una relación directa entre las propiedades del biodiesel y la composición de los ácidos grasos empleados para su producción (Puhan y col., 2010). Para determinar la idoneidad de una especie de alga para la producción de biodiesel es necesario evaluar: i) la productividad del alga, ii) la concentración de lípidos, iii) el perfil de ácidos grasos presentes en el alga; y iv) la facilidad/dificultad de extracción de los lípidos. Este último punto es especialmente importante ya que hay que tener en cuenta que el perfil de ácidos grasos es específico de cada especie, de forma que cada cepa acumula unos determinados ácidos grasos en unas cierta proporción, por lo que habrá que determinar si los ácidos grasos producidos por la especie en cuestión resultan o no interesantes para la producción de biodiesel.

En general, los lípidos de la mayor parte de las especies de microalgas son ácidos grasos poliinsaturados (PUFAs) y en una

menor proporción presentan ácidos grasos saturados y monoinsaturados. Sin embargo, para producir un biodiesel de calidad y que cumpla con las especificaciones técnicas de los motores diesel (estándar ASTM D6751 en EEUU y EN 14214 en Europa), es necesario que la mayor parte de los ácidos grasos sean saturados y en menor medida monoinsaturados y poliinsaturados. La síntesis de biodiesel a partir de lípidos principalmente saturados y en menor medida monoinsaturados y poliinsaturados produce un combustible con un mayor índice de cetano, menores emisiones de NOx, CO y humo, mayor estabilidad oxidativa y mejora la ignición; sin embargo, el combustible es más denso y viscoso, lo que puede dar lugar a problemas de solidificación en países muy fríos (Puhan y col., 2010). Por todo ello, si se quiere producir un biodiesel de calidad a partir de microalgas, es especialmente importante seleccionar aquellas especies con un perfil de ácidos grasos adecuado. Durante el desarrollo de esta tesis, se determinó el perfil de ácidos grasos de diversas especies de microalgas cultivadas en medios minerales (**Capítulo 4**) y especies cultivadas en aguas residuales de matadero (**Capítulo 6**), determinándose éstas últimas como más idóneas para la producción de biodiesel debido a su mayor concentración en ácidos grasos saturados.

Previamente a la transesterificación de los TAGs, es necesario extraer los lípidos del interior celular. Sin embargo, la eficiencia de la extracción depende fundamentalmente de tres factores: i) la concentración de lípidos del alga, ii) la especie utilizada y iii) el

método de extracción empleado (Lewis y col., 2000; Lee y col., 2010).

En la **Tabla 8** se recopilan diversos resultados en cuanto a extracción de lípidos en distintas especies tomados de la bibliografía y obtenidos en los **Capítulos 4** y **6** de la presente tesis doctoral. Se pueden clasificar los métodos de extracción en i) aquellos que emplean solventes tóxicos; y ii) aquellos que no emplean solventes tóxicos (permitiendo valorizar la biomasa resultante). La **Tabla 8** muestra como las mayores eficiencias se obtienen en algas que se han sometido a un pretratamiento físico y posteriormente a extracción con CO_2 supercrítico. También se obtuvieron rendimientos muy elevados mediante un método patentado que consiste en la aplicación de un campo electromagnético y ultrasonidos para extraer los lípidos. De las distintas técnicas que se pueden emplear para extraer TAGs, en el desarrollo de esta tesis se han analizado aquellas que ofrecen mayores rendimientos de lípidos en *Chlorella sorokiniana*, *Isochrysis* T-ISO, *Scenedesmus almeriensis, Tetraselmis* sp. (**Capítulo 4**) y en las algas filamentosas crecidas en aguas residuales de matadero (**Capítulo 6**). En estos estudios se ha observado que el proceso más interesante es la extracción supercrítica MSE ya que se alcanzan mayores rendimientos y se extraen principalmente lípidos neutros, siendo la extracción de ceras, terpenos y otros compuestos prácticamente nula (al contrario que en otros métodos), lo que evita procesos posteriores de desgomado que incrementan el precio final del biodiesel. En el proceso MSE, las microalgas se tratan brevemente

Tabla 8. Métodos de extracción de lípidos más empleados en distintas especies de microalgas: eficiencia y toxicidad.

Especie	Eficiencia extracción	Lípidos totales (% w/w)	Método de extracción	Compuestos extraídos	Compuestos tóxicos en residuo	Referencia
Chlorella pyrenoidosa	Media	52	Celulasas inmovilizadas + saponificación	Triglicéridos (AGP, AGN)	No	Fu y col., 2010
Isochrysis T-ISO	Media	23,1	Método Kochert	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	Sí	**Capítulo 4**
Tetraselmis sp.	Media	17,5	Método 2-propanol + ciclohexano	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	Sí	Li y col., 2014
Botryococcus sp.	Alta	10,2	Bead-beating + Soxhlet	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	Sí	Ryckebosch y col., 2012
Chlorella vulgaris	Alta	17	$SCCO_2$	Triglicéridos (AGN), Ps	No	Mouahid y col., 2013
Chlorella protothecoides	Alta	55	Soxhlet (metanol:cloroformo)	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	Sí	Xiong y col., 2008
Nannochloropsis oculata	Alta	45	$SCCO_2$	Triglicéridos (AGN), Ps	No	Mouahid y col., 2013
Scenedesmus almeriensis	Alta	14.8	$SCCO_2$	Triglicéridos (AGN), Ps	No	**Capítulo 4**
Tetraselmis sp.	Alta	14,8	$SCCO_2$	Triglicéridos (AGN), Ps	No	**Capítulo 4**
Tetraselmis sp.	Alta	17,5	Soxhlet (hexano:etanol)	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	Sí	Li y col., 2014
Botryococcus braunii, Chlorella sp.,	Muy alta	---	Campo electromagnético + ultrasonidos (*patentado*)	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	No	Brennan y Owende, 2010
Phaedactylum tricornutum	Muy alta	16,4	Etanol + hexano/agua	Triglicéridos (AGP, AGN), Gs, Fs, Ps, Cs	Sí	Ramírez-Fajardo, 2007
Scenedesmus almeriensis	Muy alta	15.1	Microondas + $SCCO_2$ (MSE)	Triglicéridos (AGN), Ps	No	**Capítulo 4**
Scenedesmus obliquus	Muy alta	32,5	Extracción asistida por microondas	Triglicéridos (AGN), Ps	No	Balasubramanian y col., 2011
Tetraselmis sp.	Muy alta	24,1	Microondas + $SCCO_2$ (MSE)	Triglicéridos (AGN), Ps	No	**Capítulo 4**

* Eficiencia de la extracción con respecto al total de los lípidos: Baja (<33%), Media (33-66%), Alta (66-90%), Muy alta (>90%).
** Abreviaturas: AGP (ácidos grasos polares), AGN (ácidos grasos neutros), Gs (Gomas), Fs (Fosfolípidos), Ps (Pigmentos), Cs (ceras).
*** Extracción asistida por microondas: Se realiza una extracción $SCCO_2$ mientras se lleva a cabo un tratamiento con microondas (ambos tratamientos se realizan al mismo tiempo).

con microondas para romper las paredes celulares (mejorando así la eficiencia del proceso), y posteriormente se realiza una extracción con CO_2 en condiciones supercríticas, empleando etanol como cosolvente. Tras extraer los lípidos, se puede realizar su trans-esterificación mediante una modificación del proceso propuesto por Freedman y col. (1984).

El residuo algal contiene aproximadamente un 4-5% de lípidos y la mayor parte de los carbohidratos y proteínas presentes inicialmente en la biomasa algal (**Capítulo 6**). Por lo tanto, este residuo puede ser valorizado mediante fermentación para obtener bioalcoholes, biogás o para su venta como compuesto rico en proteínas.

1.2.2.4. Producción de biogás a partir de microalgas

El uso de biomasa microalgal como sustrato para producir biogás mediante digestión anaerobia se remonta a finales de los años cincuenta (Golueke y col., 1957). Sin embargo su estudio se ha intensificado durante los últimos 20 años (Hernández y col., 1993; González-Fernández y col., 2012; Alzate y col., 2014). Este proceso consiste en la descomposición del material biodegradable en ausencia de oxígeno para dar como resultado biogás (formado mayoritariamente por CH_4) y un residuo estabilizado (digestato) (Burton y Turner, 2003). Este proceso consta de 3 etapas. En la primera de ellas (hidrólisis), los carbohidratos, lípidos y proteínas son transformados en azúcares, aminoácidos y ácidos grasos mediante bacterias hidrolíticas. En la segunda etapa, se produce la

acidogénesis-acetogénesis donde estos compuestos son transformados en ácido fórmico, láctico y butírico y a su vez en ácido acético, CO_2 y H_2. Finalmente, en la tercera etapa (metanogénesis) los microorganismos metanogénicos transforman el ácido acético en CH_4 o bien combinan el CO_2 y el H_2 para producir metano (Speece, 2008). Este biogás puede ser empleado para la generación de electricidad y calor en motores de combustión.

Los principales factores a tener en cuenta para que el proceso de metanización sea estable son (Metcalf y Eddy, 2003):

- Temperatura: En la operación del proceso de digestión anaerobia es importante identificar el rango de temperaturas óptimo. La digestión anaerobia se puede realizar a temperaturas psicrófilas (-5-20 ºC), mesófilas (8-45 ºC), termófilas (40-70 ºC) o hiper-termófilas (65-110 ºC). Durante esta tesis se llevó a cabo la digestión anaerobia a 37 ºC.
- pH: El pH óptimo para los microorganismos que llevan a cabo la digestión anaerobia debe mantenerse entre 6,5 y 8,2. Cuando el pH se encuentra por encima o por debajo del óptimo durante un tiempo prolongado la digestión anaerobia se puede inhibir completamente. Durante este proceso, la producción de CO_2 y la liberación de ácidos grasos provoca una disminución del pH. Por ello, es necesario que la alcalinidad sea igual o superior a 2000 mg $CaCO_3$/L para asegurar la estabilidad del proceso.

- Agitación: Necesaria para evitar la sedimentación y favorecer que los microorganismos mantengan un buen contacto con el substrato.
- Relación substrato/inóculo (DQOt/SV): Otro parámetro de gran influencia en el proceso de metanización es la relación entre el substrato (DQOt) y la concentración de inóculo (SV) de fango anaerobio empleado, ya que permite relacionar la materia orgánica adicionada al reactor con la cantidad de microorganismos que deben consumir dicha materia orgánica.
- Tiempo de retención hidráulico: Se corresponde con el número de días que permanece el sustrato en el digestor.
- Concentración de nitrógeno amoniacal: Una concentración elevada de nitrógeno amoniacal influye negativamente en la producción de biogás. A pH inferior o igual a 7,5 una concentración de amonio inferior a 1.500 mg $N\text{-}NH_4^+/L$ no provoca inhibición de los microorganismos (Zeng y col., 2010).

Para que el proceso de digestión anaerobia se realice en su totalidad y sin inhibición, la relación C/N del sustrato (microalgas) ha de ser próximo a 25 (Parkin y Owen, 1984). Sin embargo, en el proceso de digestión anaerobia de microalgas, existe una cierta problemática, ya que los microorganismos son susceptibles al descenso del pH debido a la producción de ácido acético y ácido propiónico, mientras que la degradación de proteínas provoca el aumento de la concentración de amonio que a altas concentraciones puede inhibir

la actividad de los microorganismos metanogénicos (Speece, 2008; Molinuevo-Salces y col., 2010). Por lo tanto, en la digestión anaerobia de microalgas, se deben buscar microalgas ricas en carbohidratos y/o lípidos, y por ende, con una baja concentración de proteínas o bien la adición de otros co-sustratos ricos en carbono (Molinuevo-Salces y col., 2010).

Los estudios realizados por Sialve y col. (2009) muestran que existe una relación directa entre la composición bioquímica de un sustrato y el rendimiento específico de metano (**Tabla 9**). Por lo tanto, conociendo la composición macromolecular del alga a digerir se puede determinar la producción teórica de metano (valores normalizados a presión de 760 mm Hg y temperatura de 0 ºC).

Tabla 9. Rendimiento específico de metano para tres compuestos orgánicos (Adaptado de Sialve y col., 2009).

Substrato	Composición	L CH_4 /kg SV
Proteínas	$C_6H_{13}O_1N_{0.6}$	0.851
Lípidos	$C_{57}H_{104}O_6$	1.014
Carbohidratos	$(C_6H_{10}O_5)n$	0.415

Según los estudios realizados por Mussgnug y col. (2010), para que el proceso de digestión anaerobia se lleve a cabo de forma completa y la relación entre el biogás producido y el biogás potencial sea lo mayor posible, es necesario que las especies de microalgas empleadas sean altamente biodegradables. En las algas, la cantidad de energía acumulada por cada kg de SV es aproximadamente 40 kJ/kg. Sin embargo, solamente un 40% de dicha energía es liberada durante la digestión anaerobia de la biomasa, debido a su escasa biodegradabilidad (Chen y Oswald, 1998). Esto es debido a

la presencia de una compleja pared celular, que impide el acceso por parte de las bacterias a los lípidos, carbohidratos y proteínas. Por ello, en muchos casos es necesario realizar pretratamientos similares a los empleados para la sacarificación de los carbohidratos, que han sido descritos en el apartado 1.2.2.2., como paso previo a la producción de biogás (Sialve y col., 2009), o bien, realizar una extracción previa de otros compuestos de interés que rompan la pared celular (**Capítulos 4** y **6**). Los pretratamientos más empleados para mejorar la biodegradabilidad son de tipo termoquímico, térmicos, físicos, biológicos, entre otros (Chen y Oswald, 1998; González-Fernández y col., 2012b; González-Fernández y col., 2013). La aplicación de pretratamientos o la extracción previa de compuestos de interés mejora el acceso de los microorganismos al interior celular, propician la ruptura de los carbohidratos en azúcares simples y las proteínas en aminoácidos, aumentando así la biodegradabilidad del sustrato algal.

En el desarrollo de esta tesis se ha llevado a cabo la digestión anaerobia de biomasa algal (**Capítulo 4**) y producida mediante el tratamiento de ARAs (**Capítulos 5** y **6**). La biomasa fue digerida anaeróbicamente obteniéndose mayor producción de metano en aquellas muestras a las que previamente se les había realizado una extracción de lípidos. En la **Tabla 10** se muestra la producción de biogás potencial y real en litros normalizados de CH_4 producidos por cada kg de SV para distintas especies de microalgas que han sido sometidas a un pretratamiento para incrementar su biodegradabilidad. La producción potencial de biogás se determinó

Tabla 10. Producción de metano a partir de microalgas previamente pretratadas.

Especie	**Pretratamiento**	**Biogás producido ($L\ CH_4$ /kg SV)**	**Biogás potencial ($L\ CH_4$ /kg SV)**	**Incremento de la biodegradabilidad (%)**	**Referencia**
Scenedesmus sp.	NaOH + 50 ºC	155	---	18	Mahdy y col., 2014a
Chlorella vulgaris	NaOH + 50 ºC	160	---	20	Mahdy y col., 2014a
Scenedesmus sp.	Tratamiento térmico (90 ºC)	170	318	26	González-Fernández y col., 2012a
Algas filamentosas	$SCCO_2$	185	377	63	**Capítulo 6**
Scenedesmus almeriensis	$SCCO_2$	203	552	20	**Capítulo 4**
Nannochloropsis gaditana	$SCCO_2$	204	541	21	**Capítulo 4**
Isochrysis T-ISO	$SCCO_2$	213	533	14	**Capítulo 4**
Tetraselmis sp.	$SCCO_2$	236	522	43	**Capítulo 4**
Chlorella vulgaris	Glucanasa + proteasa	300	353	59	Mahdy y col., 2014b
Chlamydomonas reinhardtii	Glucanasa + proteasa	312	358	17	Mahdy y col., 2014b
Scenedesmus sp.	Alta temperatura (160 ºC) + Presión (6 bar)	320	551	23	Keymer y col., 2013
Chlorococcus sp.	Enzimas de *Aspergillus lentulus*	324	364	28	Prajapati y col., 2015
Chlorella sp.	Tratamiento alcalino (pH 9)	336	---	10	Cho y col., 2013
Chlorella sp.	Ultrasonidos (130 W, 3 min)	385	---	28	Cho y col., 2013
Chlorella sp.	Tratamiento térmico (120 ºC)	405	---	33	Cho y col., 2013
Nannochloropsis salina	Tratamiento térmico (120 ºC)	570	703	285	Schwede y col., 2013

en base a la **Tabla 9**. El incremento de la biodegradabilidad se calculó siguiendo la ecuación (2):

$$\left(\frac{\text{Biogás producido con pret.} - \text{Biogás producido sin pret.}}{\text{Biogás producido sin pret.}}\right)\times 100 \qquad (2)$$

En la **Tabla 10**, se puede observar como determinados pretratamientos (enzimáticos o basados en la aplicación de presión y temperatura) permiten alcanzar producciones de metano cercanas al máximo potencial de biogás, mientras que otros como el $SCCO_2$ incrementan la biodegradabilidad hasta un 50%. Como queda reflejado en la **Tabla 10**, el tratamiento térmico a 120 ºC provoca un incremento considerable en la biodegradabilidad de todas las especies de algas estudiadas, siendo esta mejora especialmente destacable en el caso de *Nannochloropsis salina*. Sin embargo, es importante remarcar que existen importantes diferencias en la biodegradabilidad inicial, pudiéndose encontrar especies que debido a su rígida pared celular son muy poco biodegradables como es el caso de *Scenedesmus*, mientras que otras especies como *Chlamydomonas* son más biodegradables (Miranda y col., 2012).

1.2.3. Desarrollo de biorrefinerías a partir de algas

Una biorrefinería integra la producción de distintos tipos de combustibles y la extracción de compuestos químicos o biológicos de alto valor añadido a partir de biomasa (Demirbas y Demirbas, 2010). De este modo, se obtienen diferentes productos de valor a

partir de una única biomasa, generando en suma, un mayor valor que la producción de un único producto de interés.

El concepto de "biorrefinería de algas" no es nuevo. Este concepto hace referencia a la producción de distintos compuestos a partir de una única biomasa algal. De este modo, se consigue la integración de distintos bioprocesos realizados consecutivamente, incrementando la relación coste/efecto del proceso, disminuyendo su impacto ambiental y mejorando el beneficio económico del mismo (Gouveia, 2011). Una biorrefinería de algas integra diferentes tecnologías para producir distintos biocombustibles entre los que se encuentran biodiesel, bio-alcoholes, biogás, etc., además de otros compuestos de alto valor añadido como puede ser astaxantina, carotenoides, pigmentos, antioxidantes, entre otros. Aunque normalmente el concepto de biorrefinería de algas parte de una biomasa algal para producir distintos compuestos de interés, se puede considerar que el tratamiento de aguas residuales con microalgas además de reducir los costes asociados a los tratamientos convencionales, genera otro producto valorizable (agua residual tratada que puede ser reutilizada) que puede formar parte de los productos obtenidos a partir de las microalgas.

En la **Figura 8** se muestran las distintas rutas de producción de biocombustibles a partir de microalgas producidas en ARAs en el marco de una biorrefinería. Fruto del trabajo de Tesis se ha podido determinar que desde un punto de vista económico y con el objetivo de maximizar la producción de biocombustibles, resulta más

interesante llevar a cabo la extracción de lípidos y posterior producción de biogás (**Capítulo 4** y **Capítulo 6**) que realizar un único proceso. En este sentido, también se ha observado como el proceso de extracción de lípidos mediante fluidos supercríticos provoca un incremento de los azúcares fermentables y por ende una mayor producción de bioetanol si son fermentados.

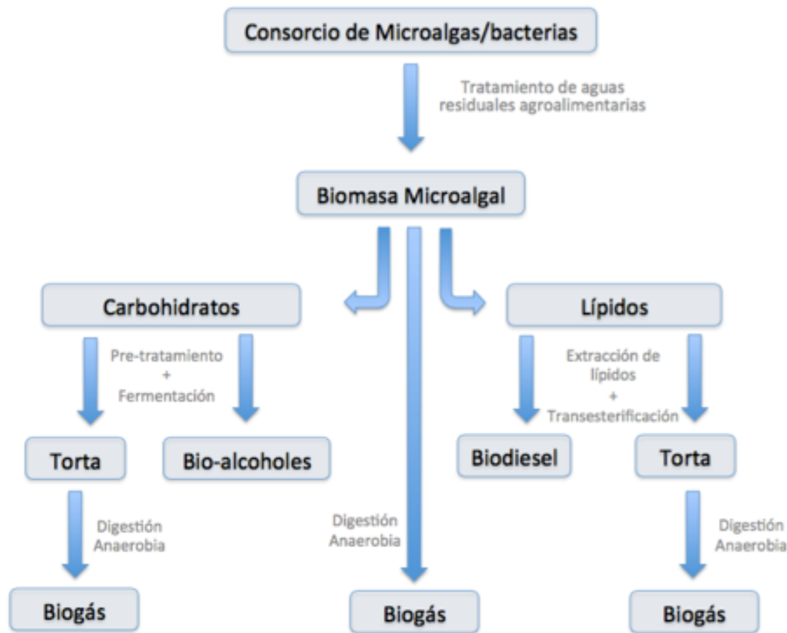

Figura 8. Concepto de biorrefinería de algas desarrollado durante la tesis doctoral

Durante los últimos años, se han llevado a cabo diversos estudios en los que se emplea la biomasa algal íntegramente con el objetivo de producir distintos biocombustibles y/o productos de alto valor añadido. La digestión anaerobia de determinadas especies conlleva producciones de metano relativamente bajas como consecuencia de las características específicas del alga como la deformación,

estructura, composición de la pared celular, liberación al medio de determinados compuestos, etc., por lo que una alternativa a la digestión anaerobia directa es la producción previa de H_2 a partir de la biomasa y la posterior producción de biogás, tal y como plantea Mussgnug y col. (2010), lo cual conlleva un incremento de la producción de biogás de un 123%, favoreciendo la viabilidad económica del proceso. Otra alternativa, es la planteada por Ramos y Carreras (2014) que llevaron a cabo la extracción de lípidos o aminoácidos de la microalga *Scendesmus* sp. y posteriormente la digestión anaerobia. La combinación de ambos procesos provoca un incremento en la producción de biogás como consecuencia de una mayor biodegradabilidad de la biomasa. Asimismo, Muñoz y col. (2015b) plantean un proceso de extracción de compuestos y producción de biocombustibles más complejo, donde inicialmente se extraen en distintas etapas las proteínas de las algas *Nannochloropsis gaditana* y *Botryococcous braunii* en medio alcalino, seguido de una extracción de lípidos para producir biodiesel. Finalmente, el residuo resultante es pirolizado a 500 ºC para producir bio-aceites. Aunque en este trabajo se muestra un proceso integral de valorización de la biomasa, la baja producción de bio-aceites recuperados en *N. gaditana* durante la pirolisis evidencia la necesidad de realizar un estudio económico del proceso global.

Aunque el uso integral de la biomasa es una cuestión clave para aumentar la viabilidad económica del proceso, al plantear un uso completo de la misma se debe tener en cuenta que cada uno de los

distintos tratamientos o transformaciones no solo se traduce en un beneficio económico (biocombustibles y/o productos de alto valor añadido), sino también en un importante gasto y una pérdida de otros componentes de la biomasa (Cerón-García y col. 2013; Ramos-Suarez y Carreras, 2014; Muñoz y col., 2015b) pudiéndose alcanzar pérdidas de compuestos de interés de un 10-50%. Por ello, se debe identificar en qué orden de prioridad deben realizarse las extracciones e hidrólisis de compuestos, para optimizar así el rendimiento global (Ramos-Suarez y Carreras, 2014).

Tal y como se muestra en diversos trabajos (Chisti, 2007; Gouveia, 2011; Olguín y col., 2012; Ward y col., 2014), es necesario plantear una biorrefinería con la biomasa microalgal, ya que permite desarrollar procesos de producción de biocombustibles y bio-compuestos a partir de microalgas técnica y económicamente viables. Por ello, en el transcurso de esta tesis se han empleado las microalgas de manera integral (**Capítulos 4**, **5** y **6**), desde su producción hasta la obtención de distintos biocombustibles empleando una única biomasa de partida (**Figura 8**). Para lograr una sustitución progresiva de los combustibles fósiles por biocombustibles procedentes de microalgas, es necesario que su producción se haga de forma sostenible, ya que de no ser así el impacto ambiental y social puede ser tan perjudicial como el de los combustibles fósiles. Para ello, es necesario tener en cuenta las siguientes consideraciones (Benemann y col., 2003; Brennan y Owende, 2010):

i) Minimizar el impacto medioambiental que tiene el cultivo de microalgas en una región o zona.
ii) Maximizar el impacto económico sobre la población de la zona.
iii) Optimizar las condiciones de operación del cultivo para maximizar la producción de biocombustible por unidad de superficie.
iv) Mejorar el caudal y la eficiencia del agua residual tratada.
v) Evitar el uso de suelos arables y fertilizantes en la producción de microalgas para evitar así la competencia con los cultivos convencionales.

Para conseguir dicha sostenibilidad, deben emplearse las microalgas en el marco de las biorrefinerías, siendo obtenidas mediante tratamiento de aguas residuales (Clarens y col., 2010; Posadas y col, 2014), y produciendo a partir de ellas biodiesel, bio-alcoholes, biogás y otros compuestos de alto valor añadido. La producción de uno u otro biocombustible dependerá de la composición macromolecular del alga en cuestión y de la mayor o menor presencia de compuestos de alto valor añadido. El cultivo de microalgas a gran escala mediante medios enriquecidos para producir biocombustibles requiere de cantidades ingentes de nutrientes, por lo que su producción supondría un impacto enorme en el medioambiente, provocando un fuerte incremento del precio de los fertilizantes. Como consecuencia, se produciría una escalada en el precio de los alimentos, compitiendo de manera indirecta con ellos (Chisti, 2008). Según las investigaciones llevadas a cabo por

Yang y col. (2011), si no se hace un reciclado de los nutrientes del agua serían necesarios 3.726 litros de agua, 0,33 kg de N y 0,71 kg de P para producir 1kg de biodiesel de microalgas. Sin embargo, reciclando los nutrientes presentes en las aguas residuales estos consumos disminuirían un 84% y un 55% para el nitrógeno y el fósforo, respectivamente.

En el desarrollo de esta tesis se han realizado dos evaluaciones económicas preliminares (**Capítulo 4** y **Capítulo 6**) para determinar si resulta más interesante producir un único biocombustible (biogás) o varios biocombustibles (biodiesel y biogás) de forma secuencial, mejorando la viabilidad económica del proceso. Para ello, se compararon los costes y beneficios económicos de ambos procesos de producción a partir de microalgas producidas por cada hectárea de terreno mediante sistemas HRAP tipo raceway. En ambas evaluaciones se determinaron los costes de extracción de lípidos mediante la tecnología de fluidos supercríticos y la digestión anaerobia (Zamalloa y col., 2011) de biomasa algal (*Isochrysis* T-ISO, *Nannochloropsis gaditana*, *Tetraselmis* sp. y *Scenedesmus almeriensis*) y biomasa crecida en ARAs (principalmente microalgas filamentosas). Es importante destacar que aunque existen otras tecnologías alternativas para extraer lípidos - método Soxhlet, método de Kochert, etc.- la mayoría de ellas emplean solventes tóxicos como metanol y cloroformo, impidiendo la posterior digestión anaerobia de la biomasa. Por ello, y por los buenos resultados obtenidos mediante la extracción con fluidos supercríticos, se descartaron dichos métodos de extracción de la

evaluación económica. Los resultados demostraron que la producción de más de un biocombustible provoca un incremento en la rentabilidad del proceso pudiendo en muchos casos, ser una cuestión esencial para hacer viable su producción. Estos resultados coinciden con la hipótesis planteada por Chisti (2007), que expuso la necesidad de emplear las algas como una biorrefinería si se quiere maximizar su rentabilidad.

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CAPÍTULO 2

JUSTIFICACIÓN, OBJETIVOS Y DESARROLLO DE LA TESIS

2.1. Justificación de la tesis

El aumento del volumen de aguas residuales agroalimentarias generado, el incremento de la concentración de nutrientes (nitrógeno y fósforo) y materia orgánica de las mismas y la mayor presencia de compuestos tóxicos (pesticidas, metales pesados y compuestos policlorados) han propiciado la búsqueda de alternativas a los tratamientos convencionales. El tratamiento de aguas residuales agroalimentarias con consorcios de microalgas-bacterias puede suponer una interesante alternativa debido a su eficacia en cuanto a la eliminación de materia orgánica y nutrientes y a la posibilidad de valorizar la biomasa producida en forma de biocombustibles u otros bioproductos de alto valor añadido. Sin embargo, es necesario profundizar en el conocimiento de este tipo de tecnología para comprender la influencia de los parámetros de operación en la depuración del agua y en la producción y composición de la biomasa. Así mismo, es necesario optimizar la extracción de compuestos de interés a partir de una única biomasa para mejorar la rentabilidad y la sostenibilidad del proceso.

2.2. Objetivos

El **objetivo principal** de esta tesis es el estudio del tratamiento de aguas residuales agroalimentarias mediante consorcios de microalgas y bacterias y el aprovechamiento de la biomasa resultante para obtener distintos biocombustibles. Para ello se evaluará la influencia de los parámetros operacionales en la eficacia

del tratamiento y en la composición bioquímica y productividad de la biomasa generada. Así mismo, se evaluará la eficacia de distintos procesos para extraer lípidos, sacarificar carbohidratos y mejorar la biodegradabilidad anaerobia de la biomasa. Para alcanzar este objetivo global se plantean los siguientes **objetivos específicos**:

1. Evaluar la producción de azúcares fermentables a partir de microalgas utilizando diferentes tratamientos físicos, químicos y enzimáticos.

2. Analizar la eficacia de diferentes métodos para extraer lípidos a partir de microalgas y la influencia que tiene esta etapa de extracción en la posterior producción de metano.

3. Recuperar nutrientes de aguas residuales agroalimentarias mediante la aplicación de consorcios de microalgas y bacterias analizando la influencia de diferentes condiciones de operación en la calidad del agua tratada, en el perfil bioquímico de la biomasa algal generada y en su posterior valorización mediante digestión anaerobia de la biomasa algal.

4. Proponer un proceso técnicamente viable para la valorización integral de la biomasa algal resultante del tratamiento de las aguas residuales agroalimentarias mediante su uso como fuente de diferentes biocombustibles, en el marco de una biorrefinería.

2.3. Desarrollo

Para alcanzar los objetivos globales y específicos de la tesis, se ha llevado a cabo el siguiente plan experimental, que se recoge en los diferentes capítulos de la tesis y que se describe a continuación.

A fin de optimizar la sacarificación de los carbohidratos de la biomasa microalgal (*Objetivo 1*), en el **Capítulo 3** se realizó un estudio comparativo de pretratamientos físicos y químicos en las especies *Chlorella sorokiniana*, *Nannochloropsis gaditana* y *Scenedesmus almeriensis*. Estos pretratamientos fueron realizados de forma simple y combinada para determinar si existía algún efecto sinérgico, y por ende, un aumento del contenido en azúcares fermentables final. Así mismo, se llevó a cabo la hidrólisis enzimática de las microalgas pretratadas y sin pretratar y se evaluó el efecto de esta etapa en la producción de monosacáridos a partir de microalgas.

Para llevar a cabo el *Objetivo 2*, en el **Capítulo 4** se realizó la extracción de lípidos en *Chlorella sorokiniana*, *Isochrysis* T-ISO, *Nannochloropsis gaditana* y *Tetraselmis* sp. mediante cuatro procesos diferentes. Se determinó qué proceso es más idóneo para la extracción selectiva de lípidos neutros, evitando a su vez la presencia de compuestos indeseables (gomas, ceras, terpenos, etc) que incrementan los costes de producción del biodiesel y afectan negativamente a su calidad. Además, se emplearon estas cuatro microalgas como sustrato para producir biogás con y sin extracción

previa de los lípidos. Se realizó una estimación de la viabilidad económica de la producción de biogás a partir de microalgas y del proceso combinado de extracción de lípidos y posterior digestión anaerobia de las microalgas.

Una vez cumplidos los *Objetivos 1* y *2*, se llevó a cabo el tratamiento de diferentes tipos de ARAs mediante el uso de consorcios de microalgas y bacterias con el fin de recuperar nutrientes y reducir la concentración de materia orgánica (*Objetivo 3*). Para analizar la viabilidad técnica del tratamiento de ARAs con consorcios microalgas-bacterias, se emplearon, en primer lugar, aguas de piscifactoría (**Anexo I**). El **Capítulo 5** se orientó al estudio del tratamiento de aguas residuales procedentes de una industria de procesado de patatas y del tratamiento biológico de purines. En este capítulo se estableció una relación entre las condiciones de operación de los fotobiorreactores, las características de las aguas residuales y la acumulación de lípidos en las microalgas. Además, se estableció la relación entre el déficit de nutrientes en las aguas residuales y la acumulación de lípidos en la biomasa algal. Asimismo, se determinó la influencia de la composición bioquímica de las microalgas en la producción de biogás.

En el **Capítulo 6** se planteó el uso integral de la biomasa microalgal desde su producción a partir de ARAs hasta su valorización en forma de lípidos y biogás (*Objetivo 4*). Para ello, se llevó a cabo el tratamiento de las aguas residuales generadas en un matadero de cerdos. A fin de mejorar la eficacia del sistema, se disminuyó el

tiempo de residencia de 15 a 10 días y se evaluaron diferentes condiciones de iluminación, determinando su influencia en la composición bioquímica de las microalgas. En base a los resultados obtenidos en el **Capítulo 4**, se recuperaron los lípidos de la biomasa y se usó el residuo como sustrato para producir biogás. El estudio se completó con una evaluación preliminar sobre la viabilidad económica de la extracción de lípidos y posterior producción de biogás a partir de la biomasa, en el marco de una biorrefinería.

El trabajo recogido en esta tesis se llevó a cabo en el marco de actividades realizadas en el ITACyL dentro del proyecto INIA RTA2010-00087-C02-01 "Evaluación de la utilización de biomasa algal en acuicultura obtenida a partir del tratamiento de aguas residuales agroalimentarias", y el proyecto RTA2013-00056- C03-01 "Obtención de bio-productos a partir de biomasa algal". Estas actividades se han realizado bajo el programa de ayudas para la formación de personal investigador en formación del Subprograma FPI-INIA en el marco del Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica 2008-2011.

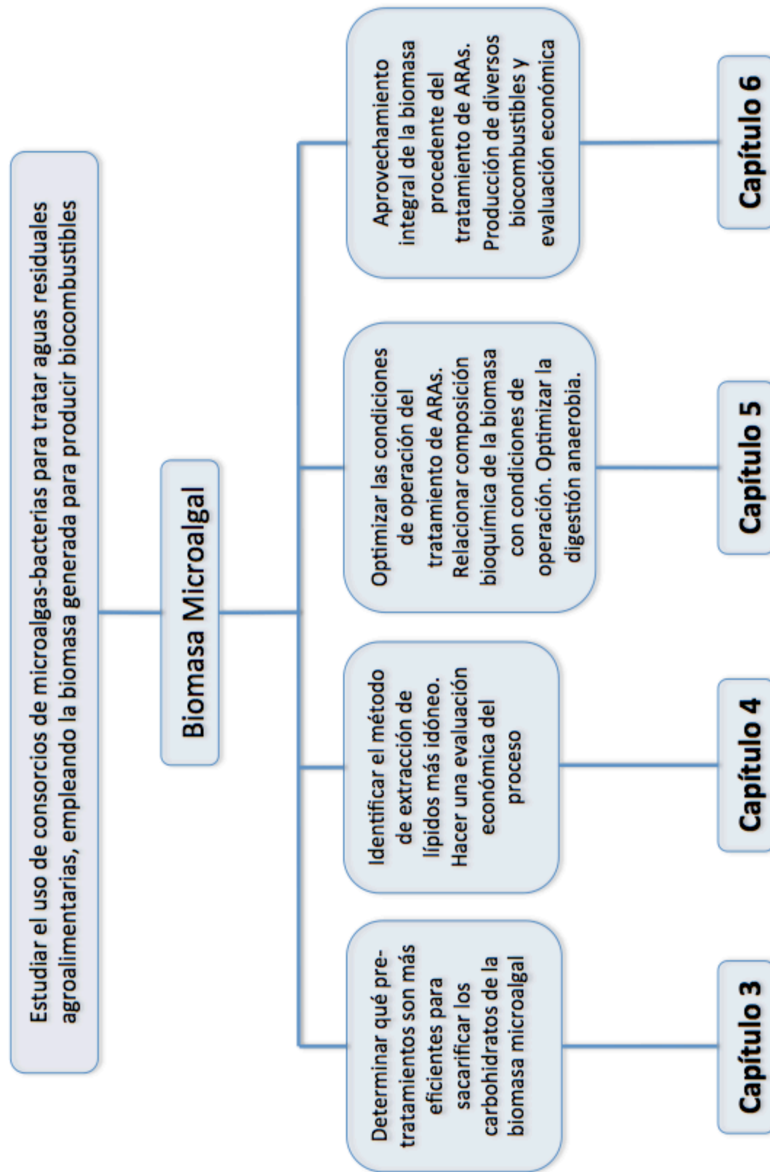

Figura 1. Organización del trabajo de tesis

CAPÍTULO 3

SACCHARIFICATION OF CARBOHIDRATES IN MICROALGAL BIOMASS BY PHYSICAL, CHEMYCAL AND ENZYMATIC PRE-TREATMENTS AS A PREVIOUS STEP FOR BIOETHANOL PRODUTION

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Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production

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HIGHLIGHTS

- Several pre-treatments were applied for carbohydrate saccharification of three microalgal species.
- Cell wall composition of microalgae determined pre-treatments efficiency.
- Microwave, autoclave and alkaline hydrolysis resulted in poor sugar release.
- Combination of pre-treatments enhanced monosaccharides release.
- Cell wall disruption was essential for enzymatic attack improving sugar release.

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ABSTRACT

Fermentation of carbohydrates to produce bioethanol is one of the pathways to produce biofuels from microalgae. This process currently needs many stages that are complex and energy consuming. Cell wall disruption and hydrolysis are two of the stages that must be carried out, since most carbohydrates are entrapped within the cell wall or intracellularly as energy storage in the form of starch. In the present work, physical, chemical, and enzymatic pre-treatments were performed on three microalgal species to disrupt and break down complex carbohydrates into simple sugars, as a preliminary stage to produce bioethanol. Pre-treatments were carried out alone and combined with each other. According to the results obtained in the present work, the highest concentration of monosaccharides per g of microalgae dry weight was achieved by the combination of pre-treatments; for *Chlorella sorokiniana* and *Nannochloropsis gaditana* the combination of acid hydrolysis followed by enzymatic hydrolysis produced 128 and 129 mg/g, respectively. In the case of *Scenedesmus almeriensis* the highest monosaccharide concentration (88 mg/g) was obtained after acid hydrolysis with sulphuric acid for 60 min at 121 °C. The results obtained proved the effectiveness of the combination of acid pre-treatment and enzymatic hydrolysis to enhance complex carbohydrates break down into simple sugars in bioethanol production process from microalgal biomass.

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1. Introduction

Currently, bioethanol is produced at commercial scale via fermentation of different carbohydrate-rich feedstocks such as corn, sugarcane and beets [1]. The main producer countries are United States and Brazil [2]. Bioethanol is particularly important since it can substitute gasoline in combustion engines, which makes it one of the most promising biofuels; expecting to reach a production of 100 billion litres in 2015 [3]. However, concerns over food safety and human demand for food poses a major challenge to the use of agricultural stocks. More sustainable fuel production alternatives are thus needed to overcome these problems. In this context, although in optimal conditions the most significant species of microalgae and cyanobacteria contain 15–25% [4] of carbohydrates, different works have identified microalgae as a suitable source of carbohydrates for bioethanol production since they are able to exhibit high carbohydrate content [5] under stress conditions like nutrient starvation [6], high salinity [7] or

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light deficit-excess [7,8]. Indeed, algae offer a number of potential advantages compared to higher plants. Microalgae have shown to be more efficient than terrestrial plants in converting sunlight to biochemical energy being its production tenfold higher [9,10]. Microalgae consume CO_2, reducing greenhouse gas emissions and their growth is not dependent on arable land availability [11,12]. However, microalgal growth requires high amounts of nutrients, mainly nitrogen (N) and phosphorous (P), being the supply of P limited and N production requires high fossil energy. One alternative to synthetic culture media is to use agro-industrial wastewater which usually presents high N and P concentration. In this sense, the interference with conventional fertilizers is avoided as fertilizers are not required [13–15]. In recent years, different microalgal genera like *Anabaena*, *Chlamydomonas*, *Chlorella*, *Porphyrydium*, *Scenedesmus*, *Spirogyra* and *Spirulina*, among others, have been used to produce bioethanol. These microalgae have been grown in synthetic medium achieving carbohydrate concentration from 8% up to 64% [4,16].

In order to produce bioethanol, a disruption of the cell wall must be carried out since most carbohydrates are entrapped within the cell wall (cellulose and hemicellulose), or intracellularly as energy storage in the form of starch [8,17]. In a further stage, it is necessary the hydrolysis of polysaccharides to release monosaccharides for its later fermentation into bioethanol. Biomass pre-treatment is also a necessary stage to increase the surface area, to enhance sugars solubility and to improve substrate digestibility [18]. Pre-treatments have been viewed as one of the most crucial and expensive processing stages in biomass conversion to fermentable sugars [19].

Different methods have been tested to disrupt and to hydrolyze cell wall carbohydrates into monosaccharides. Among others, physical methods include high-pressure homogenization, microwaving, sonication and heat [20–22]. In addition, chemical lysis using alkaline or acid reagents have been also applied to hydrolyze microalgal biomass into its constituent monosaccharides [18,23,24]. Enzymatic pre-treatment has shown to be an efficient tool to get cell wall hydrolysis in some microalgae [19]. These physical, chemical and enzymatic pre-treatments have a particular economic cost that depends on many parameters as: (i) electricity cost; (ii) alkaline or acid reagent; (iii) temperature and time reached during thermal pre-treatment; (iv) type of enzymes used; (v) addition of surfactants during enzymatic hydrolysis and (vi) type of raw material used, among others. According to pre-treatment costs, they may be ordered (from low to high costs) as (i) physical: microwaving, sonication, high-pressure homogenization and heat; (ii) chemical: alkaline and acid; and (iii) enzymatic pre-treatments: cellulases and amylases [25–27]. Pre-treatments have a great potential for improving the efficiency of fermentation and lowering costs throughout research and development. Thus, optimizing cell disruption and sugar extraction methods is essential to produce bioethanol in a cost-effective and environmentally sustainable manner. Despite of the many cell disrupting methods tested in literature for microalgal cell wall disruption, a standard pre-treatment has not been identified to treat most of microalgal species. Furthermore, data in literature concerning biomass pre-treatments are not comparable, because quite different microalgae strains, conditions and techniques are used, making it difficult to compare these results between microalgae.

In the present work, different physical, chemical, and enzymatic pre-treatments were performed on three microalgal species to study disruption and break down of complex carbohydrates into simple sugars, as a preliminary stage to produce bioethanol. *Chlorella sorokiniana* and *Scenedesmus almeriensis* were selected due to their ability to grow in wastewater containing high organic matter concentrations [15], and *Nannochloropsis gaditana* was selected because its ability to grow in high salted mediums [28]. Both characteristics minimize the appearance of competitive microalgal species and predators like rotifers and protozoa in open pond cultures, resulting in a higher growth of the selected microalgae. The methods evaluated in the present work covered physical, chemical and enzymatic processes. Sugar release (SR) was determined for each microalga under the different experimental conditions studied, analyzing monosaccharides and sugar degradation products in hydrolysates (furfural, 5-hydoxymethylfurfural (HMF), propionic acid, acetic acid, formic acid and lactic acid) to determine the effectiveness of the pre-treatment processes and the possible inhibition of the sugar degradation products on fermentative microorganisms in further sugar fermentation.

2. Materials and methods

2.1. Microorganisms

Chlorella sorokiniana was obtained from the culture collection of the University of Goettingen (Goettingen, Germany). Microalgae inoculum was cultivated in a mineral medium according to Guieysse et al. [29]. Biomass was centrifuged at 10,000 rpm (Beckman Coulter, Avanti centrifuge J-30I) for 10 min, washed with distilled water and dried in an oven (Selecta, Digitronic) to a constant weight at 80 °C. The biomass was stored at 4 °C for further use.

Nannochloropsis gaditana B-3 and *S. almeriensis* were obtained in lyophilized form from the Food Innovation and Sustainability Center (Almería, Spain). *N. gaditana* was cultured following González-López et al. [28]. *Scenedesmus almeriensis* was isolated from a fresh water pool in Almería (Spain) and cultured following Sánchez et al. [30]. Lyophilized biomass was washed with distilled water, dried in an oven to a constant weight at 80 °C and stored at 4 °C for further use.

2.2. Methods for cell disruption and sugar extraction

2.2.1. Acid hydrolysis

Acid hydrolysis of microalgal biomass was carried out using H_2SO_4 (purity greater than 96%, VWR International, Radnor, USA) at different concentrations (4%, 7%, and 10% (v/v)) at 121 °C for 30 min. The assays were carried out in 250 mL Erlenmeyer flasks (Simax, Prague, Czech Republic). The H_2SO_4 concentrations and temperature were selected according to Harun et al. [31] and Miranda et al. [24]. Acid pre-treatments were performed using 100 mL of microalgal biomass at a concentration of 30 g volatile suspended solids (VSS)/L. A control was performed using 0% H_2SO_4 followed by autoclave treatment (121 °C for 30 min).

2.2.2. Alkaline hydrolysis

The biomass was suspended in 100 mL of NaOH (purity greater than 98%, Panreac Química SLU, Barcelona, Spain), 1 M and 5 M, to set a final concentration of 30 g VSS/L. After that, samples were incubated at 90 °C for 30 min with constant agitation at 60 rpm with a magnetic stirrer (RH Basic 2, IKA, Staufen, Germany) using 250 mL Erlenmeyer flask (Simax, Prague, Czech Republic). The alkaline hydrolysis parameters in terms of NaOH concentration, temperature and incubation time were adapted from Ellis et al. [32] and Harun et al. [18]. A control was set using biomass suspended in 0% NaOH at 90 °C for 30 min.

2.2.3. Autoclaving hydrolysis

Autoclave hydrolysis assays were performed using microalgal biomass suspended in a volume of 100 mL to set a concentration of 30 g VSS/L. Two sets of experiments were carried out. In the first set, biomass was suspended in water and samples were autoclaved at 121 °C for 30, 45, 60 and 90 min. A control was performed

suspending biomass in water at 30 °C for 30 min. In the second set, microalgal biomass was suspended in 100 mL of 4% (v/v) H_2SO_4 solution and autoclaved at 121 °C for 30, 45, 60 and 90 min. A control was performed suspending microalgal biomass in 4% H_2SO_4 at 30 °C for 30 min. The autoclave hydrolysis assays were performed according to the autoclave time used by Ho et al. [17] and Miranda et al. [24].

2.2.4. Microwave hydrolysis

Microwave pre-treatment was carried out with microalgal biomass suspended in 100 mL of distilled water to set a final concentration of 200 g VSS/L. Microalgal biomass was processed in a CEM Discover Microwave 908010 (CEM Mathews N.C., USA) for 40 seconds (boiling time) and 150 W, followed by a 10 min ice bath. This procedure was repeated three times on the same sample. The microwaving time was set according to Park et al. [33], who showed that biomass degradation reaches its maximum value after boiling.

2.2.5. Enzymatic hydrolysis

In order to assess the combined effect of acid hydrolysis and subsequent enzymatic hydrolysis on sugar extraction, a set of experiments was carried out. Experiments were performed with biomass suspended in 100 mL of 4%, 7%, and 10% (v/v) H_2SO_4 solutions to reach a concentration of 30 g VSS/L, and samples were autoclaved at 121 °C for 30 min. A control was set using biomass suspended in 0% H_2SO_4 solution. After hydrolysis, the samples were cooled to room temperature and pH was adjusted using NaOH. Then, all samples were enzymatically processed with cellulases or amylases [34,35].

In accordance with Lee et al. [34] works, enzymatic treatment with cellulases were carried out using Celluclast 1.5 L (60 μL per 3 g of total solids (TS)) and Novozyme 188 (30 μL per 3 g of TS) and incubated at 55 °C and pH 4.5 for 72 h. The enzymatic loading used was 15 FPU for Celluclast 1.5 L and 15 IU for Novozyme 188 per g of DW. Hydrolysis with amylases was performed using Liquozyme SC DS (1 μL of heat-stable α-amylase per 3 g of TS) at 90 °C and at pH 6.0 for 6 h. According to Möllers et al. [35], the amylase Spirizyme Fuel (3 μL of heat-stable α-amylase per 3 g of TS) was then added and incubated at 60 °C and at pH 4.5 for 72 h. The enzymatic loading used was 240 α-amylase units and 750 amyloglucosidase units for Liquozyme SC DS and Spirizyme Fuel, respectively. Enzymes were provided by Novozymes (Denmark). The incubation times used in the enzymatic treatments were higher than those specified in the enzymatic protocols so as to ensure the completion of the enzymatic process and the release of the maximum concentration of sugars.

2.3. Analytical procedures

Total solids and VSS in microalgal biomass were determined following APHA Standard Methods [36]. Total carbohydrate content in biomass was determined by the phenol sulphuric acid method [37]. Simple sugars and sugar degradation products (furfural, HMF, propionic acid, acetic acid, formic acid and lactic acid) in hydrolysates were identified and quantified by high pressure liquid chromatography (HPLC) using a Waters system (Massachusetts, USA), equipped with an Aminex HPX-87-H column and a refraction index detector (Waters 2414). The eluent consisted of a 5 mM H_2SO_4 solution, previously filtered through a 0.20 μm membrane filter and degasified at a flow rate of 0.6 mL/min. A volume of 20 μL of sample was injected at 60 °C.

3. Results

As most of the carbohydrates present in microalgae are contained within the cell wall, its disruption and break down is necessary to free monosaccharides and make them available for fermentative microorganisms. The performance of each pre-treatment method was determined by measuring sugar release (SR) as the mass of monosaccharides in hydrolysates, determined by HPLC after each pre-treatment, per g of TS in dry weight (DW) of biomass treated. The monosaccharides determined were maltose, xylose, arabinose, glucose, ramnose, fucose, fructose and galactose.

3.1. Effect of acid hydrolysis

Acid concentration is a major operational parameter which can affect saccharification of microalgal biomass when it is conducted in autoclave, as it was evidenced by Miranda et al. [24] and Harun et al. [31]. As can be seen in Fig. 1, small differences in SR were observed when the concentration of acid raised from 4% to 7% H_2SO_4 concentration for *N. gaditana* and *S. ameriensis*; while an increase close to 3-fold was obtained in *C. sorokiniana*. The highest SR was obtained with 7% H_2SO_4 concentration and values of 84, 93, and 55 mg/g DW for *C. sorokiniana*, *N. gaditana* and *S. almeriensis*, respectively. Specifically, a small increase (<10 mg/g DW) was observed in all microalgae when H_2SO_4 concentration increased from 4% to 7%, but SR values diminished when a 10% H_2SO_4 was applied. These results concur with those reported by Miranda et al. [24], who observed that SR decreased when microalgae *Scenedesmus obliquus* was treated with H_2SO_4 solutions with a concentration above 2 N (5.4% (v/v)). The decrease observed in SR may be attributed to the degradation of monosaccharides into sugar degradation products (such as furfural, HMF, propionic acid, acetic

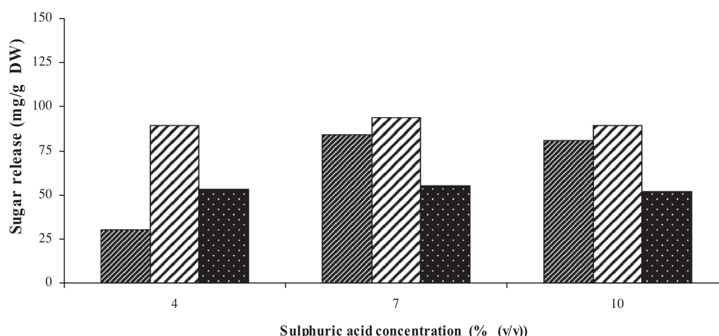

Fig. 1. Effect of acid concentration on hydrolysis of *C. sorokiniana* (▩), *N. gaditana* (▨) and *S. almeriensis* (▦).

acid, formic acid and lactic acid). The accumulation of these harmful compounds occurs as a consequence of hexoses and pentoses degradation when a high hydrolysis is performed affecting to the fermentation process [24]. In this work, due to the slight differences observed in SR when applying a concentration of 4%, 7% and 10% of H_2SO_4 and in order to avoid the production of sugar degradation products, a 4% H_2SO_4 solution was chosen for further experiments with the aim of investigating the effect of combining acid hydrolysis with other pre-treatments on sugar extraction.

3.2. Effect of alkaline hydrolysis

Alkaline pre-treatment causes extensive changes in the structure and accessibility of carbohydrates in the cell wall, allowing sugar release in the aqueous medium [18]. After pre-treatment with 1 M NaOH, SRs were lower than 4 mg/g DW in all microalgae tested (Fig. 2). When NaOH concentration increased to 5 M, sugar extraction raised 5-fold in the case of microalgae *N. gaditana* (14 mg/g DW) and *S. almeriensis* (15 mg/g DW), while SR doubled (8 mg/g DW) for *C. sorokiniana*. Despite this increase, SRs were still low compared to yields obtained when applying acid hydrolysis.

Differences between the performance of chemical hydrolysis (acid and alkaline) might be due to the fact that alkaline hydrolysis cleaves intermolecular linkages between complex polysaccharides, and releases carbohydrate fibbers and other polymeric components to the medium but does not break down complex carbohydrates into simple sugars [38]. However, in acid hydrolysis, long cellulose and hemicellulose chains are broken down into shorter oligomers and monomers that release glucose and other monosaccharides [39] and thus increasing SR. Hence, dilute sulphuric acid hydrolysis using 4% (v/v) H_2SO_4 pre-treatment was more efficient releasing simple sugar from these microalgae than strong alkaline pre-treatment with 5 M NaOH (20% (w/w)).

3.3. Effect of autoclave time

The composition of the hemicellulose backbone and the branching groups from microalgal cell wall determined the efficiency of the thermal pre-treatment. Fig. 3 depicts the effect of autoclave time (30, 45, 60 and 90 min) on the release of monosaccharides from microalgal biomass suspended in distilled water. SR resulted lower than 7 mg/g DW regardless the microalgae tested and the autoclave time. These results concur with those reported by Miranda et al. [24], who obtained SRs below 50 mg equivalent$_{glucose}$/g DW at 120 °C for 30 min using the microalgae *S. obliquus*. The effect of increasing temperature from 30 to 121 °C did not increase SR considerably (Fig. 3). The low SR obtained during thermal pre-treatment (2 bar and 121 °C) with water may be attributed to the composition of the cell wall which probably contains higher

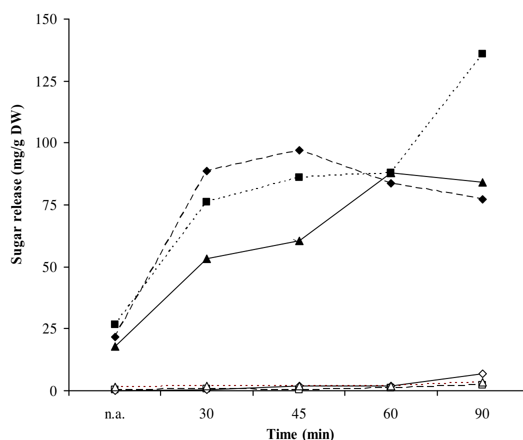

Fig. 3. Effect of autoclave time on sugar release from microalgal biomass suspended in water (open symbols) and in 4% (v/v) H_2SO_4 solution (closed symbols), for *C. sorokiniana* (■), *N. gaditana* (♦) and *S. almeriensis* (▲); n.a.: not autoclaved, corresponding to the pre-treatment at 30 °C for 30 min.

cellulose and hemicellulose and lower starch content. Thus, although this pre-treatment is able to solubilize hemicellulose, it is unable to break it down into monosaccharides [40].

The combination of acid hydrolysis (4% v/v) with autoclave resulted in a higher SR for the three microalgae studied (Fig. 3). The main reaction that occurs during acid pre-treatment at room temperature is the hydrolysis of hemicellulose, but acid pre-treatment alone is unable to solubilize carbohydrates preventing its break down [41]. However, the combination of acid hydrolysis and autoclave results in high monosaccharides as temperature solubilizes carbohydrates increasing its availability for acid. Then, H_2SO_4 breaks carbohydrates into monosaccharides. According to Fig. 3, the effect of acid hydrolysis on saccharification was particularly evident when temperature raised from 30 to 121 °C for 30 min, with SR increases higher than 4-fold in *N. gaditana* and near to 3-fold in *C. sorokiniana* and *S. almeriensis*. The highest SR was observed after 90 min of autoclave (136 mg/g DW) for *C. sorokiniana*, 45 min of autoclave (97 mg/g DW) for *N. gaditana* and 60 min of autoclave (88 mg/g DW) for *S. almeriensis*. Differences between 30–60 min of autoclave were low in *C. sorokiniana* and *N. gaditana* (below 12 mg/g DW), but a remarkable increase in SR (from 53 up to 88 mg/g DW) was obtained in *S. almeriensis*. Fig. 3 points out that after 90 min of autoclave with 4% H_2SO_4 solution, sugars from *N. gaditana* and *S. almeriensis* decreased compared to

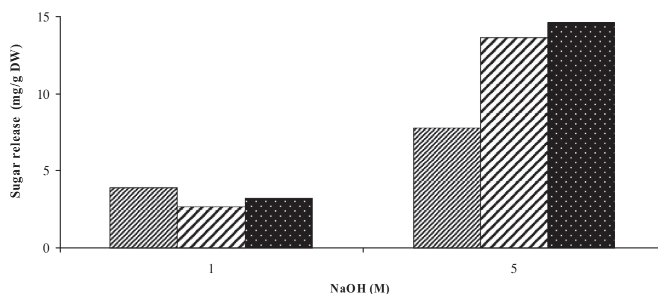

Fig. 2. Effect of NaOH concentration on sugar release for *C. sorokiniana* (▩), *N. gaditana* (▨) and *S. almeriensis* (▦).

a shorter duration. This fact could be due to its degradation into organic acids, reaching concentrations in acetic and formic acid close to 0.2 and 0.1 g/L respectively (data not shown).

3.4. Effect of microwave hydrolysis

Microwaving causes rapid alignment and realignment of dipoles in a polar solvent, resulting in heat generation, which can alter cell wall structures and break down the carbohydrates present in microorganisms [33]. The efficiency of microwave pre-treatment in sugar extraction varied considerably among the three microalgae studied. Values of SRs were 21 mg/g DW for *C. sorokiniana*, 8 mg/g DW for *N. gaditana*, and less than 2 mg/g DW for *S. almeriensis*. These values were as low as those obtained with other pre-treatments tested, i.e. alkaline hydrolysis and autoclaving with water. Budarin et al. [41] found that starch contained in microalgal biomass subjected to microwave required lower pre-treatment time and lower temperatures to be solubilized and broken down, than biomass with high cellulose content. Therefore, slight differences observed in microalgal sugar extraction using microwaving in the present work might be due to different carbohydrate composition of the studied microalgae.

3.5. Effect of enzymatic hydrolysis

Prior to enzymatic hydrolysis with cellulases or amylases, biomass was suspended in sulphuric acid (0%, 4%, 7% and 10%) and autoclaved at 121 °C for 30 min. Cellulases are enzymes that catalyze the hydrolysis of cellulose and hemicellulose breaking down β-bonds, but they are also able to break down less specifically α-bonds from carbohydrates like starch [34]. On the other hand, amylases are enzymes that catalyze the hydrolysis of starch breaking down α-bonds, but they are unable to break down β-bonds [35].

For *C. sorokiniana* suspended in 0% sulphuric acid, SR reached 67 mg/g DW and 101 mg/g DW after treatment with cellulases and with amylases, respectively (Fig. 4). These results were considerably higher than those obtained with other pre-treatments like alkaline, acid hydrolysis and microwaving. Under these experimental conditions, maximum SR obtained in the case of *N. gaditana* (15 mg/g DW) and *S. almeriensis* (26 mg/g DW) were considerably lower.

The application of an acid pre-treatment prior to enzymatic hydrolysis notably increased SR as acid allowed enzymes to access complex carbohydrate chains, increasing its break down (Fig. 4). An uniform trend of the effect of increasing the H_2SO_4 concentration on the SR for the three microalgae studied was not evident. Although results from the acid hydrolysis showed a slight decreased when H_2SO_4 concentration increased from 7% to 10%, due to monosaccharides degradation, an increase in SR was observed when using 10% H_2SO_4 previously to the enzymatic hydrolysis (Fig. 4). The increase observed in these experimental runs may be attributed to the enzymatic effect; which counteracts the monosaccharides degradation in *N. gaditana* and *S. almeriensis*.

Saccharification with cellulases was more efficient for *N. gaditana* (129 mg/g DW) and *S. almeriensis* (62 mg/g DW), while hydrolysis with amylases was more efficient (128 mg/g DW) for *C. sorokiniana*. Differences between the enzymatic hydrolysis with cellulases or amylases in *C. sorokiniana* may be attributed to a higher starch content and lower cellulose and hemicellulose concentration in the biomass, as it was hypothesized by Budarin et al. [41]. Nevertheless, SRs were also considerably high after the enzymatic hydrolysis with cellulases; probably, due to the capacity of these enzymes to break down not only β-bonds but also α-bonds present in starch. Furthermore, these results evidenced

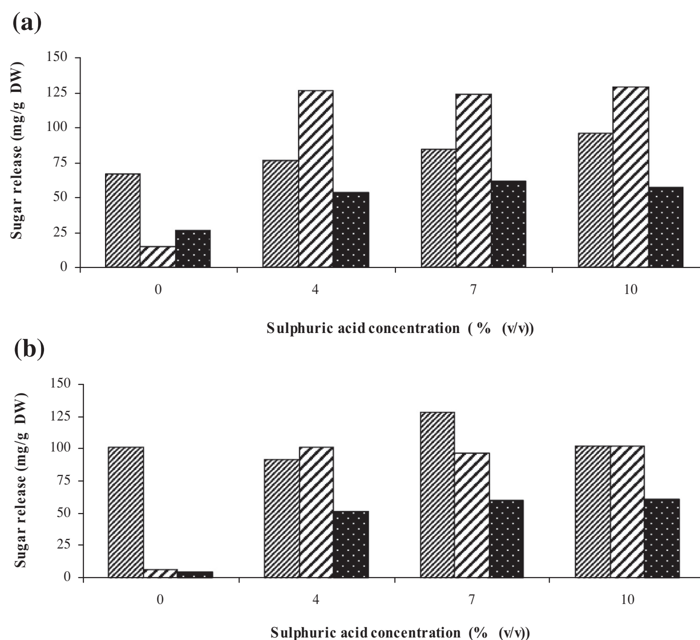

Fig. 4. Effect of (a) cellulases and (b) amylases with and without acid hydrolysis on sugar release in *C. sorokiniana* (▩), *N. gaditana* (▨) and *S. almeriensis* (▦).

Table 1
Sugar composition of microalgal biomass after optimum pre-treatments.

	Microalgae[a]		
	C. sorokiniana	*N. gaditana*	*S. almeriensis*
Total carbohydrate content (%)[b]	18.2	11.2	14.5
Maltose (%)	7.7	n.d	n.d
Glucose (%)	70.8	59.0	52.2
Xylose (%)	13.8	28.8	33.4
Ramnose (%)	6.5	6.5	6.2
Fucose (%)	1.0	4.6	7.5
Other sugars (%)[c]	0.2	1.1	0.7
Others (g/L)			
Acetic acid	0.52	0.17	0.71
Formic acid	0.26	n.d.	n.d.

n.d. Not detected.
[a] *C. sorokiniana* and *N. gaditana* pre-treated with acid hydrolysis and enzymatic hydrolysis. *S. almeriensis* pre-treated with acid hydrolysis for 60 min.
[b] Total carbohydrate content was calculated as follows: (g carbohydrates/g microalgae DW) * 100.
[c] Amount of sugars lower than 1% by mass.

that despite the phylogenetic differences between *N. gaditana* (heterokontophyta) and *S. almeriensis* (chlorophyta) and in their cell wall structure, both microalgae presented a similar response to enzymatic hydrolysis and different to that observed in *C. sorokiniana* (chlorophyta). This might be explained by the similar polysaccharide composition in the cell wall of *N. gaditana* and *S. almeriensis*, which may be rich in cellulose and hemicellulose, whereas the cell wall of *C. sorokiniana* probably contains a higher concentration of more easily degradable polysaccharides.

3.6. Hydrolysates characterization under optimal pre-treatments

To determine total carbohydrate content and composition, the optimal saccharification pre-treatment was performed for the three microalgal species. Total carbohydrate content in hydrolysates accounted for 18%, 11% and 15% for *C. sorokiniana*, *N. gaditana*, and *S. almeriensis*, respectively (Table 1). Glucose was the major monosaccharide, representing over 70% for *C. sorokiniana*, nearly 59% for *N. gaditana*, and 52% for *S. almeriensis*. Xylose was the second main monosaccharide, followed by maltose, rhamnose and fucose that were also present in lower concentrations. These percentages proved similar to those reported by Becker et al. [42] for *Chlorella* sp. and *Scenedesmus* sp., and by González-López et al. [28] for *Nannochloropsis* sp. Finally, the accumulation of sugar degradation products, mainly acetic acid, was considerably lower than 25 mg/g DW (Table 1) in all hydrolysates, thus an inhibition of fermentative microorganisms may not occur [24].

4. Discussion

From all the treatments studied, the combination of acid and enzymatic hydrolysis led to the maximum SR for *C. sorokiniana* and *N. gaditana*. These results showed the importance of applying several consecutive pre-treatments for saccharification of microalgal biomass. In the case of *S. almeriensis*, the highest SR was obtained after long autoclaving time (60 min); thus disruption and breaking down of complex carbohydrate chains were more influenced by autoclaving time than by enzymatic hydrolysis. Results obtained in the present work were in agreement with García-Cuadra et al. [43], who pointed out that each microalgal specie needs a specific pre-treatment taking into account its particular cell wall structure and carbohydrate composition.

The results presented here confirmed that *S. almeriensis* was the most resistant microalgae for sugar extraction, requiring tougher conditions to disrupt and break down its cell wall, as reported by other authors [24,44]. The low SRs obtained (a maximum of 88 mg/g DW) may be attributed not only to a thick cell wall, but also to a high content in cellulose and hemicellulose and low content in starch, as it was evidenced by the higher hydrolysis efficiency of cellulases compared with the efficiency of amylases. In the case of *N. gaditana*, the present work is the first work in carbohydrate saccharification from this microalga. The data obtained showed high SR for some of the pre-treatments tested, although total carbohydrate content was lower than in the other microalgae (Table 1). This microalgal biomass from *N. gaditana* was easier to disrupt and break down compared to other microalgae and most of pre-treatments resulted in high SR. According to enzymatic hydrolysis results, this microalga seemed to be rich in cellulose and hemicellulose with less starch content. Values of SR after enzymatic treatment with amylases on *C. sorokiniana* were high independently on the sulphuric acid hydrolysis; for that reason it can be concluded that this specie had high content in starch, which was coincident with results from Hirano et al. [45], that reported high starch content in *Chlorella vulgaris*.

From these treatments analysis it can be concluded that *C. sorokiniana* was the most suitable microalga to produce bioethanol, since it was easy to break down obtaining higher monosaccharide concentration compared to the rest of microalgae. On the other hand, *S. almeriensis* resulted to be the most inadequate microalga as all pre-treatments tested resulted in a very low monosaccharide concentration, probably due to its particularly thick cell wall.

5. Conclusions

The present study showed the efficiency of combined pre-treatments to disrupt microalgal cell wall and hydrolyze carbohydrate chains into monosaccharides in the bioethanol production process. Acid hydrolysis has proven to be efficient to disrupt the cell wall, allowing enzymes to access carbohydrates and, therefore, increasing enzymes efficiency. Thus, the combined pre-treatment of H_2SO_4 with enzymatic hydrolysis gave the highest SR in *C. sorokiniana* (128 mg/g DW) and *N. gaditana* (129 mg/g DW). In the case of *S. almeriensis*, the highest SR was obtained after acid hydrolysis with H_2SO_4 for 60 min (88 mg/g DW). The enzymatic hydrolysis with amylases of *C. sorokiniana* previously suspended in 0% sulphuric acid released a remarkable monosaccharide concentration (101 mg/g DW). Regarding microwave, autoclave in water, and alkaline hydrolysis, these pre-treatments resulted in considerably lower sugar release for all microalgae tested. The most difficult microalga to hydrolyze was *S. almeriensis* due to its cell wall structure and composition. In all assays carried out, the accumulation of sugar degradation products was considerably lower than the inhibitory concentration for fermentative microorganisms. Future studies will be focused on fermentation of the hydrolysates using the optimal conditions.

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CAPÍTULO 4

BIOFUELS FROM MICROALGAE: LIPID EXTRACTION AND METHANE PRODUCTION FROM THE RESIDUAL BIOMASS IN A BIOREFINERY APPROACH

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Biofuels from microalgae: Lipid extraction and methane production from the residual biomass in a biorefinery approach

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HIGHLIGHTS

- More lipids are extracted by $SCCO_2$ than using conventional extraction methods.
- $SCCO_2$ extracts most of neutral lipids for biodiesel production.
- $SCCO_2$ allows valorisation of the resulting microalgal biomass.
- The highest methane yield was obtained after lipid extraction by $SCCO_2$.
- $SCCO_2$ enhances microalgal biodegradability to increase methane production.

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ABSTRACT

Renewable fuels and energy are of major concern worldwide and new raw materials and processes for its generation are being investigated. Among these raw materials, algae are a promising source of lipids and energy. Thus, in this work four different algae have been used for lipid extraction and biogas generation. Lipids were obtained by supercritical CO_2 extraction ($SCCO_2$), while anaerobic digestion of the lipid-exhausted algae biomass was used for biogas production. The extracted oil composition was analyzed (saturated, monounsaturated and polyunsaturated fatty acids) and quantified. The highest lipid yields were obtained from *Tetraselmis* sp. (11%) and *Scenedesmus almeriensis* (10%), while the highest methane production from the lipid-exhausted algae biomass corresponded to *Tetraselmis* sp. (236 mL CH_4/g VS_{added}).

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1. Introduction

The search for sustainable and renewable fuels is becoming increasingly important as a direct result of climate change and rising fossil-fuel prices (Gravilescu and Chisti, 2005). In this context, liquid biofuels are expected to contribute significantly to diminish greenhouse gas emissions and fossil fuels dependence in a near future. Currently, commercial production of biodiesel involves alkaline-catalyzed transesterification of triglycerides from first generation biofuels, like oleaginous food crops mainly rapeseed in Europe and soybean in the USA (Brennan and Owende, 2010). However, their impacts in transport sector will remain limited due to competition with food and fiber production for the use of arable land, regionally constrained market structures, lack of well managed agricultural practices in emerging economies, high water and fertilizer requirements, and a need for conservation of biodiversity (Chisti, 2007).

Microalgae are considered to be one of the most promising alternative sources for biodiesel (Brennan and Owende, 2010) due to the potential high oil yields that can be obtained from them, which is about 16–70 times the oil that can be obtained from coconut, sunflower and palm (Amin, 2009). Many different species like *Chlamydomonas reinhardtii*, *Botryococcus braunii*, *Chlorella* sp., *Nannochloropsis* sp., among others, may be considered as a suitable source of lipids due to their ability to accumulate over 60% DW (dry weight) of lipids; estimating an annual biodiesel production for *Nannochloropsis* sp. between 23,000 and 34,000 L/ha (Scott et al., 2010). Microalgae are also promising due to their high growth and photosynthetic rates, enabling microalgae to capture carbon faster than terrestrial crops, and to accumulate high percentage of lipids in their biomass (Rodolfi et al., 2008). They can also be cultivated on non-arable lands, in saline water mediums

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and in agroindustrial wastewaters (Riaño et al., 2012). Moreover, they do not need herbicides or pesticides for their growth (Rodolfi et al., 2008).

Different techniques have been used to extract high value compounds from microalgae. The most important methods described in literature to extract lipids from microalgae are microwave assisted extraction, Kochert method, Soxhlet extraction, soxtec extraction, accelerated extraction and ultrasonic extraction (Balasubramanian et al., 2011; Kochert, 1978; Mendes et al., 2006). The main disadvantages of the above mentioned methodologies include high-energy inputs, the requirement of high operational temperatures and the use of organic solvents such as *n*-hexane, methanol–chloroform, that are flammable reagents and present low selectivity. An alternative method to avoid the use of toxic solvents is the use of supercritical carbon dioxide ($SCCO_2$). The $SCCO_2$ extraction technology is well known, and it is considered as a green process (Crampon et al., 2013) since CO_2 is a Generally Recognized As Safe (GRAS) solvent and not flammable. One of the main advantages of $SCCO_2$ is its high selectivity for non polar lipids such as triglycerides. In addition, it does not solubilize phospholipids, which results very useful for biodiesel applications as it avoids degumming operations (Crampon et al., 2013). Furthermore, after depressurization, CO_2 becomes gaseous and is then spontaneously separated from the extracted phase and residue, which are completely free of toxic solvent traces. This enables a direct valorization of both extracts and residues without any additional processing. In this manner, CO_2 can safely be recycled, which represents an economic and environmental benefit. Another advantage is that $SCCO_2$ does not require toxic solvents enabling a subsequent valorization of resulting microalgal biomass, for instance through anaerobic digestion. On the other hand, microalgal lipid extraction by Kochert or Soxhlet method requires toxic solvents as methanol and chloroform, inhibiting anaerobic digestion.

Chisti (2007) evidenced that many different high added value products must be obtained from microalgae (ω-3 and ω-6 fatty acids, pigments, antioxidants, biofuels) to achieve economically feasibility, and therefore it is possible apply the biorefinery concept to the complete exploitation of microalgal biomass. In the present study the concept of total valorization of microalgae to obtain fatty acids (FFA) using $SCCO_2$ and biogas through anaerobic digestion has been considered. The effect of microwave pre-treatment previous to $SCCO_2$ extraction has also been evaluated, as well as lipid extraction by Kochert and Soxhlet methods.

2. Methods

2.1. Microalgae

Microalgal biomass was obtained in lyophilized form from the Food Innovation and Sustainability Center (Almería, Spain). *Isochrysis* T-ISO and *Tetraselmis* sp. were cultured according to Fábregas et al. (1984). *Nannochloropsis gaditana* and *Scenedesmus almeriensis* were cultured according to González-López et al. (2010) and Sánchez et al. (2008), respectively. Lyophilized samples were ground and sieved before the experimental runs, obtaining a particle size distribution lower than 500 μm. The biomass was stored at 4 °C for further use.

2.2. Extraction technologies

2.2.1. Kochert method

Lipids were extracted from the lyophilized biomass using methanol–chloroform 1:2 (v/v) as solvent, following the method proposed by Kochert (1978). Once the extraction was completed, the mass extracted was quantified by gravimetric analysis at 45 °C. Experiments were carried out in duplicate and results were expressed as average values.

2.2.2. Soxhlet method

Solvent extraction was carried out by traditional Soxhlet apparatus using methanol–chloroform 2:1 (v/v) as solvent (Cheung et al., 1998). The extraction temperature was kept at 105 °C for 18 h and the extract was separated from the solvent by a rotatory evaporator (Inlabo Rotatory Evaporator EVI 68 with water bath EVI 90; Padova, Italy) at 41 ± 0.1 °C. Experiments were carried out in duplicate and results were expressed as average values.

2.2.3. Supercritical fluid extraction

Supercritical extraction tests were performed using laboratory scale equipment developed by Solana et al. (2014). The diagram of the process is shown in Fig. 1. The experiment involved several steps. Firstly, the stainless steel extraction cell (16) was filled with 0.5 g of lyophilized microalgae powder. Then, CO_2 was pumped through the extraction cell at a pressure of 30 MPa (controlled by two pressure gauges (6, 14)) and temperature of 45 °C, controlled by a thermo-resistance placed around the extraction cell. Temperature was measured in the internal flow before and after the cell (15, 17). Ethanol was used as co-solvent, pumped by an intelligent pump (Jasco PU-1580) and mixed with CO_2 before the extraction cell. After extraction, the mixture of the solvent, co-solvent and extract was expanded by a valve inserted in a water bath at 40 °C, avoiding CO_2 freezing caused by sudden pressure reduction (18). Extract samples were collected every 15 min in ethanol and they were finally separated from the ethanol by a rotatory evaporator.

The experiments of $SCCO_2$ from *Isochrysis* T-ISO, *N. gaditana*, *S. almeriensis* and *Tetraselmis* sp. were carried out at 30 MPa and 45 ± 2 °C for 90 min, with a constant CO_2 flow rate of 0.4 ± 0.05 kg/h, measured by a flow meter after depressurization. As 5% of ethanol was added as a co-solvent the critical temperature of the mixture increased to 43 °C (Mendes et al., 2006).

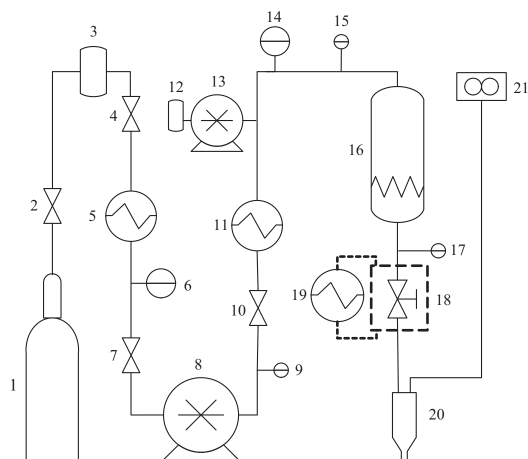

Fig. 1. Schematic diagram of supercritical extraction equipment. 1. CO_2 tank; 2, 4, 7, 10. Valves; 3. CO_2 container; 5. Cooler; 6, 14. Pressure gauges; 8. High pressure pump; 9, 15, 17. Temperature indicators; 11, 19. Heater; 12. Co-solvent container; 13. Co-solvent pump; 16. Extraction cell; 18. Depressurization valve immersed in a water bath; 20. Collector; 21. Flow meter.

2.2.4. Microwave supercritical fluid extraction

Microwave pre-treatment and subsequent supercritical extraction (MSE) were carried out with microalgal biomass suspended in 50 mL of distilled water to set a concentration of 200 g of volatile suspended solids (VSS)/L and then processed in a KOR-612R Daewoo microwave (Seoul, South Korea) at 1.2 kW and 2.45 GHz for 1 (MSE-1) and 5 min (MSE-5). Previous to $SCCO_2$ extraction, microalgae were dried in a D-6450 Heraeus air oven (Hanau, Germany) at 80 °C until constant weight. Then, $SCCO_2$ extraction was performed on pre-treated biomass following the procedure described in Section 2.2.3.

2.3. Anaerobic digestion

Anaerobic biodegradability assays were carried out at 38 ± 0.4 °C for 65 days in 0.57 L bottles. Quantities were calculated to reach a final volume of 0.30 L, allowing a headspace of 0.27 L for gas accumulation. The bottles were closed with a septum and the headspace flushed with N_2 to remove the O_2. The biogas production was measured by the overpressure in the headspace with digestion time (Hernández et al., 2013). Constant agitation and temperature was provided by a shaker at 50 rpm (Gerhardt Thermoshake TH, Königswinter, Germany).

Anaerobic sludge inoculum was collected at the municipal wastewater treatment plant of Valladolid (Spain). Anaerobic sludge presented a total solid (TS) and volatile solid (VS) concentration of 13 and 9 g/L, respectively. Two sets of experiments were performed. In the first set, lipid-exhausted biomass (LEB) obtained after $SCCO_2$ extraction was used to carry out anaerobic digestion batch assays. In the second set, non-extracted microalgae were used as biomass (NLEB) for anaerobic digestion. For the determination of endogenous methane production, blanks containing only anaerobic sludge were also tested. The substrate/inoculum ratio, measured as total chemical oxygen demand ($TCOD_{added}$)/VS ratio was 0.5 (Hernández et al., 2013), which were achieved by keeping a constant inoculum concentration of 3.0 g/L. The methane volumes were corrected by subtracting the average methane volume of the blanks (endogenous production) and were converted to standard temperature and pressure (STP, 0 °C and 760 mmHg). All experiments were carried out in triplicate and the results were expressed as average values.

2.4. Chemicals and analytical procedures

Carbon dioxide (4.0 type, purity greater than 99.9%) used as supercritical solvent was provided by Rivoira Gas (Milan, Italy). Nitrogen premier X10S (purity greater than 99.9%) and H_2 (purity greater than 99.9%) was supplied by Carburos Metálicos S.A. (Barcelona, Spain). Ethanol (⩾99.8%), methanol (⩾99.8%) and chloroform (99%) were purchased from Sigma Aldrich (Milan, Italy).

Total solids, VS, VSS, TCOD and soluble chemical oxygen demand (SCOD) were determined following APHA Standard Methods (Eaton and Franson, 2005). The FFA profile of the extracted microalgal biomass was carried out by gas chromatography, using a GC Agilent Technologies (Model 7890) with a FID detector, equipped with a HP-5MS Agilent column (30 m × 0.25 mm × 0.25 μm). Hydrogen was used as the carrier gas. The method was performed according to procedures reported by Jenkins (2010).

Biogas composition was analyzed using a gas chromatograph (Bruker 430-GC) with a thermal conductivity detector, provided by a CP-Molsieve 5A column (15 m × 0.53 mm × 15 μm) and a CP-Porabond Q column (25 m × 0.53 mm × 10 μm). Columns were obtained from Agilent technologies. Hydrogen was used as the carrier gas. The injection port temperature was set at 150 °C and the detector temperature was 175 °C.

Total volatile fatty acids (VFA) were analyzed at the end of anaerobic biodegradability assays using a gas chromatograph (Agilent 7820A) equipped with a Teknokroma 10% SP1000 capillary column and a flame ionization detector. Nitrogen was used as the carrier gas. The temperature of the injector was set at 375 °C and the oven was set isothermally at 160 °C.

3. Results and discussion

In the following, extraction yields were calculated according to Eq. (1).

$$\text{Extraction yield}(\%) = (A/B) \times 100 \quad (1)$$

where A is the weight of the total extract collected by Soxhlet, Kochert, MSE-1, MSE-5 and $SCCO_2$, determined by gravimetry, and B is the dry weight (DW) of the initial microalgae powder.

3.1. Solvent extraction yields

Table 1 summarizes the results of extraction yield, FFA conversion and lipid yield obtained by Kochert and Soxlet method. As can be seen, differences in the extraction yield were considerable despite both methods used chloroform–methanol as solvent. *Isochrysis* T-ISO, *S. almeriensis* and *Tetraselmis* sp. showed higher extraction yields performing Soxhlet method than Kochert method. These differences may be attributed to a longer extraction time (18 h) and a higher operational temperature (105 °C), compared to those applied in Kochert method that combines grinding with alumina for 5 min and lower temperature (45 °C) for 1 h. As Soxhlet extraction resulted in higher extraction yield compared with Kochert, a more detailed analysis was performed and compared only with Soxhlet and $SCCO_2$ results.

3.2. Supercritical carbon dioxide extraction yields

A set of experiments was carried out using $SCCO_2$ in order to calculate the total extraction yield and to obtain extraction curves from each microalga. Extraction curves at 30 MPa from four microalgae are represented in Fig. 2. They were fitted by using the model proposed by Sovová (2005) initially developed for lipid extraction from plants by $SCCO_2$. It must be noted that, for the application of this model, the extraction yield e is calculated as the mass of extract collected, divided by the weight of the insoluble solid, instead of the total mass of the solid. Therefore the values of e represented in Fig. 2 are slightly higher than the values calculated by the Eq. (1). This model was also used by Mouahid et al. (2013) for describing the extraction curves from the microalgae *Chlorella*, *Cylindrotheca*, *Nannochloropsis* and *Spirulina* genus. As shown in Fig. 2, the kinetics of *Isochrysis* T-ISO, *N. gaditana*, *S. almeriensis* and *Tetraselmis* sp. fitted well with the mathematical model of broken-intact cells proposed by Sovová (2005). According to the experimental results, two extraction periods were identified. During the exponential period, extraction yield e mainly depends on the solute solubility, being the period in which most of FFA were extracted, while the stationary period is governed by internal diffusion in the biological material and the recovery of FFA was minimal. The average absolute relative deviation of the kinetics ranged from 0.77 to 2.62 for all assays carried out.

As shown in Table 1, similar extraction yields were obtained by $SCCO_2$ for all the microalgae considered with values varying between 13% and 15%. The extraction yields obtained when using $SCCO_2$ were lower than those obtained by Soxhlet method in all assays carried out. Specifically, a relevant decrease in extraction yield was obtained from *Isochrysis* T-ISO (from 23% to 15%) and *S. almeriensis* (from 22% to 13%). However, further analysis of FFA

Table 1
Extraction yield, FFA conversion and lipid yield obtained after Kochert, Soxhlet and $SCCO_2$ extraction methods.

Extraction method	Pressure (MPa)	Extraction time (h)	Temperature (°C)	Extraction yield[a] (DW%)	FFA conversion[b] (DW%)	Lipid yield[c] (DW%)
Isochrysis T-ISO						
Kochert	1	1	45	12.7	–	–
Soxhlet	1	18	105	23.1	31.2	7.2
$SCCO_2$	30	1.5	45	14.7	52.0	7.7
MSE-1	30	1.5	45	15.5	61.9	9.3
MSE-5	30	1.5	45	12.6	62.1	7.8
N. gaditana						
Kochert	1	1	45	19.1	–	–
Soxhlet	1	18	105	17.7	36.2	6.4
$SCCO_2$	30	1.5	45	12.9	61.2	7.9
MSE-1	30	1.5	45	11.9	90.8	10.8
MSE-5	30	1.5	45	8.2	84.1	6.9
S. almeriensis						
Kochert	1	1	45	15.7	–	–
Soxhlet	1	18	105	22.4	35.7	8.0
$SCCO_2$	30	1.5	45	13.2	76.5	10.1
MSE-1	30	1.5	45	19.4	–	–
MSE-5	30	1.5	45	4.4	70.5	3.1
Tetraselmis sp.						
Kochert	1	1	45	14.5	–	–
Soxhlet	1	18	105	18.1	38.7	7.0
$SCCO_2$	30	1.5	45	14.8	75.0	11.1
MSE-1	30	1.5	45	4.7	–	–
MSE-5	30	1.5	45	5.2	92.3	4.8

[a] Extraction yield = (mass of microalgae extract/mass of microalgae powder) × 100.
[b] FFA conversion = (mass of FFA extracted/mass of microalga extract) × 100.
[c] Lipid yield = (extraction yield × FFA conversion)/100.

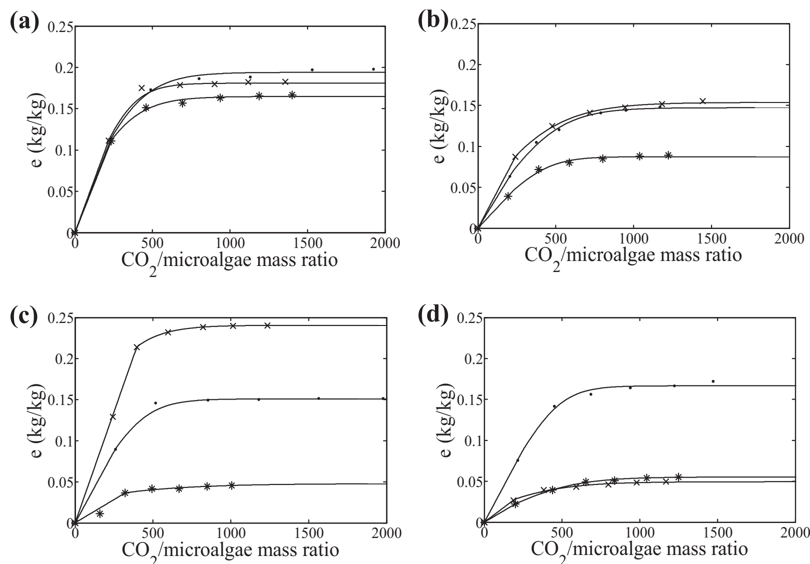

Fig. 2. Supercritical carbon dioxide extraction curves of lipids from *Isochrysis* T-ISO (a), *N. gaditana* (b), *S. almeriensis* (c) and *Tetraselmis* sp. (d). Microalgal biomass without pre-treatment (●), MSE-1 (×) and MSE-5 (*). Each point is an experimental data and curves represent the model adjustment according to Sovová (2005). The curves represent the extraction yield *e* (mass of microalgae extract / mass of insoluble solid) as a function of the CO_2/microalgae mass ratio.

content in the lipid extracted revealed that FFA conversion yields obtained by $SCCO_2$ (varying from 52% to 77%) were considerably higher than those achieved by Soxhlet method (ranging from 31% to 39%), even if the extraction temperature was much higher in the second case. The highest increase was found in *S. almeriensis*, when FFA conversion from $SCCO_2$ extraction was more than 2-folds higher than when using Soxhlet extraction. These differences may be attributed to the fact that with Soxhlet, FFA and other

components like non-polar lipids (phospholipids), pigments and waxes are extracted (Molina-Grima et al., 1994) whereas $SCCO_2$ is a more selective extraction method.

3.3. Effect of microwave pre-treatment on lipid extraction with $SCCO_2$

Microwaving causes a rapid alignment and realignment of dipoles in a polar solvent, resulting in heat generation, which can alter and break down the cell structures (Park et al., 2004), facilitating the access and diffusion of CO_2 through the microalgal cell. A set of experiments were performed over wet microalgae in order to study the influence of microwaving for 1 and 5 min on $SCCO_2$ extraction. After microwave pre-treatment, microalgae were dried in an oven and then lipids and other components were extracted by $SCCO_2$.

Results in Table 1 showed that the effect of microwave pre-treatment on the extraction yield strongly depends on the microalga tested. As illustrated in Fig. 2a, the microwave pre-treatment slightly affected the supercritical extraction from *Isochrysis* T-ISO, since the total yield *e* obtained after 90 min of extraction only decreased from 20% to 17% when a previous microwave pre-treatment for 5 min was applied. In the case of *N. gaditana* (Fig. 2b), practically no differences were found on the yield when a microwave pre-treatment for 1 min was carried out. However, after 5 min of microwave pre-treatment, the extraction yield *e* decreased from 14% to 8%. Probably, these results can be due to high temperatures reached during the microwave process (203 ± 15 °C), which could enhance thermo-oxidative FFA degradation (Vieira and Regitano, 1998) and, consequently, reduce further lipid extraction by $SCCO_2$ (Albi et al., 1997). *S. almeriensis* extraction yield *e* increased considerably (ranging from 15% to 24%) when biomass was microwaved for 1 min compared to $SCCO_2$, as shown in Fig. 2c. Such a result may be attributed to the particular characteristics of this microalga cell wall, which is reported as one of the most resistant (Miranda et al., 2012; Mussgnug et al., 2010). Thus, probably $SCCO_2$ alone was not able to break down the cell wall of *S. almeriensis*, in order to access and extract lipid content and only the microwave pre-treatment was able to break down the cell wall, enhancing lipid release. However, when microwave pre-treatments were performed for 5 min, a considerable decrease on extraction yield *e* was observed, with a 5% yield. The microalgae strain *Tetraselmis* sp. was negatively affected by the microwave regardless pre-treatment duration. Extraction yield *e* achieved without microwave pre-treatment was 17%, whereas only 5% and 6% were obtained after 1 min and 5 min of pre-treatment, respectively (Fig. 2d).

Differences in extraction yield from different microalga species may be attributed to particular characteristics of each microalgae cell wall and its easiness to be broken. Balasubramanian et al. (2011) obtained a remarkable oil yield from *Scenedesmus obliquus* with microwave assisted extraction using a solvent extraction technique, controlling temperature for not to exceed 95 °C. In the present work, lower lipid concentration was extracted by MSE-5 compared to $SCCO_2$.

3.4. Fatty acid composition: oil quality

Further analyses of the fatty acid composition were performed in all microalgae tested after supercritical extraction (Table 2). The main FFA obtained in all microalgae were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1cis), oleic (C18:1 n9c) and linolenic (C18:2) acid. Also, remarkable concentrations of eicosapentaenoic (C20:5) and palmitoleic acid were found in *N. gaditana* and *S. almeriensis*. On the other hand, more than 11% of hexadecatrienoic (C16:4) acid was determined in *Tetraselmis* sp. and 11% of stearidonic (C18:4) acid was obtained in *Isochrysis* T-ISO.

FFA profile of the extracted lipids did not show important differences, although operational temperature between Soxhlet and $SCCO_2$ methods was remarkable different (ranging from 45 °C up to 105 °C). These results were in agreement with those reported by Cheung et al. (1998), who did not find significant differences in FFA extracted by both methodologies from the macroalga *Sargassum hemiphyllum*.

Regarding the FFA composition of the lipids extracted by $SCCO_2$, microalgae presented among 31–41% of saturated acids as shown in Fig. 3. Mass extracted from *S. almeriensis* and *Isochrysis* T-ISO showed the highest percentage of saturated FFA, with values near 41%. Furthermore, *N. gaditana* and *S. almeriensis* presented 30% and 43% of monounsaturated FFA respectively, resulting about 2-fold higher than those obtained in *Isochrysis* T-ISO and *Tetraselmis* sp. Concentration of polyunsaturated FFA was higher in *Isochrysis* T-ISO (41%) and *Tetraselmis* sp. (47%) than in *N. gaditana* (35%) and *S. almeriensis* (15%). The effect of the microwave pre-treatment on the FFA composition was studied on the microalgae strains *Isochrysis* T-ISO and *N. gaditana*. Table 2 shows that a higher proportion of FFA was extracted when the microwave pre-treatment was applied, increasing from 79 to 108 mg/g for *Isochrysis* T-ISO and from 96 to 108 mg/g for *N. gaditana*. It is also noteworthy the increase of polyunsaturated fatty acids when a previous microwave pre-treatment was applied in the microalgae strain *Isochrysis* T-ISO. Results from Table 2 show that monounsaturated and polyunsaturated FFA extracted using MSE-5 were considerably lower than those extracted using $SCCO_2$ alone. These results evidenced that each microalga has a different FFA profile, being useful for different applications depending on the concentration of saturated, monounsaturated and polyunsaturated FFA.

Fig. 3 shows FFA composition of the oil extracted by $SCCO_2$, which is important to determine the most suitable microalgae for biodiesel production. According to Puhan et al. (2010), the synthesis of biodiesel fuel with oils containing more saturated and monounsaturated FFA results in higher cetane number, lower hydrocarbon emissions, lower nitrogen monoxide, smoke and carbon monoxide emissions, and shorter ignition delay; although it has also higher viscosity and lower density. In the present work, and according to these authors' conclusions, the most suitable microalga for biodiesel production was *S. almeriensis* due to its high content of saturated (42 mg oil/g microalgae DW) and monounsaturated (44 mg oil/g microalgae DW) FFA and relative low (16 mg oil/g microalgae DW) polyunsaturated FFA content (Fig. 3). *N. gaditana* was also interesting since it contained a similar concentration of saturated and monounsaturated FFA, but it also had near to 2-fold polyunsaturated FFA (27 mg oil/g microalgae DW) in comparison to *S. almeriensis*.

3.5. Anaerobic digestion of microalgal biomass

3.5.1. Biogas production and methane yields

Methane yields were calculated according to Eq. (2).

$$\text{Methane yield} = \text{corrected methane volume}/VS_{added} \quad (2)$$

Fig. 4 shows the accumulated methane production at a substrate/inoculum ratio of 0.5 g TCOD/g VS_{added} for the four microalgae studied with and without $SCCO_2$ lipid extraction. According to this figure, lipid exhausted biomass (LEB) presented higher methane yield than non lipid exhausted biomass (NLEB), with 236 mL CH_4/g VS_{added} for *Tetraselmis* sp. and 203 mL CH_4/g VS_{added} for *S. almeriensis*, respectively. These data were in the range of previous results obtained by Zamalloa et al. (2012) who accomplished methane yields of 360 and 240 mL CH_4/g VS_{added} by digesting *Phaeodactylum tricornutum* and *S. obliquus* biomass without lipid extraction.

According to Sialve et al. (2009), lipids are the microalgal component which result in higher methane yield (1.014 L CH_4/g VS_{added}), compared to proteins (0.851 L CH_4/g VS_{added}) and carbohydrates

Table 2
Composition of fatty acids (mg FFA/g DW) extracted from microalgae by different techniques.

Fatty acids	*Isochrysis* T-ISO				*N. gaditana*				*S. almeriensis*				*Tetraselmis sp.*			
	Soxhlet	$SCCO_2$	MSE-1	MSE-5	Soxhlet	$SCCO_2$	MSE-1	MSE-5	Soxhlet	$SCCO_2$	MSE-1	MSE-5	Soxhlet	$SCCO_2$	MSE-1	MSE-5
Saturated																
C 14:0	14.1	13.6	14.6	13.1	4.0	4.0	6.5	13.4	6.3	9.4	–	2.4	3.0	3.6	–	1.1
C 16:0	10.2	14.4	13.4	11.6	16.5	16.7	23.8	11.8	22.6	25.0	–	6.7	16.8	25.2	–	9.9
C 18:0	n.d.	2.6	n.d.	n.d.	n.d.	0.7	1.5	n.d.	n.d.	2.0	–	0.4	n.d.	0.8	–	n.d.
Others[a]	1.9	0.5	3.3	3.3	2.2	6.4	3.0	2.9	5.4	5.1	–	2.2	2.0	3.9	–	1.6
Monounsaturated																
C 16:1 n9 ω-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.9	n.d.	n.d.	n.d.	–	0.5	2.5	3.9	–	1.4
C 16:1cis ω-7	5.0	4.6	5.1	5.4	17.6	18.7	28.0	5.5	26.6	34.5	–	7.5	5.6	8.9	–	3.0
C 18:1 n9c ω-9	11.0	8.0	8.7	7.5	5.2	3.0	4.5	7.7	3.7	4.7	–	1.6	3.2	8.1	–	2.9
C 18:1 n7c ω-9	0.8	n.d.	1.0	0.9	0.5	n.d.	n.d.	0.9	n.d.	n.d.	–	n.d.	0.8	n.d.	–	0.5
Others[a]	1.7	0.7	1.6	1.6	1.8	2.0	3.1	2.9	3.3	4.7	–	1.0	1.8	4.2	–	1.0
Polyunsaturated																
C 16:2 ω-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	0.3	2.4	3.0	–	1.3
C 16:3 ω-3	0.8	n.d.	2.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	0.4	3.3	4.2	–	2.6
C 16:4 ω-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	0.8	7.4	11.7	–	5.5
C 18:2 (LA) ω-6	3.7	3.8	5.1	4.0	1.7	2.5	3.9	3.0	2.6	2.9	–	1.3	4.4	6.8	–	3.1
C 18:3 (ALA) ω-3	5.0	5.4	9.9	5.4	n.d.	3.5	7.6	4.0	n.d.	n.d.	–	1.9	11.0	17.4	–	9.9
C 18:4 ω-3	11.2	11.4	15.3	12.4	7.2	7.3	11.7	9.3	n.d.	n.d.	–	0.4	1.7	3.1	–	1.2
C 18:5 ω-3	1.4	1.5	2.1	1.2	0.8	0.9	1.6	0.9	n.d.	n.d.	–	n.d.	n.d.	n.d.	–	n.d.
C 20:4 ω-6	n.d.	n.d.	n.d.	n.d.	1.1	1.6	1.8	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–	n.d.
C 20:5 (EPA) ω-3	n.d.	n.d.	n.d.	0.8	3.0	5.8	4.7	0.8	5.9	7.9	–	2.2	1.1	2.1	–	1.0
C 22:6 (DHA) ω-3	2.5	2.9	4.9	3.7	n.d.	5.2	n.d.	1.0	n.d.	n.d.	–	n.d.	n.d.	n.d.	–	n.d.
Others[a]	2.3	6.0	5.1	5.1	2.3	0.5	5.2	4.9	3.4	4.9	–	1.5	2.8	4.2	–	1.6

n.d.: not detected.
[a] Amount of fatty acids lower than 1% by mass.

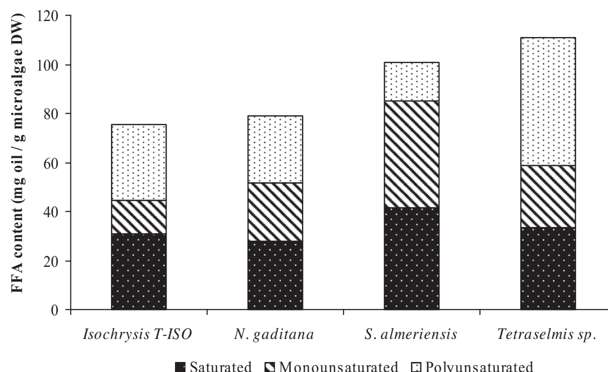

Fig. 3. Composition of saturated, monounsaturated and polyunsaturated FFA extracted from microalgae by $SCCO_2$.

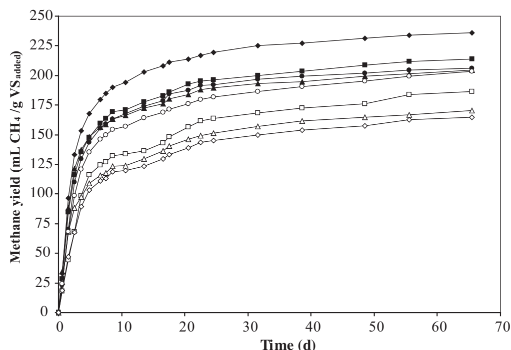

Fig. 4. Accumulated CH_4 production in *Isochrysis* T-ISO (■), *N. gaditana* (▲), *S. almeriensis* (●) and *Tetraselmis* sp. (♦). Closed symbols correspond to lipids-exhausted biomass (LEB). Open symbols correspond to biomass that has not been used for lipid extraction (NLEB).

Table 3
Initial and final pH, NH_4^+–N and VS in anaerobic digestion tests of lipid exhausted biomass (LEB) and non lipid exhausted biomass (NLEB).

	pH		NH_4^+-N (mg/L)		VS (mg/L)	
	Initial	Final	Initial	Final	Initial	Final
LEB						
Isochrysis T-ISO	7.22	7.21	236	441	5.65	4.27
N. gaditana	7.41	7.48	207	385	3.97	3.29
S. almeriensis	7.38	7.35	222	417	6.45	4.49
Tetraselmis sp.	6.99	7.44	210	428	6.12	4.03
NLEB						
Isochrysis T-ISO	7.35	7.61	228	392	4.65	3.64
N. gaditana	7.36	7.42	210	361	4.15	3.70
S. almeriensis	7.42	7.20	222	398	5.48	4.08
Tetraselmis sp.	7.12	7.18	233	402	8.06	6.11

(0.415 L CH_4/g VS_{added}). However, higher methane yield was obtained in LEB compared to NLEB for all microalgae tested despite of in NLEB lipids were extracted by $SCCO_2$ and its content was close to zero and content in proteins and carbohydrates were equal. Moreover, TCOD removal from LEB ranged between 43% and 59%, while in NLEB TCOD removal ranged between 31% and 42%. Also, LEB resulted in higher VS removal efficiency than in NLEB in all microalgae tested. Volatile solid removal efficiency ranged between 17% and 34% in LEB, while it varied between 11% and 26% in NLEB. The highest VS removal in LEB was achieved by *Tetraselmis* sp. (34%) and by *S. almeriensis* (26%) in NLEB. These results may be attributed to $SCCO_2$ extraction could break down not only microalgal cell wall but also proteins into aminoacids and carbohydrates into monosaccharides, being more available for anaerobic bacteria and thus increasing CH_4 production. However, unbroken cells in NLEB may hinder access to lipids, proteins and carbohydrates by anaerobic bacteria resulting in lower CH_4 production. Thus, after anaerobic digestion, microalgal biomass could not be completely digested resulting in lower methane production. $SCCO_2$ is a suitable methodology not only to extract lipids from microalgae but also to increase biodegradability.

3.5.2. Process stability

Table 3 shows initial and final values of pH, NH_4^+–N and VS concentration before and after anaerobic digestion. All final pH values ranged from 7.2 to 7.4, being compatible with normal anaerobic microorganism growth. Also, high ammonia ratios may influence in biogas production, affecting to acetate-utilizing methanogenic *Archaea*, hydrogen-utilizing methanogens and syntrophic bacteria (Zeng et al., 2010). The highest concentration of ammonia was reached by *Isochrysis* T-ISO (441 mg NH_4^+–N/L), which is above inhibitory threshold levels (Stams et al., 2003). Finally, no volatile fatty acids were found after anaerobic digestion, indicating that the process finished in all treatments. Thus, these results evidenced that $SCCO_2$ enhanced biodegradability of microalgal biomass, resulting in higher methane production in LEB than in NLEB.

3.6. Preliminary economic considerations

A preliminary economic evaluation of the $SCCO_2$ extraction and anaerobic digestion technologies applied to algal biomass is presented in this section, based only on the operational costs and incomes associated to these processes. The aim of this qualitative analysis was to estimate the benefits of carrying out the anaerobic digestion of the residual biomass obtained from the $SCCO_2$ extraction. The microalga *Tetraselmis* sp. was selected as feedstock in this analysis, since it presented the highest FFA extraction yield by $SCCO_2$, and also the highest methane production during anaerobic digestion. It has to be emphasized that the cost calculations are only approximate, and a rigorous economic analysis should consider, among others, microalgae production costs, initial investment, prices at plant scale, maintenance and labor costs.

For $SCCO_2$ extraction, operational costs include energy costs and reagents required. Considering an electrical consumption of

400 kWh/t and a price of 0.133 €/kWh (Fiori, 2010), the operational costs of the electricity energy to process 100 t/year from *Tetraselmis* sp. were estimated around 50 €/t, what was always referred to the dry algal biomass. Regarding the reagents, their average prices were 0.20 €/kg CO_2 (Fiori, 2010) and 0.22 €/kg ethanol 96% (v/v) (Montañés et al., 2012). A percentage of 2% of the total amount of CO_2 and ethanol was considered to be lost during the process. Therefore, around 2 €/t of CO_2 and 6 €/t of ethanol were necessary to perform the $SCCO_2$ extraction. To calculate the incomes, it was assumed a sale price of the oil extracted by $SCCO_2$ of 0.39 €/L, the same proposed by Chisti (2007) for the crude palm oil, as no literature data of microalgae oil extracted by $SCCO_2$ was found. Hence, the profits from this process were expected to be around 40 €/t.

The estimation of operational costs for anaerobic digestion was performed according to Zamalloa et al. (2011), considering a combined heat and power cogeneration system with a 40% electric energy conversion efficiency and a 45% thermal energy conversion efficiency. The operational costs of the anaerobic digestion of the lipid exhausted biomass, taking into account these literature data and the experimental data presented in this paper, would be around 65 €/t and 1 €/t from thermal and electricity energy, respectively. The profits obtained from biogas production were estimated to be 115 €/t for thermal energy and 102 €/t for electricity energy. Hence, the operational costs of the complete process ($SCCO_2$ + anaerobic digestion of the lipid exhausted biomass), were around 125 €/t and the profits were about 260 €/t for the non lipid exhausted biomass, the operational costs of the anaerobic digestion process were estimated to be 80 €/t, whereas the incomes were around 180 €/t, obtaining a benefit of 100 €/t.

From this qualitatively analysis it can be concluded that the extraction of FFA from *Tetraselmis* sp. and further anaerobic digestion of the residual biomass could provide an economic benefit of 35 €/t higher than the direct anaerobic digestion of this microalga without previous lipid extraction.

Similar benefits were estimated for the application of these processes when using *Isochrysis* T-ISO and *N. gaditana* as feedstocks. However, in the case of *S. almeriensis* the highest revenue was obtained by direct anaerobic digestion. Nevertheless, the FFA composition of *S. almeriensis* oil would allow producing a higher quality biodiesel compared to the other microalgae extracted oil, and, therefore, it can be expected a higher sale price of the oil extracted. This fact would enhance economic benefit of the $SCCO_2$ technology in this particular case.

4. Conclusions

$SCCO_2$ showed to be the most efficient methodology to extract FFA from microalgae for biodiesel production compared to Soxhlet and Kochert method. According to lipid yield and oil composition, *S. almeriensis* resulted to be the most suitable microalga for biodiesel production, extracting 101 mg FFA/g. Regarding energy recovery, anaerobic digestion evidenced that lipid exhausted biomass presented higher CH_4 yield than non lipid exhausted biomass for all microalgae. Thus, $SCCO_2$ has shown to be a suitable methodology to extract lipids and also to increase microalgal biodegradability for further anaerobic digestion, increasing economic viability of the process.

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CAPÍTULO 5

TREATMENT OF AGRO-INDUSTRIAL WASTEWATER USING MICROALGAE-BACTERIA CONSORTIUM COMBINED WITH ANAEROBIC DIGESTION OF THE PRODUCED BIOMASS

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Treatment of agro-industrial wastewater using microalgae–bacteria consortium combined with anaerobic digestion of the produced biomass

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HIGHLIGHTS

- ▶ Two agroindustrial wastewaters are used as culture medium for microalgae production.
- ▶ Phosphorous limitation triggers lipid accumulation in produced biomass.
- ▶ Anaerobic batch experiments are carried out using the produced biomass.
- ▶ Lower TCOD/VS ratio (0.5) promotes higher methane yield.
- ▶ The higher lipid content (30%), the higher methane yield.

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ABSTRACT

Two combined processes were studied in order to produce second generation biofuels: microalgae biomass production and its further use to produce biogas. Two 5 L photobioreactors for treating wastewater from a potato processing industry (from now on RPP) and from a treated liquid fraction of pig manure (from now on RTE) were inoculated with *Chlorella sorokiniana* and aerobic bacteria at 24 ± 2.7 °C and 6000 lux for 12 h per day of light supply. The maximum biomass growth was obtained for RTE wastewater, with 26.30 mg dry weight $L^{-1} d^{-1}$. Regarding macromolecular composition of collected biomass, lipid concentration reached 30.20% in RPP and 4.30% in RTE. Anaerobic digestion results showed that methane yield was highly influenced by substrate/inoculum ratio and by lipids concentration of the biomass, with a maximum methane yield of 518 mL CH_4 g COD^{-1}_{added} using biomass with a lipid content of 30% and a substrate/inoculum ratio of 0.5.

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1. Introduction

The potential of microalgae as an alternative to biofuels is currently subjected to strong research (Sialve et al., 2009). In fact, algae have a high number of potential advantages compared to higher plants: (1) it is estimated that the production of algae is ten-fold higher than those of higher plants; (2) algae growth is independent from arable lands, attenuating food and feed competition (Rittman, 2008; Stephens et al., 2010); (3) algae biomass is rich in lipids, proteins and starch, which could be converted into energy using thermochemical processes, biochemical processes and esterification of fatty acids to produce biodiesel (Markou and Georgakakis, 2011). Products obtained through these processes can be considered "first generation" biofuels. However, the use of the resulting biomass to obtain "second generation" biofuels such as methane, is the optimal strategy from the energetic and economic point of view (IEA, 2010).

The first study on energetic recovery from algal biomass was published by Golueke et al. (1957), in which the energetic recovery was carried out throughout anaerobic digestion of the biomass. This research effort was reintroduced in the 1970s and 1980s due to the first global energy crisis, and nowadays there is a renewed interest on anaerobic digestion motivated again by the actual fuel crisis and the ability to treat and to convert a wide range of organic wastes into renewable energy, including microalgae biomass.

However, microalgae production requires high amount of nutrients, for which environmental and economic impact may not be suitable (Halleux et al., 2008; Sialve et al., 2009). One alternative to synthetic culture media is to use wastewaters, especially those derived from agro-industrial facilities which usually present high nutrient concentration (Markou and Georgakakis, 2011). In this sense, microalgal–bacterial systems for agro-industrial wastewater treatment have been gaining special attention in last years. In

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these systems, microalgae produce oxygen during photosynthesis that is used by bacteria metabolism whereas bacteria release CO_2 needed for microalgae growth. Microalgal–bacterial systems for wastewater treatment avoid the external oxygen supplementation compared to conventional processes, allow nutrients recovery into biomass and reduce CO_2 emissions to the atmosphere by its microalgae use contributing to CO_2 mitigation (Molinuevo-Salces et al., 2010). Therefore, the re-use of this kind of substrates can improve the feasibility to produce microalgae biomass for its further valorization, like anaerobic digestion (González-Fernández et al., 2011).

The aim of the study herein was to evaluate the integrated system of combining a microalgal–bacterial system for wastewater treatment with anaerobic digestion of the produced biomass. For this purpose, two agro-industrial wastewaters (treated liquid fraction of pig manure and potato processing wastewater) were selected for feeding separately two photobioreactors. The selection of these wastewaters was based on the different phosphorous concentration. The performance of the photobioreactors was evaluated in terms of organic matter and nutrient removal efficiency together with biomass production and biochemical composition. Additionally, anaerobic batch experiments were carried out using the produced biomass in both photobioreactors. Finally, the influence of the substrate/inoculum ratio was determined in terms of methane yield.

2. Methods

2.1. Photobioreactors and culture conditions

2.1.1. Microalgae–bacteria inoculum

Chlorella sorokiniana was obtained from the culture collection of the University of Goettingen (Goettingen, Germany). Microalgae inoculum was prepared according to Guieysse et al. (2002). The average temperature was 24 ± 2.7 °C. Before inoculation, microalgae were centrifuged (4000 rpm; Centrifuge 5810R, Eppendorf) for 20 min and resuspended in distilled water. The aerobic sludge was obtained from an activated sludge reactor of the municipal wastewater treatment plant of Valladolid (Spain).

2.1.2. Substrate composition

Treated liquid fraction of pig manure (TE) was collected from a pig manure treatment plant located in Cuellar (Segovia, Spain). Treatment consisted of a solid-liquid separation (with addition of coagulants and flocculants) and a treatment of the liquid fraction by nitrification–denitrification. Potato processing wastewater (PP) was obtained from a potato industry located in Medina del Campo (Valladolid, Spain). Wastewaters were homogenized mechanically and stored at 4 °C for further use. Chemical characterization of TE and PP is shown in Table 1.

Table 1
Characterization of treated piggery effluents (TE) and potato processing wastewater (PP). Standard deviation is shown in brackets.

	TE	PP
pH	7.5 (0.3)	5.8 (0.2)
TS ($mg\ L^{-1}$)	3319 (147.9)	1603 (388.2)
VS ($mg\ L^{-1}$)	1031 (96.5)	903 (320.2)
TCOD ($mg\ L^{-1}$)	616 (44.8)	1536 (529.1)
SCOD ($mg\ L^{-1}$)	465 (38.5)	745 (227.2)
BOD_5 ($mg\ L^{-1}$)	63.0 (18.3)	917 (166.9)
TKN ($mg\ L^{-1}$)	32.9 (8.0)	33.7 (10.1)
NH_4^+–N ($mg\ L^{-1}$)	12.3 (1.7)	12.1 (1.7)
NO_3^-–N ($mg\ L^{-1}$)	53.8 (6.1)	n.d.
NO_2^-–N ($mg\ L^{-1}$)	131.7 (5.7)	n.d.
TP ($mg\ L^{-1}$)	50.1 (9.0)	4.2 (0.01)
SP ($mg\ L^{-1}$)	47.5 (4.4)	3.4 (0.6)

n.d.: not detected.

2.1.3. Experimental set-up

The experimental set-up consisted in two photobioreactors, which are open to the atmosphere for a treated liquid fraction of pig manure (RTE) and for a wastewater from a potato processing industry (RPP), with a total working volume of 5 L (17 cm wide, 30 cm long, 10 cm high). Each photobioreactor was illuminated using four fluorescent lamps at 6000 lux (Philips 50 W) for 12 h per day. The lighting of the photobioreactors also provided heating for the cultivation medium. The average temperature was 24.1 ± 2.7 °C. The cultures were gently agitated with magnetic stirrers at 60 rpm. The volume was daily checked and the water lost due to evaporation was corrected by adding distilled water (lower than 4% of culture broth volume). Dissolved oxygen (DO), pH and temperature were monitored *in situ*.

Both photobiorreactors were initially filled with distilled water and inoculated with 25 and 12 mg volatile suspended solids (VSS) L^{-1} of microalgae *C. sorokiniana* and aerobic sludge, respectively. Right after inoculation, the photobiorreactors were fed with TE (photobioreactor RTE) and PP (photobioreactor RPP). The hydraulic retention time (HRT) was 10 days, corresponding to an ammonium loading rate (ALR) of 1.2 mg NH_4^+–N $L^{-1}\,day^{-1}$ for both photobioreactors.

Culture broths were collected separately in two settlers for biomass sedimentation. Samples of the influent and effluent from the top of the settlers were collected periodically in order to determine total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), total solids (TS), volatile solids (VS), total suspended solids (TSS), VSS, total Kjeldahl nitrogen (TKN), soluble phosphorus (SP), ammonia (NH_4^+–N), nitrites (NO_2^-–N) and nitrates (NO_3^-–N). In addition, TS and VS were monitored periodically in culture broths. Biomass was purged from the bottom of the settlers at the end of the experimental time. These purges were analysed for TS, VS and TKN determination. Moreover, a fraction of collected biomass was lyophilized (Lyoquest 85 Plus Eco, Spain) for lipid content determination.

2.2. Anaerobic biodegradability experimental set-up

Anaerobic biodegradability assays were carried out at 37 ± 0.4 °C for 50 days in 0.57 L bottles. Quantities were calculated to reach a final volume of 0.25 L, allowing a headspace of 0.32 L for gas accumulation. The bottles were closed with a septum and the headspace flushed with N_2 to remove the O_2. The biogas production was measured by the overpressure in the headspace with time frequency (Colleran et al., 1992). Constant agitation was provided by a shaker at 200 rpm.

Anaerobic sludge inoculum was collected at the municipal wastewater treatment plant of Valladolid (Spain). Anaerobic sludge presented a TS and VS concentration of 20.0 and 10.2 $g\,L^{-1}$, respectively. For these assays, microalgal–bacterial biomass collected from the bottom of the two settlers described in Section 2.1.3 at the end of the experimental period was used. Specifically, biomass was concentrated by centrifugation (8000 rpm; Beckman Coulter, Avanti centrifuge J-30I) for 10 min. For the determination of endogenous methane production, blanks containing only anaerobic sludge were also tested.

The influence of the substrate/inoculum ratio (from now on TCOD/VS ratio) was evaluated according to González-Fernández and García-Encina (2009). TCOD/VS ratios ranged between 0.5 and 2.0, which were achieved by keeping a constant inoculum concentration of 2.2 $g\,L^{-1}$. Treatments T1 to T4 were performed using microalgal biomass produced in photobioreactor RTE, whereas treatments T5–T8 were performed using biomass produced in pho-

tobioreactor RPP. All experiments were carried out in duplicate and the results were expressed as means.

2.3. Analytical procedures

TS, VS, TSS, VSS, TCOD, SCOD, biological oxygen demand (BOD_5) TKN and SP were analysed in duplicates according to APHA Standard Methods (2005). NH_4^+–N, NO_2^-–N and NO_3^-–N concentrations were determined using electrodes, Orion 900/200 (Thermo Electron Corporation, Beverly, USA). DO, pH and temperature in the reactors were determined using a multi-probe system model YSI 556 MPS (YSI incorporated, USA).

Lipids were extracted from the lyophilized biomass with chloroform–methanol following the method proposed by Kochert (1978). Once the extraction was done, the lipids were quantified by gravimetric analysis. Proteins were calculated by multiplying the TKN by 5.95 (González-López et al., 2010). Carbohydrates were estimated by subtracting the percentage of ashes, lipids and proteins out of 100% (Wilkie and Mulbry, 2002).

Microalgae identification was carried out by microscopical examination (OLYMPUS IX70, USA) of culture broth samples fixed with formaldehyde at 0.5% and stored at 4 °C prior to analysis, according to Phytoplankton Manual (Sournia, 1978).

Biogas composition was analyzed using a gas chromatograph (Bruker 430-Gc) with a thermal conductivity detector, provided by a CP-Molvsieve5A column (15 m × 0.53 mm × 15 μm) followed by a CP-Porabond Q column (25 m × 0.53 mm × 10 μm). Hydrogen (13.6 $mL\,min^{-1}$) was used as carrier gas. The injection port temperature was set at 150 °C and the detector temperature was 175 °C.

Total volatile fatty acids (VFA) were analyzed at the end of the assays using a gas chromatograph (Agilent 7820A) equipped with a Teknokroma 10% SP1000 capillary column and a flame ionization detector. Carrier gases were nitrogen, hydrogen and air and the temperature of the injector was 375 °C. The temperature of the oven was set at 160 °C.

3. Results and discussion

3.1. Photobioreactor performance

3.1.1. Organic matter removal

The organic loading rates (OLR) applied to photobioreactors during the whole experimental time were 0.06 and 0.15 g TCOD $L^{-1}\,day^{-1}$ for RTE and RPP, respectively. The highest COD removal efficiencies were observed when treating potato processing wastewater. More specifically, TCOD removal was 62.3% for RTE and 84.8% for RPP (Table 2). SCOD removal efficiencies accounted for 58.1% and 86.1% for RTE and RPP, respectively. As stated by other authors, higher COD removal efficiency observed in photobioreactor RPP could be attributed to the higher influent strength (Molinuevo-Salces et al., 2010; Wang et al., 2012). Additionally, BOD_5/TCOD ratio in PP wastewater (0.59) was higher than in TE (0.10) and therefore, organic matter was more degradable. As shown in Fig. 1a, DO was never limited in reactor RTE. Therefore, the remaining COD was acting as recalcitrant for this system. COD removal efficiencies observed in reactor RTE were slightly lower than those reported by De Godos et al. (2009) when treating piggery wastewaters (76%). These better COD removal behaviour could be probably promoted by higher pig manure biodegradability. In the case of photobioreactor RPP, DO decreased during the experimental time up to values below 1 mg O_2 L^{-1} at day 23 (Fig. 1a), which indicated that microalgae limited the system and higher COD removal efficiencies could be achieved with a proper consortia development.

Table 2
COD removal, ammonium removal, ammonium removed by stripping, nitrification, denitrification and soluble phosphorous removal in the two photobioreactors. Standard deviation is shown in brackets.

	RTE%	RPP%
Removed TCOD	62.3 (2.0)	84.8 (3.2)
Removed SCOD	58.1 (4.5)	86.1 (2.6)
Removed NH_4^+–N	82.7 (3.0)	>95
Removed by stripping	25.4 (8.0)	2.9 (1.3)
Removed by nitrification	75.7 (11.8)	–
Removed by denitrification	53.8 (10.1)	–
Removed SP	58.0 (7.5)	80.7 (12.3)

3.1.2. Nutrient removal

ALR was similar in both photobioreactors (1.2 mg NH_4^+–N $L^{-1}\,d^{-1}$). Ammonium was removed up to 82.7% in RTE and it was almost exhausted in RPP (Table 2). These high removal efficiencies were expected since ALR was low in comparison with those applied in other studies (Sialve et al., 2009; Xin et al., 2010; Wang et al., 2012). In order to quantify NH_4^+–N stripping, the free ammonia concentration was calculated according to Hansen et al. (1998). The results indicated that ammonia volatilization was not the main mechanism for ammonium removal since ammonia stripping accounted for 25% in RTE and for 3% in RPP (Table 2). This difference was attributed to the higher pH achieved in reactor RTE (8.8) compared to RPP (8.0), as a consequence of the higher SCOD removals in photobioreactor RPP (Fig. 1b), and therefore to higher bacterial activity in this reactor. Therefore, biomass nitrogen assimilation was determined by the daily TKN increase, being higher in RPP (0.25 mg TKN g^{-1} TS d^{-1}) than in RTE (0.08 mg TKN g^{-1} TS d^{-1}).

Photobioreactor RTE showed a nitrite removal efficiency higher than 75%. Even when ammonia stripping and assimilation were indicated as the main mechanisms for nitrogen removal in open reactors (Molinuevo-Salces et al., 2010), the present study demonstrates that denitrification also occurs when pH ranges between 8.0 and 8.8. In this manner, denitrification accounted for 53.8% in RTE. It should be noted that denitrification requires low DO in the medium and RTE presented DO concentration up to 4 mg $O_2\,L^{-1}$ (Fig. 1a). Nevertheless, DO could be much lower in the flocks formed during the treatment (De Kreuk et al., 2005; Molinuevo-Salces et al., 2010). Additionally, even when nitrate is not the preferred nitrogen form for microalgae uptake (Travieso et al., 2006), this process could also have contributed to the nitrogen removal from the culture broth. In photobioreactor RPP, NO_2^-–N or NO_3^-–N was not detected during the whole experimental time, which indicated that nitrification process did not occur.

As shown in Table 2, SP removal efficiency was 58.0% and 80.7% in RTE and RPP, respectively. These results were similar to those from Wang et al. (2012) who obtained a SP removal efficiency of 60.6% when treating diluted piggery wastewater with similar COD concentration using the microalgae *Chlorella pyrenoidosa*. These differences in phosphorous removal could be attributed to the higher SP loading rate applied in RTE (4.75 mg $L^{-1}\,d^{-1}$) than in RPP (0.34 mg $L^{-1}\,d^{-1}$). SP removals achieved in present study were high compared with those reported by De Godos et al. (2009), who obtained efficiencies of 10% working with high rate algal ponds. In this sense, it should be noticed that high pH achieved in both reactors may be involved in PO_4^{3-} precipitation (Nurdogan and Oswald, 1995).

3.1.3. Biomass productivity and biochemical profile

Biomass growth was measured as the dry weight (total solids) of produced biomass per day and litre of the photobioreactor. RTE produced 26.3 mg dry weight (DW) $L^{-1}\,d^{-1}$ while RPP produced 18.8 mg DW $L^{-1}\,d^{-1}$. Hence, the higher phosphorous availability in RTE resulted in higher biomass growth. These results

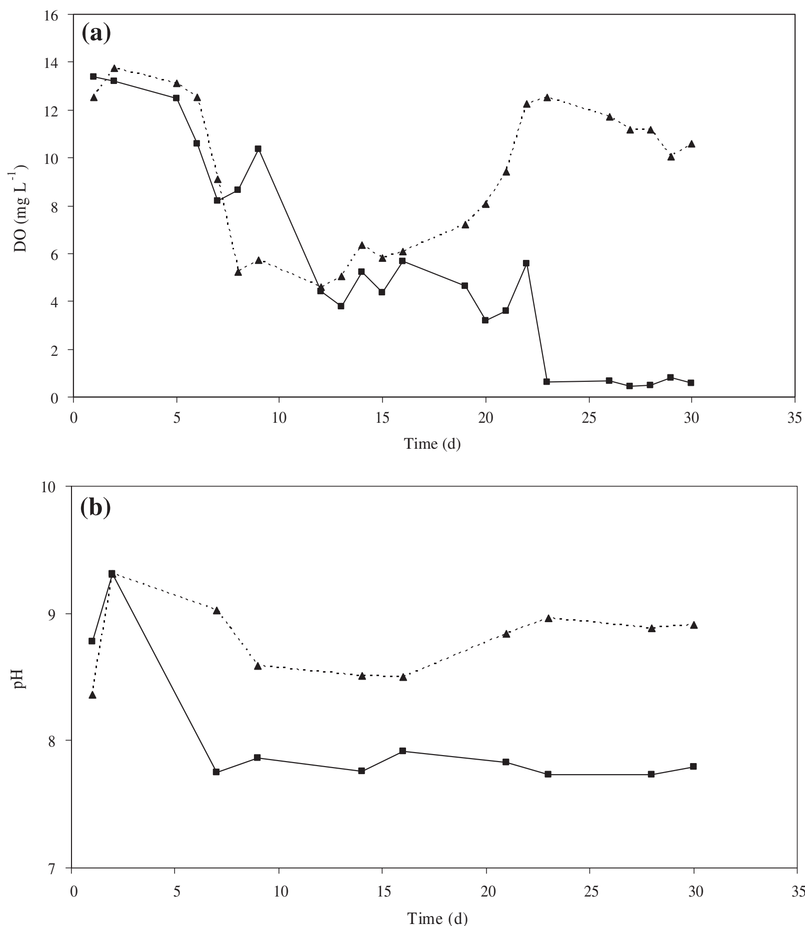

Fig. 1. (a) Dissolved oxygen and (b) pH measured *in situ* in photobioreactor RTE (discontinuous line) and RPP (continuous line).

were in agreement with those reported by Riaño et al. (2011), who observed an increase in biomass productivity concomitantly with increasing nitrogen and phosphorous loading rate when treating fish processing wastewater in open ponds.

The carbon and nutrients contained in wastewater were assimilated by the biomass and converted to organic macromolecules.

There is a great interest in microalgal biomass as a renewable energy source due to their availability to accumulate substantial quantities of lipids. In the present study, significant differences were found in lipids content of biomass produced in photobioreactor RTE (4.3%) and RPP (30.2%). Nutrient limitation is an efficient trigger to increase lipid content per algal biomass (Xin et al., 2010; Wang et al., 2012). In this context, it should be stressed that although this experiment was conducted under similar ALR in both reactors, SP concentration was almost 14-folds higher in TE wastewater than in PP wastewater (Table 1). This fact may cause higher lipid accumulation in biomass produced in RPP. The results herein were in agreement with those reported by Goldberg and Cohen (2006), who observed that under the condition of phosphorous limitation, the total cellular lipid content increased from 6.5% up to 39.3%.

Nitrogen is directly implicated in aminoacid synthesis and hence in protein formation (Sialve et al., 2009). Percentage of proteins was 8.6% for photobioreactor RTE and 26.6% for RPP. These differences could be attributed to higher ammonia volatilization in photobioreactor RTE and, therefore lower ammonia assimilation by biomass in this reactor. These results were in agreement with those reported by Piorreck et al. (1984), who obtained that increasing ammonia concentrations from 30 to 1000 mg L^{-1} NH_4Cl, led to

Table 3
Methane yields and total solid removal efficiency obtained after anaerobic process of biomass produced in RTE (T1–T4) and RPP (T5–T8).

	Substrate/inoculum ratio (g TCOD/g VS)	% TS removal	Methane yield (mL CH_4 g $COD^{-1}{}_{added}$)
T1	0.5	12.24 (0.37)	329.8
T2	1.0	18.36 (2.92)	207.2
T3	1.5	17.17 (2.08)	172.0
T4	2.0	21.33 (1.80)	200.5
T5	0.5	15.81 (5.09)	517.5
T6	1.0	23.32 (1.48)	408.2
T7	1.5	24.14 (1.34)	460.1
T8	2.0	24.97 (1.82)	404.6

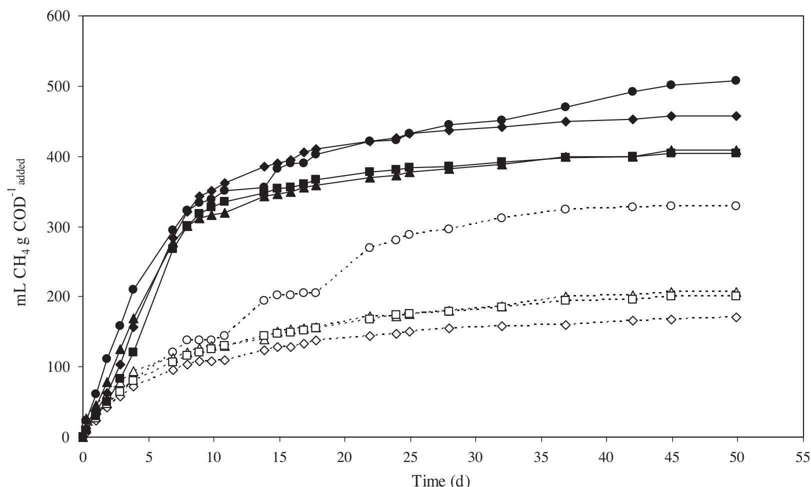

Fig. 2. Net methane yields in the evaluated treatments for RTE (discontinuous line) and for RPP (continuous line) at substrate/inoculum ratio of 0.5 (●), 1.0 (▲), 1.5 (◇) and 2.0 (■) g TCOD/g VS.

a significant protein content reduction (from 8% up to 54% of proteins) in different microalgae species.

In this study, carbohydrates were the main cellular component obtained for both photobioreactors. Percentage of carbohydrates was found to be 2-fold higher in biomass produced in RTE than in RPP (data not shown), as a consequence of the lower content in lipids and proteins. Therefore, it was detected that the characteristics of wastewater presented a high influence on macromolecular composition of microalgal–bacterial biomass produced. Specifically, the use of a substrate with low phosphorous concentration was elucidated as an important factor affecting percentage of lipids in biomass, and therefore, it could determine the further valoration of this added-value product.

3.2. Overall anaerobic biodegradability performance

3.2.1. Biogas production and methane yields

Anaerobic experiments lasted for 50 days. Table 3 shows the accumulated biogas production at substrate/inoculum ratio from 0.5 to 2.0 g TCOD/g VS when using biomass produced in photobioreactor RTE (T1–T4) and RPP (T5–T8). The methane volumes were corrected by subtracting the mean methane volume of the blanks (endogenous production) and were converted to standard temperature and pressure (STP, 0 °C and 760 mm Hg). Methane yields were calculated by dividing the corrected methane volume by TCOD added to each digester. As seen in Table 3 and Fig. 2, the highest methane yields were achieved by the treatments T1 and T5 corresponding to a substrate/inoculum ratio of 0.5 g TCOD/g VS ratio. The rest of treatments showed a similar methane production, varying between 172.0 and 207.2 mL CH_4 g $COD^{-1}{}_{added}$ in the case of the digestion of the biomass produced in photobioreactor RTE and between 404.6 and 460.1 mL CH_4 g $COD^{-1}{}_{added}$ for biomass from RPP. According to González-Fernández and García-Encina (2009), high COD/VS ratios were responsible for methane production delay due to the accumulation of VFA. Therefore, the substrate/inoculum ratio can be an essential parameter to influence the methane yield in the batch anaerobic digestion of microalgal–bacterial biomass. The same conclusion was obtained by previous research using different substrates (Raposo et al., 2006, 2008; Foster-Carneiro et al., 2008).

Biochemical composition of microalgal-biomass also determined methane yield obtained. Specifically, methane yield increased between 157% and 268% in the case of the biomass from RPP in comparison to biomass obtained from RTE (Fig. 2). This fact could be explained by the lower lipid content of biomass from RTE than from RPP as stated before. Regarding this, several authors reported that lipids showed a higher biogas production potential compared with proteins and carbohydrates (Cirne et al., 2007; Li et al., 2002). The results herein demonstrated that an increase in lipid content of digested, also increases the potential methane yield. However, an excess of the percentage of lipids in biomass could lead to VFA accumulation, causing the inhibition of the anaerobic process (Park and Li, 2012).

The percentage of methane in biogas varied between 71.9% and 76.1% for biomass from RTE and between 76.5% and 77.0% for biomass from RPP (data not shown). These results revealed a good conversion of the microalgal–bacterial biomass into methane. High methane content in anaerobic digester implies a steady balance of methane and carbon dioxide, which are products of methanogenesis and acetogenesis, respectively (Park and Li, 2012). The values obtained in present study were similar to those reported by Sialve et al. (2009).

3.2.2. Solid removal efficiency

TS removal efficiency improved with the increase of substrate/inoculum ratio (Table 3). For biomass produced in RTE, TS removal

Table 4
pH and NH_4^+–N during anaerobic process of biomass produced in RTE (T1–T4) and RPP (T5–T8).

	pH		NH_4^+–N ($mg L^{-1}$)	
	Initial	Final	Initial	Final
T0	8.0	7.7	153.0 (1.4)	162.5 (2.9)
T1	7.9	7.8	168.0 (1.4)	190.0 (1.5)
T2	8.0	7.7	161.5 (0.7)	198.0 (2.6)
T3	8.0	7.8	175.0 (0.0)	236.0 (0.5)
T4	8.1	7.6	166.5 (0.7)	241.0 (3.0)
T5	7.6	7.6	155.0 (0.0)	167.0 (1.6)
T6	7.6	7.4	165.0 (0.0)	181.5 (1.0)
T7	7.5	7.3	160.5 (0.7)	210.8 (5.1)
T8	7.4	7.4	172.5 (0.7)	229.8 (1.7)

efficiency increased from 12.2% to 21.3% when substrate/inoculum ratio increased from 0.5 to 2.0. In the case of biomass produced in reactor RPP, TS removal efficiency increased from 15.8% to 25.0% when substrate/inoculum ratio increased from 0.5 to 2.0. These findings were in accordance with previous results obtained by González-Fernández et al. (2011), who accomplished TS removal efficiencies of 14.7–32.9% by co-digesting algal biomass with swine manure.

3.2.3. Process stability

As seen from Table 4, all final pH values ranged from 7.3 to 7.8. These values were compatible with the normal growth of anaerobic microorganisms. Ammonia could mainly influence the anaerobic digestion by affecting acetate-utilizing methanogenic *Archaea*, hydrogen-utilizing methanogens and syntrophic bacteria (Zeng et al., 2010). The inhibitory concentrations of ammonia are reported to be between 1.7 and 5 g $NH_4^+{-}N\,L^{-1}$ (Stams et al., 2003). From Table 4, initial and final ammonia concentrations are too low to inhibit anaerobic digestion. Finally, many authors have observed that VFA are one of the most important parameter for the accurate control of anaerobic digestion, having a direct relation with the digester performance (Zeng et al., 2010). In the present study, no VFA were detected in the samples after digestion, which indicated that the anaerobic digestion process was complete in all treatments.

4. Conclusions

Microalgae–bacteria consortia presented high organic matter and nutrient removal efficiencies in agro-industrial wastewater treatment. Low phosphorous concentration in wastewater led to an increase in the lipid content of produced biomass. Moreover, batch anaerobic digestion assays indicated that methane yield was determined by lipid content and by substrate/inoculum ratio. The highest methane yield (518 mL CH_4 g $COD^{-1}{}_{added}$) was obtained using biomass with a lipid percentage of 30% and a substrate/inoculum ratio of 0.5.

In conclusion, the selection of a suitable agro-industrial wastewater for microalgae growth, attending to the nutrient concentration, could determine biomass macromolecular composition and, therefore, its potential valorization for biofuel production.

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CAPÍTULO 6

MICROALGAE CULTIVATION IN HIGH RATE ALGAL PONDS USING SLAUGHTHERHOUSE WASTEWATER FOR BIOFUEL APPLICATIONS

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MICROALGAE CULTIVATION IN HIGH RATE ALGAL PONDS USING SLAUGHTERHOUSE WASTEWATER FOR BIOFUEL APPLICATIONS

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Abstract

The performance of two 75-L high rate algal ponds (HRAPs) treating slaughterhouse wastewater was evaluated for 115 days with the aim of growing microalgae to produce biofuels (biodiesel, methane). One HRAP was placed indoors under controlled conditions of temperature (25 ± 2 ºC) and light supply, while the other was placed in a greenhouse under 20 ± 6º C and 9 fold higher light supply. The hydraulic retention time (HRT) was decreased from 15 to 10 days. High removal efficiencies were achieved in HRAP placed indoors (92 and 71%) and placed in the greenhouse (86 and 91%) for total chemical oxygen demand and soluble phosphorous, respectively. The maximum biomass production obtained was 12.7 g volatile suspended solids (VSS)/m^2day. High quality fatty acids (FFA) were extracted by supercritical carbon dioxide, obtaining 142 mg FFA/g biomass. The highest CH_4 productions were obtained from lipid exhausted biomass corresponding to greenhouse HRAP at a HRT of 10 days (195 mL CH_4 /g VSS_{added}).

Keywords: Microalgae, Slaughterhouse Wastewater, Lipid Extraction, Supercritical Carbon Dioxide, Anaerobic Digestion.

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1. Introduction

The use of microalgal-bacterial systems for agro-industrial wastewater treatment has shown to be an efficient alternative to conventional treatment plants. In these systems, microalgae provide oxygen during photosynthesis that is used by bacteria metabolisms, whereas bacteria release CO_2 needed for microalgae growth. Thus, microalgal-bacterial systems avoid external oxygen supplementation, decreasing energy costs and allow the recovery of nutrients and CO_2 as a valorizable biomass [1-4].

Reports on the use of microalgal-based systems for agro-industrial wastewater treatment are mainly focused on the influence of operating conditions, such as temperature, light supply and hydraulic retention time, on organic matter and nutrient removal efficiency [1,5-7]. However, photobioreactor efficiencies should be assessed taking into account the biomass chemical profile in order to valorize the resulting biomass by the extraction of high-added value products such as biofuels.

Different techniques have shown to be efficient to extract lipids from microalgae [8-10], but most of them require high-energy inputs, high operational temperatures or the use of toxic solvents for lipid extraction. Recent studies evidenced that supercritical carbon dioxide extraction ($SCCO_2$) have a several advantages in comparison with other techniques. These advantages are: 1) $SCCO_2$ is a suitable technique to extract fatty acids (FFA) from microalgae; 2) it does not require toxic solvents; 3) it has high selectivity for non polar lipids such as triglycerides and; 4) it is recognized as a green process [11-12]. Besides, in comparison with other techniques, $SCCO_2$ avoids further degumming operations as it does not solubilise non-polar lipids (phospholipids) and waxes from microalgal biomass [13]. The absence of toxic solvents allows further valorization of lipid exhausted biomass (LEB) by fermentation into bioethanol, by anaerobic digestion to produce biogas or by the extraction of high-added value components [11]. In addition, the direct conversion of microalgal biomass into bioethanol or methane is often limited by the high resistance of the microalgal cell wall [14-15]; thus the application of a previous pre-treatment may enhance further biofuel production, increasing economic benefit from microalgae [16]. The use of microalgae for anaerobic digestion has been studied for the last 50 years since initial works developed by Golueke et al. [17]. In recent years, due to the need to substitute fossil fuels, anaerobic digestion of microalgal biomass has achieved increasing interest [6,18-19]. Preliminary studies of anaerobic digestion carried out on microalgae have shown that despite the high biochemical methane potential, productivity was considerably low, as a result of the strong microalgal cell walls that hinder the bacterial attack. In this context, a pre-treatment of microalgal biomass may enhance biodegradability and thus increase methane production through extraction of FFA by $SCCO_2$, as previously reported by Ehimen et al. [19] and Hernández et al. [16].

Slaughterhouse wastewater (SW) represents one of the most abundant industrial wastewater; it is estimated that European slaughterhouse industry produced 145 millions m^3 per year of wastewater [20] that needs to be treated previously to be discharged into rivers or municipal wastewater networks. The use of an alternative

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treatment for this wastewater by microalgal-based systems can provide advantages, mainly depuration, recovery of nutrients and exploitation of the produced biomass.

The research goal of the work herein is to valorize the nutrients from SW in the form of microalgal biomass, for further valorization of the biomass (extraction of lipids and production of biogas). To achieve this, an integrated system based on the biorefinery concept was evaluated. Two high rate algal ponds (HRAPs) production systems were compared, one operating under greenhouse conditions of temperature and light supply and the other placed indoors under controlled conditions. The performance of wastewater treatment in both production systems were evaluated in terms of organic matter and nutrient removal, biomass productivity and composition of the biomass produced. Then, lipids were extracted by $SCCO_2$ and, with the extracted biomass, a determination of monosaccharides and an anaerobic biodegradation study to produce biogas were carried out. Finally, a preliminary economic evaluation was performed.

2. Materials and methods

2.1. Experimental set-up

Slaughterhouse wastewater was collected from a pig slaughterhouse located in Segovia (Spain). This wastewater was generated during washing operations after sacrifice and butchering of porks. The wastewater was mechanically homogenized and subsequently stored at 4 ºC for further use. Prior to experimentation, SW was diluted 3 times using tap water to feed the HRAPs. The main characteristics of raw slaughterhouse wastewater were: TS = 753 ± 18 mg/L, TCOD = 1621 ± 81 mg/L, NH_4^+-N = 9.2 ± 0.5 mg/L, TP = 1.4 ± 0.1 mg/L. Other characteristics are shown in Table 2.

The experimental set-up consisted in two identical open-pond raceway HRAPs (from now on R1 and R2) which were constructed in flexible PVC. Both HRAPs presented the same raceway configuration and the same dimensions: total working volume of 75 L with a surface of 0.43 m^2 (1.25 m long, 0.6 m wide and 0.18 m deep). Culture mixing was achieved by a six bladed paddle-wheel driven by a motor operated at 15 rpm (Kelvin K2000), which supported a liquid velocity of 31 cm/s. R1 was operated indoors and illuminated 12 h using eight fluorescent lamps at 4500 ± 150 lux (Phillips Master TLD, 18W) located 20 cm over the surface. The average temperature in R1 was 25 ± 2 ºC. R2 was operated in a greenhouse located in the Technological Agricultural Institute of Castilla y León (41.07º N, 4.71º W). The average lighting during the day was 40000 ± 7500 lux for a period of 13 h. The average temperature was 20 ± 6 ºC, but temperatures reached during the day were close to 30 ºC (with a maximum temperature of 37 ºC) and during the night were close to 15 ºC. The volume of the raceway ponds was checked daily and water lost due to evaporation was replenished with tap water. Dissolved oxygen (DO), pH and temperature in the ponds were monitored *in situ* using a multi-probe system model YSI 556 MPS (YSI Incorporated, USA).

The HRAPs were initially filled with tap water and inoculated with 25 and 12 mg

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volatile suspended solids (VSS)/L of microalgae and aerobic sludge, respectively. Right after inoculation, the HRPAs were fed with diluted SW at an HRT of 15 days (period I) and 10 days (period II). The operational conditions were not changed until steady-state performance was reached. In this manner, the change took place after 75 days for a HRT of 15 days and 40 days for a HRT of 10 days.

Effluent sedimentation was carried out in a 30 L settler located at the outlet of each pond. Influent and effluent samples from the top of the settler were collected periodically in order to determine total solids (TS), volatile solids (VS), total suspended solids (TSS), VSS, total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), total Kjeldahl nitrogen (TKN), soluble phosphorous (SP), ammonium (NH_4^+–N), nitrites (NO_2^-–N) and nitrates (NO_3^-–N). Biomass was withdrawn from the bottom of the settlers at the end of each period. This purge was analyzed for TS, VS and TKN determination. Then, biomass was lyophilized (Lyoquest 85 Plus Eco, Spain), ground and sieved before carrying out the experimental runs for FFA extraction, obtaining a particle size distribution lower than 500 μm. The biomass was stored at 4 ºC for further use. The whole biomass produced during each experimental period was used for biochemical characterization, lipid extraction and anaerobic digestion.

2.2. Microorganisms

Microalgae used to inoculate R1 and R2 were collected from a storage lagoon containing aerobically treated swine manure located in Segovia (Spain). The inoculum was composed by a mix of microalgae, whose population structure was *Chlamydomonas subcaudata*, *Anabaena* sp. and *Nitzschia* sp. (Table 1). Microalgae identification and quantification were carried out by microscopic examination (Olympus IX70, USA) of culture broth samples fixed with formaldehyde at 0.5% and stored at 4 ºC prior to analysis. Quantification was performed following the Phytoplankton Manual [21]. The aerobic sludge was obtained from an activated sludge reactor of the municipal wastewater treatment plant of Valladolid (Spain). Prior to inoculation, microalgae and bacteria were centrifuged (4,000 rpm; Centrifuge 5010R, Eppendorf) for 20 min and resuspended in distilled water.

2.3. Supercritical fluid extraction

Supercritical carbon dioxide extraction ($SCCO_2$) tests were performed using a laboratory scale equipment developed by Solana et al. [13]. The schematic diagram and the process were described in a previous work [16]. Two sets of experiments were carried out: in the first set, FFA were extracted from biomass R1 for period I and II; in the second set, FFA were extracted from biomass from R2 for period I and II. For each test, 0.5 g of lyophilized biomass powder was placed inside the extraction cell. Ethanol was used as co-solvent and mixed with CO_2 before the extraction cell. As 5% of ethanol was added as a co-solvent, the critical temperature of the mixture increased to 43 ºC [8]. The pressure was set at 30 MPa and the temperature was fixed at 45 ± 2 °C for an extraction time of 90 min. Extract samples were collected every 15 min in ethanol. A constant CO_2 flow rate of 0.4 ± 0.05 kg/h, measured by a flow meter after

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depressurization, was maintained. The extracts were finally separated from ethanol by a rotatory evaporator.

2.4. Anaerobic digestion

Anaerobic biodegradability assays were performed at 38 ± 1 ºC for 42 days in 0.57 L bottles. Quantities were calculated to reach a final volume of 0.30 L, allowing a headspace of 0.27 L for gas accumulation. The bottles were closed with a septum and the headspace flushed with N_2 for 5 min to remove O_2. The biogas production was measured by the overpressure in the headspace with time frequency [22]. Constant agitation and temperature was provided by a shaker at 50 rpm (Gerhardt Termoshake TH, Königswinter, Germany).

Anaerobic sludge presented a TS and VS concentration of 14 and 13 g/L, respectively. The anaerobic biodegradability of the biomass was studied in lipid-exhausted biomass (LEB) obtained after $SCCO_2$ extraction and in non lipid exhausted biomass (NLEB). For the determination of endogenous methane production, blanks containing only anaerobic sludge were also tested. The substrate/inoculum ratio measured as $TCOD_{added}$/VS ratio was 0.5; this ratio was selected according to Hernández et al. [23] who used ratios from 2 to 0.5 obtaining the highest biogas production using 0.5. This ratio was achieved by keeping a constant inoculum concentration of 3.0 g/L. The methane volumes were corrected by subtracting the average methane volume of the blanks (endogenous production) and were converted to standard temperature and pressure values (STP, 0º C and 760 mmHg). All experiments were carried out in duplicate and the results were expressed as average values.

2.5. Chemicals and analytical procedures

Carbon dioxide (4.0 type, purity greater than 99.9%) used as supercritical solvent was provided by Rivoira Gas (Milan, Italy). Nitrogen premier X10S (purity greater than 99.9%) and H_2 (purity greater than 99.9%) was supplied by Carburos Metálicos S.A. (Barcelona, Spain). Ethanol (≥ 99.8%), methanol (≥ 99.8%) and chloroform (99%) were purchased from Sigma Aldrich (Milan, Italy).

Total solids, VS, TSS, VSS, TCOD, SCOD, TKN, SP, NH_4^+–N and alkalinity were analysed in duplicates following APHA Standard Methods [24]. The partial pressure of dissolved CO_2 (DCO_2) was measured by the determination of the alkalinity and pH according to Moore et al. [25]. NO_2^-–N and NO_3^-–N concentrations were determined using a Continuous Flow Autoanalyzer equipment (Bran and Luebbe, Analytical AA3, Norderstedt, Germany). The FFA profile of the extracted biomass was carried out by gas chromatography, using a GC Agilent Technologies (Model 7890) with a FID detector, equipped with a HP-5MS Agilent column (30 m x 0.25 mm x 0.25 µm). Hydrogen was used as the carrier gas. The method was performed according to procedures reported by Jenkins [26]. The concentration of carbohydrates in biomass was determined by the phenol sulphuric acid method [27]. Monosaccharides were identified

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and quantified by high pressure liquid chromatography (HPLC) using a Waters system (Massachusetts, USA), equipped with an Aminex HPX-87-P column and a refraction index detector (Waters 2414). The eluent consisted of Mili Q water, previously filtered through a 0.20 μm membrane filter and degasified at a flow rate of 0.6 mL/min at 80 ºC. A volume of 20 μL of sample was injected.

Biogas composition was analyzed using a gas chromatograph (Bruker 430-GC) with a thermal conductivity detector, provided by a CP-Molsieve 5A column (15 m x 0.53 mm x 15 μm) and a CP-Porabond Q column (25 m x 0.53 mm x 10 μm). Columns were obtained from Agilent technologies. Hydrogen was used as the carrier gas. The injection port temperature was set at 150 ºC and the detector temperature was 175 ºC. Total volatile fatty acids (VFA) were analyzed at the end of the assays using a gas chromatograph (Agilent 7820A) equipped with a Teknokroma 10% SP1000 capillary column and a flame ionization detector. Carrier gases were nitrogen, hydrogen and air and the temperature of the injector was 375 °C. The temperature of the oven was set at 160 °C.

3. Results and discussion

3.1. HRAPs performance

The characteristics of SW used to feed HRAPs during this experiment are shown in Table 2. The organic loading rate (OLR) applied to both photobioreactors during the period I and II were 36 and 54 mg TCOD/L per day, respectively. As it is shown in Table 2, the highest TCOD removal efficiencies were observed in R1-PII (92%) and in R2-PII (86%). Furthermore, SCOD removal efficiencies accounted for 90% for R1-PII and 84% for R2-PII (Table 2). The higher removal efficiencies observed during period II for both HRAPs may be attributed to a higher biomass production and thus higher COD removal. The results from Table 2 evidenced a higher COD removal efficiency in R1 compared to R2 during both periods. These slight differences may be attributed to a different growing behaviour between HRAPs as a consequence of low light supply in R1. According to Mahapatra et al. [28], although autotrophic microalgae do not use organic matter as a source of carbon, in some stress conditions as a deficit of light supply, microalgae may turn into mixotrophic. In the present experiment, R1 probably had a mixotrophic behaviour and thus, removing higher COD than in R2, which mainly behaved as autotrophic microalgae. This hypothesis could explain that despite R1 produced less biomass it removed more COD than R2. Although microalgae from R1-PII have a mixotrophic behaviour and microalgae from R2-PII have an autotrophic behaviour, a higher dissolved oxygen is not observed in R2-PII as could be expected (Fig. 1a). This result may be attributed to the higher temperatures reached in R2 during the noon (close to 30 ºC) and the subsequent stripping of the dissolved gases (CO_2 and O_2) to the atmosphere.

Microalgae require nitrogen as an essential constituent of amino acids, nucleic acids, pigments such as chlorophyll and other biomolecules. Dissolved NH_4^+-N is the most biologically accessible form of inorganic N as it is already in the chemical form

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available for microorganisms, and it does not require reduction reactions for its assimilation. Besides, the microalgae-bacteria consortium is able to transform the nitrogen from proteins into ammonium form, that then it is assimilated by microalgae in their biomass or nitrified by bacteria to NO_3^-–N. The ammonium loading rate (ALR) during PI and PII were 0.2 and 0.3 mg NH_4^+–N/L per day, respectively. In the case of R1, ammonium was removed up to 80 and 73 % while in R2 it was removed up to 71 and 79% during PI and PII, respectively (Table 2). The ammonium removal efficiencies obtained in the work herein were very similar to those obtained by Hernández et al. [23] applying an ALR of 1.2 mg NH_4^+–N/L per day when using microalgae-bacteria consortium for agro-industrial wastewater treatment from a potato processing industry and from a treated liquid fraction of pig manure.

Due to the uptake of inorganic carbon by autotrophic organisms, pH of the medium increased reaching values close to 8.2 and 8.5 for R1 and R2, respectively. This fact combined with high temperatures reached in outdoors systems during summer contributes to enhance ammonia stripping. Abiotic ammonia loss was quantified theoretically according to Hansen et al. [29]. The results from Table 2 evidenced that volatilization of ammonia was not the main mechanism for ammonium removal, since the average ammonia stripping accounted for 1.0 ± 0.1% in R1 and 3.9 ± 0.2% and R2. The low ammonia stripped was a consequence of the pH achieved in both HRAPs during the whole experimental time, ranging between 7 and 8.5, as can be seen in Fig. 1a. Also, DO was higher than 1 mg/L, avoiding denitrification processes (Fig. 1b). Hence, although during the whole experimental set up the concentrations of NH_4^+–N and NO_3^-–N were low, these low values may be attributed to most of nitrogen was rapidly assimilated by the microalgal-bacterial consortium. The assimilation of NH_4^+–N and NO_3^-–N in biomass may be corroborated by the daily TKN increase in the biomass, which accounted for 1.2 and 0.9 mg TKN/g TS per day in R1 and R2, respectively (data not shown). Therefore, the present study demonstrated that the biomass assimilation was the main mechanism for ammonium and nitrate removal, as it was previously reported by Posadas et al. [4], who treated fish farm and domestic wastewater with microalgal-bacteria consortium in two HRAPs.

Soluble phosphorous in the form of phosphate is used by microorganisms for metabolic activity. As shown in Table 2, the highest SP removal efficiencies were achieved in R2 (80 and 91%) in comparison with R1 (71 and 57%), for PI and PII, respectively. This maximum SP uptake in R2 may be attributed to higher biomass productivity in the greenhouse HRAP, as shown in Fig. 2. These results are in agreement with Riaño et al. [7], who pointed out that nutrient removal (N and P) was directly linked to photosynthetic activity and biomass production of microalgal biomass.

3.2. Biomass productivity and microalgal identification

Both HRAPs were fed with the same type of wastewater and operating at the same HRT and nutrient and organic loading rates; however remarkable differences were observed in areal biomass productivity (g VSS/m^2 d) between both HRAPs (Fig. 2). According to the experimental results, two phases were identified in PI; firstly a growth phase was observed followed by a stationary phase. Specifically, during PI, average

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biomass productivity was 4.4 ± 1.2 for R1 and 7.4 ± 1.6 g VSS/m^2 per day for R2 (Fig. 2).

At the beginning of period II, a remarkable decrease in productivity (from 7.3 to 4.7 g VSS/m^2 day) was observed during the first 15 days in R1, probably due to the inability of biomass to adapt fast to the new OLR conditions and also because of the increase in wastewater colour that diminished light penetration. On the opposite, this phenomenon was hardly observed in R2, probably due to the higher light supply received. In this vein, Kuei-Ling et al. [5] described the key role of light supply in the microalga *Chlorella vulgaris* ESP-31 under different regimes of illumination and found that was highly affected not only by the quantity of light but also by the quality of this light (type of fluorescent lamp). As a consequence of the deficit of light supply in R1-PII, microalgae must adapt to new conditions. Most of the members of chlorophyta, cyanophyta and heterokontophyta may switch over from autotrophy to mixotrophy metabolism, requiring an acclimation time to be able to use residual organic matter as a carbon source instead of DCO_2. These results are in agreement with Mahapatra et al. [28] who treated municipal wastewater with microalgae and observed a variation from autotrophy to mixotrophy after a change in OLR. The increase in productivity in R1-PII occurred despite of the probable change in microalgal population from autotrophy to mixotrophy. Biomass was able to adapt to the new conditions and productivity was recovered achieving 9.2 ± 0.3 g VSS/m^2 d at the end of period II. In R2, areal biomass productivity achieved values of 12.7 ± 0.4 g VSS/m^2 at the end of the experimental time.

To determine the evolution of different microalgal species, a study of the community composition of each pond at the end of period I and II was carried out. The microalgal concentration was constant during the whole experiment (2.3 x 10^9 cells/L) with the exception of R1-PII, where the concentration was 10 fold lower. The microalgal inoculum was mainly composed by the genus *Chlamydomonas* and *Anabaena* (Table 1). During the whole experiment, cyanophyta dominated over chlorophyta and heterokontophyta in both HRAPs with the exception of R1-PI. After inoculation, the genus *Chlamydomonas* was close to disappear in both ponds; while *Teilingia*, *Anabaena* and *Nitzschia* were the dominant taxon in R1-PI, and *Anabaena* and *Phormidium* were the main genus in R2. In PII, an important variation in microalgal population was carried out; most of heterokontophyta microalgae disappeared. The microalga *Phormidium tergestirum* was the most abundant specie (94.5%) in R1 and *Anabaena* sp. (97.4%) in R2; thus, filamentous microalgae showed an increasing trend from initial inoculum to period II. Both species are facultative mixotrophic and depending on nutrient concentration and light supply their metabolism can be autotrophic, heterotrophic or mixotrophic [6]. The sum of both species accounted over 99% of microalgae present in both HRAPs at the end of period II.

Data obtained herein shows a variation in concentration of different microalgal species through the period I and II, being minor the variation in microalgal population in R2. These differences might probably be attributed to the fact that R1 received 9-fold lower light supply than R2. In addition, the lack of light in R1 together with the increase in OLR elicited the switch from autotrophic to mixotrophic nutrition, increasing the acclimation period, as previously described by Mahapatra et al. [28]. For this reason,

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and as it was previously pointed out, biomass productivity decreased at the beginning of period II in R1. Nevertheless, the relation between nutrient availability, environmental conditions and evolution of microalgal community is not well understood. Riaño et al. [30] reported that the response of the same species to similar nutrient concentration varied among studies; thus OLR, variations in temperature, light supply and interaction between microcosms were responsible for species diversity of microalgal populations [1,31-32]. Diverse species with differential interactions/competition also contributes to the system stability with enhanced biomass growth and efficient removal of nutrients.

3.3. Characterization of biomass produced

Chemical characterization of biomass was performed to determine if operational conditions in both HRAPs had an effect on biomass composition. The results shown in Table 3 evidenced that differences in protein concentration between both HRAPs were close to 10%, showing higher accumulation in R1 than in R2 for both periods. Probably, a lack in SP could occur in R2 as a consequence of higher biomass productivity, preventing protein accumulation and thus increasing carbohydrate content. Indeed, total carbohydrate content was higher in R2 (25 and 22%) than in R1 (16 and 13%) for period I and II, respectively; while a similar concentration of lipids (from 13 to 15%) was obtained in both photobioreactors. This difference in carbohydrate content may also be due to an excess of light supply in R2, triggering carbohydrate accumulation. Similar results were described by Kuei-Ling et al. [5] who evidenced accumulation of carbohydrates in *Chlorella sorokiniana* ESP-31 under light stress conditions. Furthermore, Rodolfi et al. [33] pointed out that nitrogen and phosphorous are required to protein synthesis and its deprivation leaded carbohydrate and/or lipid accumulation in the microalgae *Chlorella, Nannochloropsis*, *Porphiridium* and *Scenedesmus* among others.

3.3.1. Extraction of fatty acids by $SCCO_2$ and further characterization

A set of experiments was carried out using $SCCO_2$ in order to calculate extraction yield and to obtain the extraction curves from biomass grown in R1 and R2 during both periods. Supercritical carbon dioxide method was selected for FFA extraction as it takes out very efficiently the neutral lipids from biomass, impeding the extraction of non-polar lipids (phospholipids), pigments and waxes and avoiding thus degumming operations during biodiesel production process [11]. Furthermore, after depressurization, CO_2 becomes gaseous and it is then spontaneously separated from the extracted phase and residue, which are completely free of toxic solvent traces. This enables a direct valorisation of both extracts and residues without any additional processing. In this manner, CO_2 is safely recycled representing and economic and environmental benefit. Extraction yields were calculated according to Eq. (1).

$$\text{Extraction yield (\%)} = (A/B) \times 100 \qquad (1)$$

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where A was the weight of the total extract collected by $SCCO_2$, determined by gravimetry, and B was the dry weight of the initial biomass powder.

The overall extraction curves at 30 MPa and 45 °C are represented in Fig. 3. They were fitted by using the model proposed by Sovová [34] initially developed for lipid extraction from plants by $SCCO_2$, but recently used also for $SCCO_2$ lipid extraction from microalgae [13,16]. In this model, the extraction yield *e* is calculated as the mass of extract collected, divided by the weight of the insoluble solid, instead of the total mass of the solid. For that reason, the values of *e* represented in Fig. 3 were slightly higher than the values calculated by the Eq. (1). As shown in Fig. 3, the extraction kinetics fitted well with the mathematical model of broken-intact cells proposed by Sovová [34]. The extraction curves have two extraction periods; firstly an exponential period where extraction yield *e* mainly depended on the solute solubility, being the period in which most extract was obtained; followed by a stationary period which was governed by internal diffusion in the biological material and the recovery of extract was minimal. The relative average deviation of the kinetics ranged from 0.5 to 1.7 for all assays performed. The overall extraction curves represented in Fig. 3 showed a similar behaviour in all biomasses, with the exception of biomass grown in R2-PI, whose final extraction yield *e* was considerably lower. The highest extraction yield was obtained by biomass grown in R1-PI reaching 21% and the lowest one by biomass from R2-PI with 13%. However, results from Table 4 corroborate a similar FFA concentration with values ranging between 128-148 mg FFA/g DW for biomass grown in both HRAPs during the two experimental periods.

After supercritical fluid extraction, further analyses of the FFA composition were performed in all biomass samples. As shown in Table 4, the main FFA obtained were palmitic (C16:0), stearic (C18:0), oleic (c18:1 n9c) and linolenic (C18:2) acids, being the palmitic acid the predominant FFA in all the samples. Looking at Fig. 4, it can be seen that the composition of the extracts was more than 50% of saturated FFA, around 30% of monounsaturated and between 9 and 15% of polyunsaturated FFA, as shown in Fig. 4. According to Puhan et al. [35], the synthesis of biodiesel fuel with oils containing more saturated and monounsaturated FFA results in higher cetane number, lower hydrocarbon emissions, lower nitrogen monoxide, smoke and carbon monoxide emissions and shorter ignition delay; although viscosity of the biodiesel produced is higher and density is lower. Hence, composition of saturated, monounsaturated and polyunsaturated FFA of microalgal biomass (Fig. 4) is essential to determine the potential biodiesel quality and it makes necessary to determine the most suitable culture conditions to increase synthesis of saturated and monounsaturated FFA to produce higher quality biodiesel. According with these considerations, the most suitable culture conditions to produce biodiesel were stated in HRAP located in the greenhouse (R2) operating at a HRT of 10 days, since produced biomass presented the maximum saturated and monounsaturated FFA concentration, accounted for 90 and 38 mg FFA/g DW, respectively.

3.3.2. Determination and characterization of carbohydrates

In a biorefinery approach, not only lipids must be extracted from biomass (section 3.3.1) but also carbohydrates in the form of fermentable sugars. Microalgal carbohydrates are difficult to break down by weak pre-treatments due to rigid microalgal cell wall as shown by Hernández et al. [36] with the microalgae *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus almeriensis*. To evaluate the influence of lipid extraction by $SCCO_2$ on carbohydrates break down, monosaccharide composition in LEB and NLEB biomass was analyzed (Table 5). Glucose and xylose were the major monosaccharides, representing close to 40% in all samples. A higher concentration of glucose was found in all biomass tested with the exception of NLEB biomass from R1-PI, in which concentration of xylose (37 mg/g DW) was higher than glucose (34 mg/g DW). The third main monosaccharide was mannose (ranging from 16 up to 31%), followed by ramnose, arabinose and fucose. These results were similar to those reported by Hu et al. [37], who observed that glucose, xylose and mannose were the main monosaccharides in the filamentous microalgae *Microcoleus vaginatus*, *Scytonema javianucum*, *Phormidium tenue* and *Nostoc* sp., with a monosaccharide concentration similar to the one reported in the present work. Hence, despite of the different operating conditions in both studies were different, monosaccharide composition was similar.

Experimental results evidenced an increase in monosaccharides content (ranging between 7 and 46%) in LEB in comparison with NLEB as a result of supercritical extraction, that can promote breakdown of carbohydrates into monosaccharides, being more available for anaerobic digestion to produce CH_4 and also for a further use in fermentation processes to obtain ethanol; both cases would increase the profitability of the process.

3.4. Anaerobic digestion of biomass

Anaerobic digestion experiments of exhausted and non exhausted biomass took 42 days. During this process, the production of biogas was regularly determined.

Methane yields were calculated according to Eq. (2).

$$\text{Methane yield} = \text{corrected methane volume}/VS_{added} \quad (2)$$

The accumulated methane production at a substrate/inoculum ratio of 0.5 g $TCOD_{added}$/g VS for the biomass studied with and without previous $SCCO_2$ lipid extraction is represented in Fig. 5. According to the results, LEB presented higher methane yield than NLEB for all the assays performed. The maximum methane production was obtained for LEB (195 mL CH_4 /g VS_{added}) and for NLEB (146 mL CH_4/g VS_{added}) both grown in R2-PI. Similar results were obtained by Hernández et al. [16] (2014) who accomplished methane yields of 236 mL CH_4/g VS_{added} for LEB *Tetraselmis* sp. after lipid extraction by $SCCO_2$ and 203 mL CH_4/g VS_{added} for NLEB *Scenedesmus almeriensis* biomass.

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Lipids are the microalgal component which result in higher methane production (1.014 mL CH_4/g VS_{added}), compared to proteins (0.851 mL CH_4/g VS_{added}) and carbohydrates (0.415 mL CH_4/g VS_{added}) according to Sialve et al. [38]. Although LEB did not contain lipids as a consequence of $SCCO_2$ extraction, this biomass resulted in higher methane yield compared to NLEB in all assays. At this point, it should be indicated that proteins and carbohydrate content before and after $SCCO_2$ extraction were equal in all samples. TCOD removal from LEB ranged between 40 and 54%, while in NLEB TCOD removal ranged between 39 and 49% (data not shown). In addition, anaerobic digestion of LEB resulted in higher VS removal efficiency (ranging between 47 and 61%) compared to NLEB (ranging between 45 and 55%). These results could be attributed to $SCCO_2$ extraction, which would break down not only microalgal cell wall but also proteins into aminoacids allowing anaerobic microorganisms to access more easily to biomass and enhancing biodegradability. Furthermore, an increase in monosaccharides content, as pointed out in section 3.3.2, could increase biogas production in LEB. Therefore, although theoretically NLEB had a greater methane production potential than LEB, unbroken cells may hinder access to lipids, proteins and carbohydrates by anaerobic bacteria resulting in lower CH_4 production. The results obtained in the work herein confirmed the hypothesis that $SCCO_2$ extraction was a suitable methodology not only to extract lipids but also to enhance biodegradability of proteins and carbohydrates that remained in the LEB, being more available to processes like anaerobic digestion or fermentation.

Regarding to the process stability, Table 6 shows pH, NH_4^+–N and VS concentration before and after anaerobic digestion. All final pH values ranged from 7.2 to 7.8, being compatible with normal anaerobic microorganism growth. The concentration of ammonium obtained after anaerobic digestion process ranged from 173 up to 235 mg NH_4^+–N/L. These concentrations could not inhibit microorganism according to Stams et al. [39]; thus, it did not affect to methanogenic bacteria activity. Furthermore, no volatile fatty acids were found after anaerobic digestion, and, therefore, the anaerobic digestion was complete. In conclusion, these results confirmed that $SCCO_2$ enhanced microalgal biomass biodegradability, resulting in higher methane production in LEB in comparison with NLEB.

3.5. Economic considerations

A preliminary economic evaluation of the production of microalgal biomass as well as the $SCCO_2$ extraction and anaerobic digestion technologies applied to R1-PII and R2-PII was carried out based on the operational costs and incomes associated to these processes. The selection of these biomasses was performed according to their high biomass production and to their content in saturated and monounsaturated FFA. The purpose of this analysis was to estimate the benefits of carrying out the anaerobic digestion of intact biomass in comparison with performing anaerobic digestion of the residual biomass obtained from the $SCCO_2$ extraction. It has to be highlighted that the cost calculations are only approximate, and a rigorous economic analysis should consider, among others, initial investment, prices at plant scale, maintenance and labour costs. On the other hand, it is important to note that the economic and environmental benefit related to wastewater treatment was not determined in this work. Conventional

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biological treatments such as activated sludge involve high energy inputs associate with O_2 supply.

The costs associated to the production of biomass grown using slaughterhouse wastewater and under greenhouse conditions (R2) only included electrical energy demand to move paddle wheels (10.9 €/t) and water pumping (0.6 €/t) and they were determined according to Fiori [40]. The costs associated to $SCCO_2$ extraction included energy and reagents. Fiori [40] established an electrical consumption of 400 kWh per tonne of feedstock treated and a price of 0.133 €/kWh; thus an electrical cost of 53 € per tonne was estimated. The average prices of reagents were 0.20 €/kg CO_2 [40] and 0.22 €/kg ethanol 96% (v/v) [41]. To perform $SCCO_2$ extraction, a lost of 2% of the total amount of CO_2 (2 €/t) and ethanol (6 €/t) was considered. In order to determine incomes, it was assumed a sale price of the extracted oil of 0.48 €/L, as it was proposed by Gallagher [42] for microalgal oil. Hence, the profits from this process were expected to be around 54 €/t for R1-PII and 68 €/t for R2-PII.

The evaluation of operational costs for anaerobic digestion were estimated according to Zamalloa et al. [43], considering a combined heat and power cogeneration system with a 40% electric energy conversion efficiency and a 45% thermal energy conversion efficiency. In the present work a fixed feed-in-tariff of €0.133 kW/h was assumed for both electricity and thermal energy for the entire lifetime of the project, when assessing the profitability of the process. According to the literature and to the data obtained in the present work, the operational costs of the anaerobic digestion of LEB would be 1 €/t for electricity and 66 €/t for thermal energy. The profits obtained from biogas production were estimated to be 87 and 89 €/t for thermal energy and 77 and 79 €/t for electricity energy for R1-PII and R2-PII, respectively. Hence, the operational costs of the complete process (biomass production+$SCCO_2$+anaerobic digestion of the lipid exhausted biomass), can be estimated around 133 €/t and the profits about 231 and 236 €/t for R1-PII and R2-PII, respectively. Thus, the benefit obtained of the whole process is 98 €/t for R1-PII and 103 €/t for R2-PII. In the case of the non lipid exhausted biomass, the operational costs for the anaerobic digestion would be 90 and 79 €/t, while the incomes would be close to 141 and 122 €/t for R1-PII and R2-PII. In this sense, NLEB ensures a lower revenue and net energy produced than LEB. From this qualitatively analysis it can be concluded that the extraction of FFA from microalgae and further anaerobic digestion of the residual biomass could provide an economic benefit of 51 and 43 €/t higher than the direct anaerobic digestion without previous lipid extraction.

The results obtained from the economic evaluation shown in Table 3 suggested that although lipids content in the biomass were low (between 13-15%), its extraction using $SCCO_2$ affect other biomass compounds like carbohydrates, increasing its biodegradability and thus further methane production, as well as increasing total incomes. A suitable strategy to valorise algal biomass could focus on the combination of extraction techniques to recover valuable compounds and energy, being the opposite to obtain algal biomass rich in a specific valuable compound.

The estimated costs associated to the biomass production process (light and heat supplementation) for R1 were extraordinary high, resulting in significant economic losses, evidencing the unfeasibility to carry out the process under controlled conditions of light and temperature. Hence, it may be concluded that more revenue may be

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obtained from biomass grown in greenhouse conditions than in controlled conditions, when a combination of processes ($SCCO_2$+anaerobic digestion) is carried out.

Thus, this work has evidenced that two main economic benefits may be obtained by the use of microalgal-bacteria consortium; firstly this system has shown to be an efficient tool for slaughterhouse wastewater treatment, but also it allows producing high amounts of microalgal biomass using wastewater, avoiding the use of synthetic culture medium and decreasing costs of the microalgal production.

4. Conclusions

The use of microalgae-bacteria consortia presented high capacity to remove organic matter and nutrients from slaughterhouse wastewater. The highest biomass production (12.7 g VSS/m^2 day) was achieved by biomass grown in the HRAP located in a greenhouse operating at a HRT of 10 days. This biomass had a FFA content of 142 mg FFA/g and it was the most suitable to produce biodiesel. Carbohydrates and proteins composition of microalgal biomass was affected by operational conditions. The highest CH_4 yield was achieved by lipid exhausted biomass, evidencing that $SCCO_2$ extraction increases biodegradability for further biogas production, increasing the economic viability.

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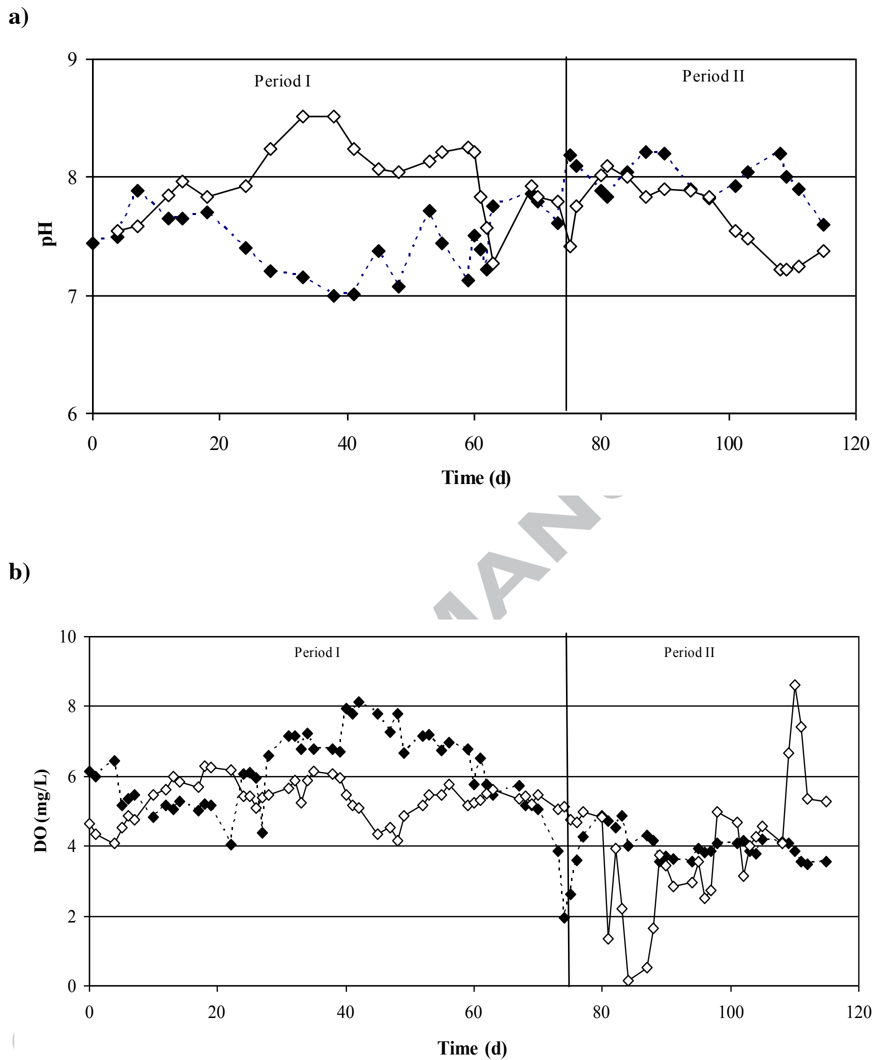

Figure 1. pH (a) and dissolved oxygen (b) measured in situ in the photobioreactors during period I and II. R1 (closed symbols) and R2 (open symbols).

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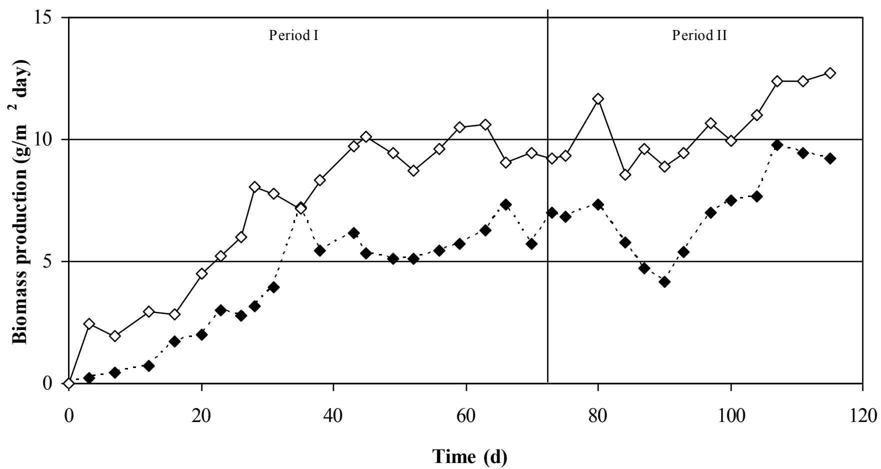

Figure 2. Biomass production through the period I and II in both photobioreactors. R1 (closed symbols) and R2 (open symbols).

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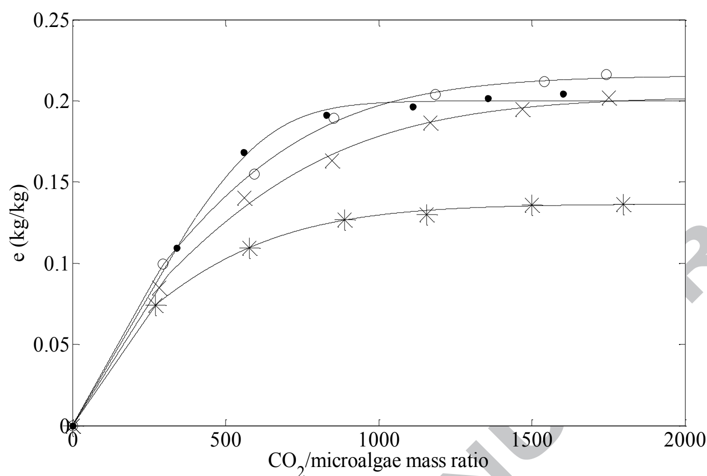

Figure 3. Supercritical carbon dioxide extraction curves of lipids from biomass grown in R1 during PI (●) and II (×), and from biomass grown in R2 during PI (∗) and PII (○). Each point is an experimental data and curves represent the model adjustment according to Sovová (2005). The curves represent the extraction yield *e* (mass $_{extract}$/mass $_{insoluble\ solid}$) as a function of the CO_2/ mass ratio.

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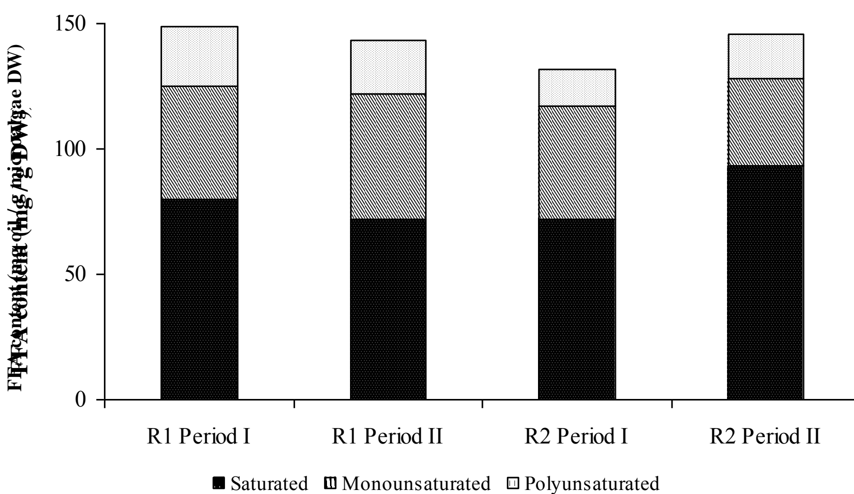

Figure 4. Composition of saturated, monounsaturated and polyunsaturated FFA extracted from biomass grown in R1 and R2 during periods I and II.

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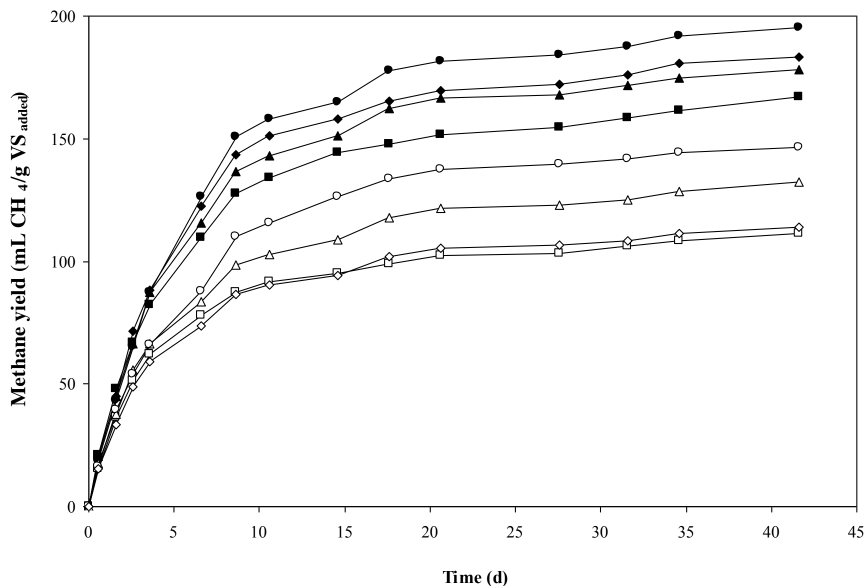

Figure 5. Accumulated CH_4 production during anaerobic digestion of biomass grown in R1 during period I (■) and period II (▲), and in R2 during period I (●) and period II (◆). Open symbols correspond to biomass that has not been used for lipid extraction (NLEB). Closed symbols correspond to lipid-exhausted biomass (LEB).

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Table 1. Evolution of microalgal community composition in R1 and R2 during period I and II.

Microorganisms (% of cells/L)	Initial inoculum	R1		R2	
		Period I	Period II	Period I	Period II
Chlorophyta					
Chlamydomonas subcaudata	92.0	0.1	---	0.3	---
Teilingia sp.	---	56.0	---	0.8	---
Chlorophyta contribution	92.0	56.1	0	1.1	0
Cyanophyta					
Anabaena sp.	7.5	13.1	5.5	66.9	97.4
Phormidium tergestinum	---	---	94.5	31.7	2.3
Cyanophyta contribution	7.5	13.1	100	98.6	99.4
Heteronkontophyta					
Nitzschia frustulum	---	---	---	0.1	0.3
Nitzschia sp.	0.5	28.9	---	0.2	0.3
Pinnularia sp.	---	1.9	---	---	---
Heterokontophyta contribution	0.5	30.8	0	0.3	0.6

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Table 2. (A) COD removal efficiencies through period I and period II in the two photobioreactors. (B) Ammonium removal, ammonium removed by stripping and soluble P removal through period I and period II in the two photobioreactors. Standard deviation is shown in brackets.

Reactor	Period	Total COD reduction (%)	Soluble COD reduction (%)	
(A)				
R1	I	85.8 (14.9)	84.7 (14.1)	
R1	II	91.7 (13.1)	89.7 (11.7)	
R2	I	84.3 (6.5)	80.2 (14.8)	
R2	II	86.4 (7.5)	83.4 (11.3)	
(B)		NH_4^+-N removal (%)	NH_4^+-N removed by stripping (%)	SP removal (%)
R1	I	80.2 (28.0)	1.0 (0.1)	70.8 (32.1)
R1	II	73.0 (12.5)	0.9 (0.1)	56.7 (21.3)
R2	I	70.8 (20.2)	3.1 (0.2)	79.7 (29.5)
R2	II	78.7 (10.3)	4.7 (0.1)	90.7 (24.6)

Initial characteristics of raw slaughterhouse wastewater were: pH = 7.3 ± 0.1, TS = 753 ± 18 mg/L, VS = 702 ± 19 mg/L, TCOD = 1621 ± 81 mg/L, SCOD = 1009 ± 54 mg/L, TKN = 149.2 ± 12 mg/L, NH_4^+-N = 9.2 ± 0.5 mg/L, NO_3^- -N = 0.44 ± 0 mg/L, NO_2^- -N = 0.03 ± 0 mg/L, TP = 1.4 ± 0.1 mg/L, SP = 1.3 ± 0.1 mg/L.

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Table 3. Biomass production and biomass composition through the period I and II in both photobioreactors. Standard deviation is shown in brackets.

Reactor	Period	Biomass production ($gVSS/m^2$ day)	Biomass composition (%)			
			Lipids	Carbohydrates	Proteins	Ashes
R1	I	4.4	14.8 (1.1)	15.8 (0.5)	57.8 (5.5)	4.1 (0.1)
R1	II	7.1	13.9 (0.9)	12.6 (0.3)	57.7 (6.1)	5.2 (0.1)
R2	I	7.4	12.8 (1.0)	25.0 (0.5)	45.1 (4.9)	5.3 (0.2)
R2	II	10.7	14.2 (1.0)	21.7 (0.4)	46.5 (4.9)	5.5 (0.1)

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Table 4. Composition of fatty acids of lipid exhausted biomass (LEB) and non lipid exhausted biomass (NLEB) during periods I and II in both photobioreactors.

	R 1		R 2	
Fatty acids composition (mg FFA/g DW)	**Period I**	**Period II**	**Period I**	**Period II**
Saturated				
C 14:0	4.0	6.5	3.2	7.7
C 15:	n.d.	4.4	n.d.	n.d.
C 16:0	55.1	45.6	46.2	56.7
C 18:0	15.8	11.0	16.9	17.5
Others[a]	6.6	7.5	7.7	8.1
Monounsaturated				
C 16:1 n9 ω-9	n.d.	3.8	1.8	n.d.
C 16:1cis ω-7	4.4	14.5	3.6	3.3
C 18:1 n7	5.6	2.8	n.d.	3.0
C 18:1 n9c ω-9	26.6	16.7	29.3	25.3
C 18:1n7c ω-9	4.3	3.9	2.8	3.5
Others[a]	3.6	4.8	4.5	3.1
Polyunsaturated				
C 16:2 ω-6	n.d.	2.6	1.7	n.d.
C 16:3 ω-3	2.8	n.d.	n.d.	n.d.
C 18:2 (LA) ω-6	12.7	9.5	7.3	10.1
C 18:3 (ALA) ω-3	4.0	3.5	1.5	2.6
Others[a]	2.4	1.9	1.5	1.4

n.d.: not detected.
[a]Amount of fatty acids lower than 1% by mass.

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Table 5. Composition of monosaccharides in biomass during period I and II in both photobioreactors.

Reactor	Period	Monosaccharides (mg monosaccharide/g DW)						
		Glucose	Xylose	Ramnose	Arabinose	Fucose	Manose	Other sugars
					LEB			
R1	I	40.8	39.8	15.2	2.5	5.0	47.5	0.4
R1	II	34.5	27.5	8.0	1.4	3.7	17.6	0.2
R2	I	79.0	50.8	20.8	3.3	1.8	30.5	0.2
R2	II	46.9	38.7	11.3	2.5	1.9	28.9	0.5
					NLEB			
R1	I	34.1	36.8	7.8	1.2	2.9	20.7	0.1
R1	II	37.4	26.7	6.3	1.0	3.6	11.6	0.2
R2	I	59.2	44.8	19.5	3.0	1.1	30.4	0.3
R2	II	42.4	35.7	12.6	2.1	1.1	22.8	0.2

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Table 6. Initial and final pH, NH_4^+–N and VS in anaerobic digestion tests of lipid exhausted biomass (LEB) and non lipid exhausted biomass (NLEB). Standard deviation is shown in brackets.

Reactor	Period	pH		NH_4^+-N (mg/L)		VS (g/L)	
		Initial	Final	Initial	Final	Initial	Final
				LEB			
R1	I	7.64 (0.08)	7.81 (0.16)	145 (16)	235 (18)	2.63 (0.2)	1.41 (0.2)
R1	II	7.38 (0.07)	7.56 (0.19)	140 (14)	227 (25)	2.78 (0.2)	1.27 (0.1)
R2	I	7.52 (0.09)	7.23 (0.23)	134 (17)	185 (17)	3.20 (0.4)	1.25 (0.1)
R2	II	7.49 (0.11)	7.39 (0.31)	121 (9)	200 (12)	2.88 (0.2)	1.29 (0.1)
				NLEB			
R1	I	7.35 (0.09)	7.51 (0.28)	134 (21)	208 (16)	2.66 (0.3)	1.46 (0.1)
R1	II	7.43 (0.08)	7.32 (0.35)	132 (17)	201 (7)	2.82 (0.2)	1.27 (0.0)
R2	I	7.42 (0.12)	7.51 (0.24)	136 (19)	173 (14)	1.84 (0.2)	1.01 (0.0)
R2	II	7.18 (0.10)	7.45 (0.34)	136 (18)	189 (11)	2.58 (0.3)	1.18 (0.1)

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CAPÍTULO 7

CONCLUSIONES Y TRABAJO FUTURO

El **objetivo principal** de esta tesis es el estudio del tratamiento de aguas residuales agroalimentarias mediante consorcios de microalgas y bacterias y el aprovechamiento de la biomasa resultante para obtener distintos biocombustibles. Para ello, se operó con fotobiorreactores abiertos y diferentes tipos de aguas residuales, procedentes de: i) la industria de procesado de patatas, ii) el tratamiento biológico secundario de purines, iii) un matadero industrial de cerdos y iv) una industria de procesado de pescado. La biomasa producida durante el proceso fue caracterizada y, posteriormente, se extrajeron compuestos de interés de la misma (carbohidratos, lípidos) y se analizó la producción de biogás.

Los resultados obtenidos en la presente tesis confirman el potencial de los consorcios de microalgas y bacterias para depurar aguas residuales agroindustriales y recuperar los nutrientes en forma de biomasa algal valorizable. Así mismo, se ha demostrado como esta tecnología puede suponer una alternativa a los tratamientos aerobios, anóxicos y anaerobios convencionales.

En el **Capítulo 3** se demostró que la combinación de tratamientos físicos, químicos y enzimáticos produce una mayor liberación de azúcares fermentables que la consecución de dichos tratamientos de forma individual. Los mayores rendimientos de liberación de monosacáridos se obtuvieron mediante el pretratamiento ácido y posterior hidrólisis enzimática, alcanzando producciones de 128, 129 y 88 mg de monosacáridos/g peso seco de alga, para las microalgas *Chlorella sorokiniana*, *Nannochloropsis gaditana* y

Scenedesmus almeriensis, respectivamente. Como consecuencia de la degradación de los carbohidratos durante los pretratamientos, se produjeron compuestos potencialmente inhibidores de la fermentación como ácido acético y ácido fórmico, pero las concentraciones fueron significativamente inferiores a aquellas que provocan la inhibición de la fermentación. No se detectó la presencia de otros compuestos de degradación como furfural y 5-hidroximetilfurfural.

En el **Capítulo 4** se trabajó con distintos métodos para extraer lípidos neutros de las microalgas *Isochrysis* T-ISO, *Nannochloropsis gaditana*, *Scenedesmus almeriensis* y *Tetraselmis* sp. Los mayores rendimientos se obtuvieron mediante el tratamiento de la biomasa con microondas y posterior extracción con CO_2 supercrítico. En comparación con otros métodos estudiados, el incremento de la extracción de lípidos fue del 15-25% mediante este proceso. Como consecuencia de la extracción de los lípidos se produjo un incremento de la biodegradabilidad de la biomasa algal en todas las especies analizadas, obteniéndose mayores producciones de biogás que durante el uso de la biomasa intacta. Así mismo, la estimación económica del proceso llevada a cabo corroboró un incremento de la rentabilidad económica próximo a un 30% al realizar de forma combinada la extracción de lípidos y una posterior digestión anaerobia de la biomasa resultante.

En el **Capítulo 5** se demostró la capacidad de los consorcios de microalgas-bacterias de tratar eficientemente ARAs procedentes de

una industria de fritura de patata y del tratamiento biológico secundario de purines, siendo capaz de degradar materia orgánica recalcitrante y alcanzando niveles de eliminación de DQO, nitrógeno y fósforo superiores al 62, 80% y 58%, respectivamente. Además se recuperaron los nutrientes (nitrógeno y fósforo) presentes en el agua en forma de biomasa que posteriormente fue valorizada mediante digestión anaerobia para la producción de biogás. También se demostró que la disminución del tiempo de residencia (de 15 a 10 días) provoca un incremento de la productividad de las microalgas, sin afectar a la calidad del agua residual tratada. Además, se demostró que el déficit de fósforo en el agua residual favorece la acumulación de lípidos en la biomasa y que, como consecuencia, se produce un incremento en la producción de biogás, demostrándose así que existe una relación directa entre la composición macromolecular de la biomasa y la producción de metano. Así mismo, se optimizó la relación substrato/inóculo obteniéndose un incremento significativo de la producción de metano.

Con el objetivo de analizar la viabilidad del proceso de depuración a mayor escala, bajo condiciones ambientales reales, en el **Capítulo 6** se realizó el tratamiento de aguas residuales de matadero en dos reactores HRAP raceway de 75 L alcanzándose concentraciones finales en el efluente tratado de DQOt, SV, nitrógeno total y fósforo total muy bajas. A continuación, se llevó a cabo la valorización de la biomasa producida mediante extracción de los lípidos con CO_2 supercrítico y posterior uso de la torta resultante en un proceso de

digestión anaerobia. Se demostró el efecto hidrolítico que tiene dicha extracción sobre los carbohidratos presentes en la biomasa, provocando un incremento de los azúcares fermentables. Se evaluó económicamente el proceso integral de valorización de la biomasa algal y se determinó una mejora económica del mismo al realizar la extracción de lípidos y posterior digestión anaerobia del residuo resultante, corroborando la hipótesis inicial donde se planteaba que es más interesante obtener biocombustibles de algas en el marco de una biorrefinería, que mediante la obtención de un único biocombustible.

Por lo tanto, el sistema de tratamiento de aguas residuales agroalimentarias con consorcios de microalgas y bacterias ha demostrado ser una alternativa viable para tratar las aguas residuales empleadas durante la presente tesis. Además, esta tecnología permite la valorización de los nutrientes del agua residual en forma de biomasa algal que puede ser posteriormente empleada para la producción de biocombustibles (biodiesel, bioalcoholes y biogás) lo cual puede suponer no solo una disminución de los costes de tratamiento, sino una importante fuente de ingresos, fruto de la producción de biocombustibles a partir de la biomasa en el marco de una biorrefinería.

Para asegurar el éxito de la implementación de esta tecnología a escala industrial, se seguirá trabajado en la optimización de ciertos parámetros que resultan esenciales cuando se emplean fotobiorreactores de gran tamaño, como por ejemplo:

- Realizar un estudio sobre la influencia de la lámina de agua en los fotobiorreactores con el objetivo de maximizar la cantidad de luz recibida por el cultivo, permitiendo así un incremento significativo de la productividad y por lo tanto una mejora de la calidad del ARA tratada.
- Llevar a cabo el aporte y la optimización de CO_2 en el cultivo, evitando así su déficit y mejorando la productividad de las microalgas.

Respecto a la valorización de la biomasa algal, el trabajo futuro se centrará en los siguientes aspectos:

- Fermentación de los azúcares obtenidos a partir de las microalgas *Chlorella sorokiniana, Nannochloropsis gaditana* y *Scenedesmus almeriensis* tras haber sido sometidas al tratamiento de hidrólisis óptimo.
- Optimizar el tiempo y la potencia del pretratamiento con microondas para maximizar la extracción de lípidos mediante CO_2 supercrítico.
- Realizar la co-digestión de la biomasa algal (rica en nitrógeno) con un residuo rico en carbono para equilibrar el balance C/N y obtener mayores producciones de biogás.

CAPÍTULO 8

BIOGRAFÍA

8. Biografía

David Hernández González (Murcia, 1986).

Estudios

- **Ingeniero Técnico Agrícola** en la especialidad de Explotaciones Agropecuarias (2004-2007) en la Universidad de Salamanca.
- **Licenciado en Biotecnología** (2008-2010) en la Universidad Francisco de Vitoria.
- **Master en Biotecnología Agroforestal** (2010-2011) en la Universidad Politécnica de Madrid.

Experiencia laboral

- Laboratorio "Plant Virus Biotechnology" bajo la supervisión del Dr. Fernando Ponz Ascaso. CBGP-INIA, España (Junio-Septiembre, 2009)
- Laboratorio "Interacción molecular Planta-Insecto" bajo la supervisión de la Dra. Isabel Díaz Rodríguez. CBGP-UPM, España (Octubre, 2009-Julio, 2011).
- Laboratorio de "Tratamiento de residuos ganaderos y de la industria agroalimentaria" bajo la supervisión de la Dra. María Cruz García González. ITACyL, España (Septiembre, 2011- Noviembre 2015).

Participación en proyectos de I+D

- Caracterización del perfil proteolítico del coleóptero *Tomarus villosus* para identificar posibles dianas necesarias en su control. Proyecto financiado por la Universidad Politécnica de Madrid y la Universidad Francisco de Vitoria (Octubre, 2009-Julio, 2010), Investigador Principal (IP): Dra. Isabel Díaz Rodríguez.
- Propéptidos de proteasas C1A de cebada: Actividad reguladora de las proteasas. Proyecto financiado por la Universidad Politécnica de Madrid (Julio, 2010- Junio, 2011). Investigador principal (IP): Dra. Isabel Díaz Rodríguez.
- Valorización de la biomasa microalgal obtenida mediante tratamiento fotosintético de aguas residuales agroindustriales para su futuro uso en acuicultura. Proyecto financiado por INIA, España. Participantes: Instituto Tecnológico Agrario de Castilla y León, y la Universidad de Valladolid (Enero, 2010-Abril, 2013). Investigador principal (IP): Dra. María Cruz García González.
- Valorización de efluentes agroindustriales mediante producción y aprovechamiento integral de microalgas para la obtención de bioproductos. Proyecto financiado por INIA, España. Participantes: Instituto Tecnológico Agrario de Castilla y León, Universidad de Valladolid, y la Universidad de León (Enero, 2015-Diciembre, 2018).

Investigador principal (IP): Dra. María Cruz García González.

Becas de investigación

- FPI-INIA "Producción de biocombustibles y bioproductos a partir de la biomasa algal obtenida mediante el tratamiento de residuos ganaderos y de la industria agroalimentaria" (2011-2015), realizada en el Instituto Tecnológico Agrario de Castilla y León (ITACyL). Valladolid, España.

CAPÍTULO 9

OTRAS PUBLICACIONES Y MÉRITOS

9. Otras publicaciones

Publicaciones en revistas internacionales:

- ✓ Riaño, B., **Hernández, D.**, García-González, MC., (2012). Microalgal-based systems for wastewater treatment: Effect of applied organic and nutrient loading rate on biomass composition Ecol. Engineer. 49, 112-117.

Publicaciones en congresos internacionales

- ✓ **Hernández, D.**, Riaño, B., García-González, MC. Microalgae-bacteria consortia for wastewater treatment: nutrient recovery and biomass composition. International Congress on Water, Waste and Energy Management (Salamanca, España), 23-25 Mayo 2012 (**Poster**).

- ✓ **Hernández, D.**, Riaño, B., Coca, M., García-González, MC. Physical pre-treatments of three microalgal species as a previous step to produce bioethanol. Alg n´Chem 2014, Which Future for Algae in Industry? (Montpellier, Francia), 31 Marzo-3 Abril 2014 (**Poster**).

- ✓ Solana, M., **Hernández, D.**, García-González, MC., Bertucco, A. Microwave as a pre-treatment to facilitate the supercritical extraction of lipids from microalgae. 14th

European Meeting on Supercritical Fluids (Marsella, Francia), 18-21 Mayo 2014 (**Poster**).

- ✓ **Hernández, D.**, Riaño, B., Solana, M., Coca, M., García-González, MC., Bertucco, A. Growth of microalgal biomass in raceway using agroindustrial wastewater: Biomass productivity and fatty acid accumulation. 10th International Conference on Renewable Resources and Biorefineries (Valladolid, España), 4-6 Junio 2014 (**Poster**).

- ✓ Riaño, B., **Hernández, D.**, García-González, MC. Anaerobic digestión of microalgae-bacteria biomass growth in fish processing wastewater: impact of substrate/inoculum ratio. 10th International Conference on Renewable Resources and Biorefineries (Valladolid, España), 4-6 Junio 2014 (**Poster**).

- ✓ **Hernández, D.**, Riaño, B., Coca, M., García-González, MC. Recovery and valorization of nutrients from slaughterhouse wastewater through microalgae cultivation in high rate algal ponds. Nutrient Removal and Recovery: Moving Innovation into Practice (IWA) (Gdansk, Polonia) 18-21 Mayo 2015 (**Poster**).

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Asistencia a cursos

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9.1. ANEXO I

MICROALGAL-BASED SYSTEMS FOR WASTEWATER TREATMENT: EFFECT OF APPLIED ORGANIC AND NUTRIENT LOADING RATE ON BIOMASS COMPOSTION

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Microalgal-based systems for wastewater treatment: Effect of applied organic and nutrient loading rate on biomass composition

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ABSTRACT

Microalgal-based systems are currently receiving increasing attention due to their ability to treat wastewater, whilst also providing a high-added value product in the form of biomass. The potential of the microalgae *Chlorella sorokiniana* and *Spirulina platensis* to use fish processing wastewater as a growth medium in symbiosis with aerobic bacteria was evaluated. Microalgae were cultivated separately in two semi-continuously fed photobioreactors under lab conditions. Soluble organic loading rate (OLR) and ammonium loading rate (ALR) increased almost 10-fold in the photobioreactors when fed with higher strength wastewater. Soluble chemical oxygen demand (SCOD) removal efficiencies were similar regardless of microalgal population, and an increase of up to 54% was detected with the load applied. Ammonium was completely exhausted in both photobioreactors regardless of operating conditions. Removal of soluble phosphorous (SP) increased from 47% to up to 67% in both systems with the applied load increase. Microscopic analysis showed that initial microalgae completely disappeared, and that the predominant group in both photobioreactors was cyanophyta, ranging from 53% to 76% of biovolume during the whole experimental period. Moreover, percentage of lipids in biomass doubled from approximately 9% to 18% in both photobioreactors with applied load increase. These findings suggest the importance of culture conditions rather than selected microalgae in biomass production using wastewater treatment as feed for microalgal growth.

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1. Introduction

The use of microalgal-bacterial systems for domestic and industrial wastewater treatment has increased in recent years (Bordel et al., 2009; Medina and Neis, 2007). In these processes, microalgae provide oxygen that can be used by aerobic bacteria to degrade organic matter while, in return, consuming the carbon dioxide produced in bacterial respiration. Thus, these systems constitute an economical alternative to conventional aerobic technologies since aeration is avoided (Guieysse et al., 2002; Muñoz and Guieysse, 2006). Moreover, the nutrients (nitrogen and phosphorous) contained in wastewater could be accumulated in the biomass during the removal process (Su et al., 2011). Furthermore, the use of microalgal biomass for biofuel production is being considered as the most suitable energy alternative in the current economic climate (Singh et al., 2011a).

Previous reports by our research group focus on the use of microalgal-based systems for fish processing wastewater treatment and the influence of operating conditions, such as temperature and hydraulic retention time, on organic matter and nutrient removal efficiency, using wildly grown microalgae as inoculum (Riaño et al., 2011). However, photobioreactor efficiencies should be assessed taking into account the biomass chemical profile in order to evaluate the potential production of high-added value products.

The chemical composition of microalgae depends on the species, culture medium and operating conditions used. Microalgae can be rich in lipids, carbohydrates or proteins or have a balanced content thereof. Various studies have addressed the impact of environmental conditions on productivity or the chemical composition of microalgae cultivated in synthetic medium. However, to our knowledge, little effort has been made to investigate the effect of environmental conditions on microalgae culture growth using agro-industrial wastewater. Olguín et al. (2001) studied the effect of low light flux and nitrogen deficiency on the chemical composition of *Spirulina* cultivated in sea-water supplemented with anaerobically digested pig manure, concluding that these culture conditions resulted in lipid or polysaccharide enrichment,

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depending on the specific light flux. Kebede-Westhead et al. (2004) studied how the elemental composition of harvested biomass varied using different loading rates of anaerobically digested flushed dairy manure. Such works provide relevant information since reduced production costs due to the use of low cost culture medium might prove a key factor in developing economical process for obtaining high-added value products.

The main objective of the present work was to compare two semi-continuously fed photobioreactors inoculated with the green microalgae *Chlorella sorokiniana* and the cyanobacteria *Spirulina platensis* by means of organic matter and nutrient removal efficiencies, biomass productivity and biomass composition. Additionally, this study aims to evaluate the effect of the load applied on biomass composition, in terms of lipid, protein and carbohydrate accumulation.

2. Materials and methods

2.1. Photobioreactors and culture conditions

The experimental set-up consisted of two open to the atmosphere photobioreactors with a total working volume of 3 L (17 cm wide, 31 cm long, 6.5 cm high). These reactors were called RS (reactor inoculated with *Spirulina platensis*) and RC (reactor inoculated with *C. sorokiniana*). Photobioreactors were continuously illuminated using four fluorescent lamps at $94.5\,\mu E\,m^{-2}\,s^{-1}$ (Philips 50 W). The average temperature was $24.0 \pm 2.9\,^{\circ}C$ and $26.3 \pm 3.3\,^{\circ}C$ for RS and RC, respectively. In order to avoid nutrient gradients, light limitation and biomass sedimentation, the culture broth was gently suspended by means of magnetic stirrers. Photobioreactors may therefore be considered completely-mixed reactors. Reactor volume was checked daily and any water loss due to evaporation was corrected.

The RS photobioreactor was initially filled with tap water and inoculated with 35 and 54 mg volatile suspended solids (VSS) L^{-1} of aerobic sludge and microalgae *S. platensis*, respectively. The same operation was performed to inoculate the RC reactor with 35 mg VSS L^{-1} of aerobic sludge and 58 mg VSS L^{-1} of *C. sorokiniana*. Immediately after inoculation, the two reactors were fed daily with fish processing wastewater (FPW) at a hydraulic retention time (HRT) of 10 days. The HRT chosen was based on previous studies (Riaño et al., 2011). The substrate was fed into the photobioreactors in two different periods (Table 1). During period II, the soluble organic loading rate (OLR) and ammonium loading rate (ALR) was almost 10-fold higher than in period I.

Photobioreactor temperature, dissolved oxygen (DO) and pH were periodically monitored *in situ*. Influent and samples from each reactor's culture broth were withdrawn twice a week to monitor pH, total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), soluble phosphorous (SP), total phosphorous (TP), total Kjeldahl nitrogen (TKN), ammonium (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N). Additionally, the culture broth was monitored for total and volatile suspended solids (TSS and VSS).

Table 1
Characterization of fish processing wastewater (FPW) for period I (batch I) and for period II (batch II). Standard deviation is shown in brackets.

	FPW	
	Batch I	Batch II
SCOD (mg L^{-1})	181 (51)	1100 (276)
TS (mg L^{-1})	732 (131)	2196 (368)
TKN (mg L^{-1})	27.6 (5.2)	159.8 (22.0)
NH_4^+-N (mg L^{-1})	8.1 (2.0)	76.6 (34.8)
SP (mg L^{-1})	1.1 (0.3)	20.1 (5.8)

The culture broth was collected and centrifuged (2469 *g*; Centrifuge 5810R, Eppendorf) for 5 min in order to harvest the biomass at the different loading rates. This biomass was analysed to determine total solids (TS), volatile solids (VS), TKN and TP and was lyophilized (Lyoquest 85 Plus Eco, Spain) to determine lipid content.

2.2. Substrate composition

Photobioreactors were fed with FPW collected from a fish farm located in Segovia (Spain). This wastewater was generated during washing operations after rainbow trout (*Onkhorynchus mykiss*) fileting. Two batches of FPW were used in the present study: batch I was used in period I (1–36 days) whereas batch II was used in period II (37–60 days). The average composition of the two batches is shown in Table 1. The differences observed in wastewater characterization might be attributed to the inherent temporal variability in agro-industrial activity. The substrate was homogenized and subsequently stored at $4\,^{\circ}C$ for further use. At this point, it is worth mentioning that the sole feeding source was FPW and that no external carbon dioxide was provided.

2.3. Microorganisms

S. platensis and *C. sorokiniana* were obtained from the Culture Collection of Algae of the University of Goettingen (SAG) (Goettingen, Germany). Microalgae inoculum was prepared following Guieysse et al. (2002). These microalgae species were selected for this study depending on their tolerance to highly polluted environments. Thus, *Chlorella* sp. evidences high ammonia tolerance whereas *Spirulina* sp. exhibits low ammonia tolerance (De Godos et al., 2010).

The aerobic bacterial culture was collected from an activated sludge reactor in the Valladolid municipal wastewater treatment plant (Spain). Prior to inoculation, algae and bacteria were centrifuged (2469 *g*; Centrifuge 5810R, Eppendorf) for 20 min and resuspended in distilled water.

2.4. Analytical procedures

TS, VS, TSS, VSS, TCOD, SCOD, TKN, TP and SP were analysed in duplicate in accordance with APHA Standard Methods (2005). NH_4^+-N, NO_2^--N and NO_3^--N concentrations were determined using electrodes, Orion 900/200 (Thermo Electron Corporation, Beverly, USA). pH, temperature and DO in the reactors were determined using a multi-probe system model, YSI 556 MPS (YSI Incorporated, USA).

Lipids were extracted from the lyophilized biomass with chloroform-methanol following the method proposed by Kochert (1978). Once extraction was performed, the lipids were quantified by gravimetric analysis. Proteins were calculated by multiplying the TKN by 5.95 (González-López et al., 2010). Carbohydrates were estimated by subtracting the percentage of ashes, lipids and proteins from 100% (González-Fernández et al., 2010).

Microalgae identification and quantification were carried out by microscopic examination (OLYMPUS IX70, USA) of culture broth samples fixed with formaldehyde at 0.5% and stored at $4\,^{\circ}C$ prior to analysis. Quantification was performed following the Phytoplankton Manual (Sournia, 1978).

Table 2
Microalgal community in the RS and RC photobioreactor during periods I and II (in % biovolume).

Genus	RS			RC		
	Initial	Period I	Period II	Initial	Period I	Period II
Chlorophyta						
Ankira sp.	–	–	2.34	–	0.61	1.18
Chlorella sp.	–	–	–	100	–	–
Chodatella sp.	–	–	–	–	0.91	–
Microspora sp.	–	–	–	–	3.34	–
Scenedesmus longispina	–	12.33	–	–	10.61	23.53
Scenedesmus obliquus	–	–	43.66	–	4.85	–
Scenedesmus sp.	–	10.40	0.78	–	2.07	0.29
Chlorophyta contribution	–	22.73	46.78	100	22.39	25.00
Cyanophyta						
Chroococcus limneticus	–	27.73	44.44	–	49.12	74.12
Cyanophyta cocal	–	0.91	–	–	–	0.88
Dactylococcopsis sp.	–	6.16	5.85	–	1.01	–
Phormidium sp.	–	–	2.92	–	3.03	–
Spirulina platensis	100	–	–	–	–	–
Stigeoclonium sp.	–	41.60	–	–	8.89	–
Cyanophyta contribution	100	76.40	53.21	–	62.05	75.00
Bacillariophyta						
Fragilaria sp.	–	–	–	–	15.56	–
Navicula sp.	–	0.87	–	–	–	–
Bacillariophyta contribution	–	0.87	–	–	15.56	–

3. Results

3.1. Biomass productivity

Areal biomass density was chosen to quantify biomass productivity, measured as the VSS of biomass produced per day and square metre of the photobioreactor. Areal biomass density evidenced significant differences in RS and RC. More specifically, during period I, areal biomass density was 1.270 g VSS m^{-2} d^{-1} for the RC photobioreactor and 0.613 g VSS m^{-2} d^{-1} for RS. The same trend was observed during period II. In this period, areal biomass density increased up to 1.540 and 1.102 g VSS m^{-2} d^{-1} for RS and RC, respectively. In period I, average biomass concentration measured in culture medium was 0.6 and 0.5 g VSS L^{-1} for the RC and RS photobioreactors, respectively. Period II showed an increase in VSS concentration (1.9 g VSS L^{-1} in both reactors). The results displayed the same tendency as for biomass growth. Therefore, biomass productivity increased concurrently with higher loading rates in both photobioreactors, as a result of higher carbon and nutrients (N and P) availability. These biomass productivities proved lower in comparison to that reported by De Godos et al. (2009), who observed a maximum productivity of 27.7 g m^2 d^{-1} in high rate algal ponds treating piggery wastewaters. These differences could be partially attributed to the higher ammonium loading rates (21.4 mg L^{-1} d^{-1}) applied by those authors. Likewise, low biomass growth in open photobioreactors might also be affected by other factors, including evaporation losses, inefficient mixing and light limitation (Brennan and Owende, 2010).

The evaluation of algal cell density evidenced the influence of operating conditions in both photobioreactors on microalgal community. In period I, RS showed an algal cell density of 16.8×10^8 cells L^{-1} increasing up to 20.9×10^8 cells L^{-1} during period II. In RC, algal cell density accounted for 5.8×10^8 cells L^{-1} during period I and increased about four-fold (up to 22.1×10^8 cells L^{-1}) in period II. The composition of microalgal communities was studied and is given in Table 2. Microscopic analyses indicated that initially inoculated microalgae (*S. platensis* in RS and *C. sorokiniana* in RC) completely disappeared at the end of period I. As regards biovolume, green algae (chlorophyta) and cyanobacteria (cyanophyta) were the major groups of algae in both photobioreactors during the whole experimental period. Nevertheless, cyanophyta dominated over chlorophyta in the two photobioreactors. Thus, in period I, chlorophyta only represented 22.7% in RS and 22.4% in RC, whereas cyanophyta represented 76.4% in RS and 62.1% in RC. In this period, consortia were dominated by the taxons *Stigeoclonium* sp. in RS and *Chroococcus limneticus* in RC. The presence of diatoms (Bacillariophyta) was not significant in the two photobioreactors (0.9% and 15.6% in RS and RC, respectively). During period II, chlorophyta increased up to 46.8% in RS while remaining approximately constant in RC (25.0%). Cyanophyta was again the predominant group, whereas bacillariophyta completely disappeared in both reactors. *C. limneticus* was the dominant taxon in the two reactors during period II. Additionally, the data obtained herein showed a decrease in microalgal species diversity in both reactors with the applied load increase (Table 2). These findings did not concur with those obtained by De Godos et al. (2009), who observed increased diversity when increasing the applied load. Nevertheless, the relation between nutrient availability and microalgal community in these systems is not well understood. Chinnasamy et al. (2010) reported that the response of the same species to similar nutrient concentration varied among studies. In this context, variables such as the organic load of the receiving wastewater, species interaction, seasonal environmental conditions, competition and interaction within the microcosms were responsible for species diversity of microalgal populations (Mara and Pearson, 1986; Fukami et al., 1997; Chinnasamy et al., 2010).

3.2. Organic matter and nutrient (N and P) removal

Two different batches of wastewater were used, giving an OLR of 0.02 and 0.11 g SCOD L^{-1} d^{-1} for period I and period II, respectively. SCOD was used to compare organic matter removal efficiency in order to avoid the influence of biomass growth on TCOD concentration. As can be seen in Table 3A, SCOD removal was approximately 30% for both reactors during period I, while during period II SCOD removal efficiency was 54% in RC and 59% in RS. Organic matter

Table 3
(A) Average SCOD, NH_4^+-N and SP removal efficiency in the RS and RC photobioreactors during periods I and II. B) Percentages of ammonia stripped in the RS and RC photobioreactors during periods I and II. Standard deviation is shown in brackets.

	% SCOD		% NH_4^+-N		% SP	
	RS	RC	RS	RC	RS	RC
(A)						
Period I	30.0 (29.7)	29.0 (29.5)	>99	>99	47.8 (24.1)	48.4 (15.6)
Period II	59.2 (0.1)	54.0 (0.1)	>99	>99	76.5 (5.9)	67.3 (16.1)

	% NH_4^+-N stripped	
	RS	RC
(B)		
Period I	37.6 (24.5)	44.4 (24.9)
Period II	1.1 (0.5)	1.1 (0.8)

removal was thus very similar for both reactors even when microalgal populations differed.

Nutrient (N and P) removal is directly linked to photosynthetic activity and biomass production. ALR was 0.81 mg NH_4^+-N L^{-1} d^{-1} for period I, increasing almost 10-fold for period II (7.66 mg NH_4^+-N L^{-1} d^{-1}). Ammonium was completely exhausted in both photobioreactors during the two experimental periods (Table 3A). This result was expected since the applied ALRs were very low in comparison to those applied in other works (Molinuevo-Salces et al., 2010). Neither NO_2^--N nor NO_3^--N were detected in either photobioreactor during either of the two periods, which indicated that nitrification did not occur. Due to photosynthetic activity, CO_2 of the medium was rapidly consumed during period I and, as a consequence, the pH increased markedly (Fig. 1). The high pH reached during period I resulted in ammonia stripping. In order to theoretically quantify the fraction of NH_4^+-N that was stripped, the free ammonia concentration was calculated following Hansen et al. (1998). Ammonia volatilization accounted for 37.6% and 44.4% for RS and RC, respectively (Table 3B). During period II, pH decreased to values ranging between 6.9 and 7.5 in both photobioreactors (Fig. 1), indicating that a more balanced activity between microalgae and bacteria was reached. Thus, in period II nitrogen biomass assimilation increased in comparison to period I, in accordance with higher biomass productivity in period II in both photobioreactors. Accumulation of nitrogen in biomass during the experimental period is shown in Fig. 2. In the RS photobioreactor, the nitrogen content in dry biomass increased from 12.9 to a mean content of 21.4 ± 6.4 mg g^{-1} during period I, reaching an average of 54.7 ± 1.2 mg g^{-1} in period II. In RC, nitrogen content in dry biomass increased from 12.1 to 23.5 ± 3.7 mg g^{-1} in period I, reaching an average of 56.0 ± 0.4 mg g^{-1} in period II. Nitrogen concentrations in this work were comparable to those reported by Mulbry et al. (2005), who obtained biomass with a nitrogen content ranging between 3 and 8%.

Phosphate is used by microorganisms for metabolic activities. Phosphorous assimilation by biomass includes the formation of phospholipids, nucleotides and nucleic acid for microorganism growth. As seen in Table 3A, during period I both photobioreactors exhibited low PO_4^{3-} removal (approximately 48% for RS and RC). In period II, PO_4^{3-} removal efficiency increased up to 76.5% in RS photobioreactor and 67.3% in RC. This was in accordance with high nitrogen assimilation by biomass observed during period II, and consequently, with increased biomass productivity. Moreover, in our experimental set-up, pH was below 9.0 in both photobioreactors (Fig. 1) and, therefore, abiotic phosphorous precipitation did not occur (De Godos et al., 2009). The low PO_4^{3-} removal efficiencies observed in the present study might be due to nitrogen limitation in the culture growth. Thus, NH_4^+-N:SP ratios in FPW ranged between 5:1 and 8:1 (Table 1), whereas the stoichiometric ratio of N and P in phytoplankton is 16:1 (Redfield, 1958). Compared to conventional wastewater technologies, nutrients (N and P) in microalgal-based systems are converted into algal biomass that can be sustainably recycled (Singh et al., 2011b). In this context, in the RS photobioreactor, phosphorous content in dry biomass remained approximately constant (near to 3.0 mg g^{-1}) during period I (Fig. 2). During period II, TP content increased up to 7.2 mg g^{-1} concomitantly with increased PO_4^{3-} removal efficiency. The same trend was observed in phosphorous accumulation in biomass in the RC photobioreactor.

3.3. Biomass chemical profile

The biochemical profile in terms of carbohydrates, lipids and proteins in the microalgal-bacterial biomass is shown in Table 4.

Microalgae are known to produce and accumulate lipids within their cell mass which are similar to those found in many vegetables (Singh et al., 2011a). In period I, the percentage of lipids accounted for 8.8% in RS and 9.5% in RC. These results showed that lipid content was similar regardless of the microalgal population present in the photobioreactor. As shown in Table 4, during period II, a significant increase in lipid content was observed, similar values being reached both reactors (approximately 18%). In the present study, the percentage of lipids concurred with those found in the literature, ranging from low (10%) to moderate (25–30%) lipid content (Pittman et al., 2011). In our particular case, the higher organic matter and ammonium loading rate resulted in a higher lipid accumulation in biomass. This result was not in agreement with those reported by Rodolfi et al. (2009), who observed an increase in both lipid content and areal lipid productivity through nutrient starvation in an outdoor algal culture using thirty microalgal strains. However, several authors have proposed that lipid accumulation may not be dependent on nitrogen starvation but on excess carbon in the culture medium. In autotrophic or heterotrophic cultures, accumulation might therefore be due to exogenous carbon

Table 4
Biochemical profile of the macromolecules contained in the biomass obtained in the RS and RC photobioreactors during periods I and II.

	Period I		Period II	
	RS	RC	RS	RC
% Lipids	8.8	9.5	18.9	18.2
% Proteins[a]	38.3	23.3	30.8	43.4
% Carbohydrates[b]	41.4	59.4	38.4	33.3
% Ashes	11.6	7.8	4.4	4.5

[a] These values were calculated by multiplying the TKN value by 5.95 (González-López et al., 2010).
[b] These values were calculated by subtracting the percentage of lipids, ashes and proteins to 100% (González-Fernández et al., 2010).

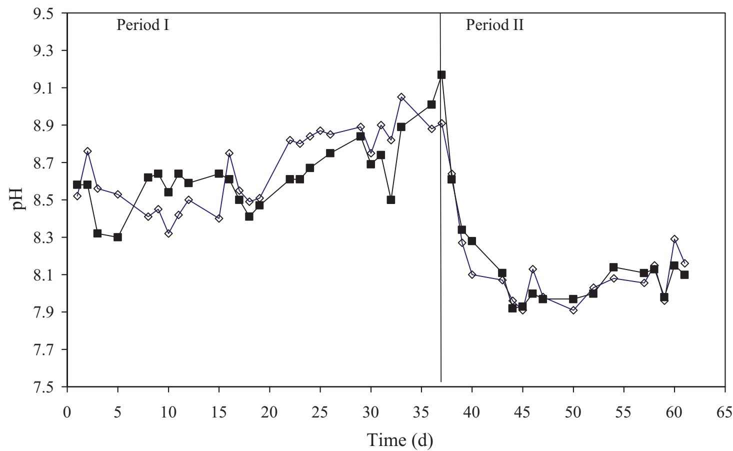

Fig. 1. pH measured *in situ* in the RS (■) and RC (◊) photobioreactors during periods I and II.

source consumption at a higher rate than the rate of cell division, which promotes conversion of carbon to lipids (Chen and Johns, 1991; Ratledge, 2004; Pérez-García et al., 2011). Thus, the increased SCOD removal efficiency observed in period II could be partially attributed to this mechanism, which may cause lipid accumulation in biomass at the same time. Other factors have been reported to affect lipid content in microalgal biomass, such as the strain used (Chinnasamy et al., 2010), cell growth phase (Lv et al., 2010), light supply (Olguín et al., 2001) or feed mode (Griffiths and Harrison, 2009). At this point it should be stressed that in the present study, the two photobioreactors performed under the same conditions and that the same trend was observed in both, even when different microalgal populations developed. Therefore, the applied load was deemed an important factor affecting lipid content in microalgal-bacterial biomass under these experimental conditions.

Proteins comprise a large fraction of the biomass of actively growing microalgae and cyanobacteria (González-López et al., 2010). In period I, the percentage of proteins was 38.3% for the RS photobioreactor and 23.3% for RC. In period II, the increase in the ammonium loading rate resulted in an increase in protein content for RC (up to 43%). Unexpectedly, in the RS photobioreactor, nitrogen assimilation decreased slightly (to 30.8%) with increased ammonium availability. These percentages proved low compared to those reported by González-Fernández et al. (2010), who obtained a protein content ranging from 43.5 to 55.1% when treating anaerobically digested swine slurry in open and enclosed photobioreactors. It is even known that in certain species protein content can reach values as high as 50–60% by dry weight biomass (Renaud et al., 2002). As stated before, the low protein content in the present study might be due to the low ALR applied.

Microalgae contain complex long-chain sugars (polysaccharides) in their cell walls that account for a large proportion of the carbon contained in these microorganisms (Parker, 2009). As shown in Table 4, the percentage of carbohydrates in biomass was lower during period II than period I, as protein and lipid content increased.

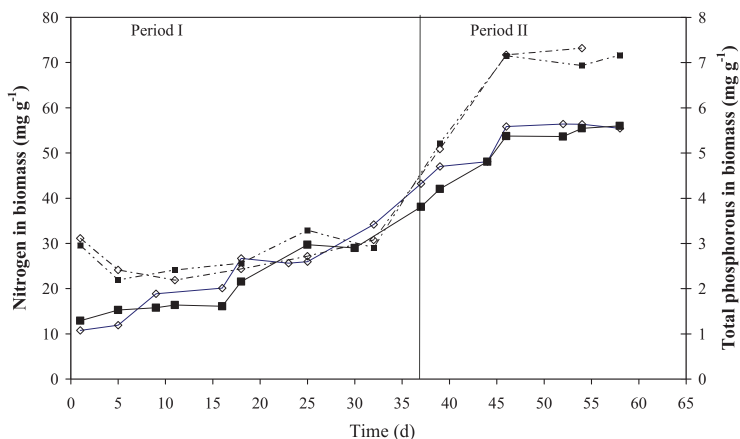

Fig. 2. Nitrogen (continuous line) and phosphorous (discontinuous line) content in biomass in the RS (■) and RC (◊) photobioreactors during periods I and II.

4. Conclusions

Two photobioreactors exhibited similar organic matter and nutrient removals when degrading fish processing wastewater, regardless of the microalgal population in their respective culture broths. Microalgae species did not show a significant effect in terms of macromolecular cellular components of the produced biomass. Nevertheless, the biochemical profile of biomass exhibited a direct relation with the load applied to the system. In this context, the higher the load applied the higher the lipid content. To conclude, controlling operational conditions, particularly the organic matter and nutrient loading rate applied, might prove helpful in obtaining the desired macromolecular cellular components.

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9.2. ANEXO II

OTRAS PUBLICACIONES

Microalgae-bacteria consortia for wastewater treatment: nutrient recovery and biomass composition

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Introduction

Conventional aerobic wastewater technologies are effective for organic matter degradation; however high energy inputs are required for O_2 supply. Recently, the interest in microalgal-bacterial consortia for wastewater treatment is increasing because bacteria supply the CO_2 needed by microalgae whereas microalgae produce the O_2 needed by bacteria to oxidize the organic matter, reducing aeration needs and CO_2 emissions.

Furthermore, microalgal-bacterial consortia allows to recover nitrogen and phosphorus from wastewater and, depending on protein, lipid and carbohydrate content, biomass can be used for different uses.

The objective of the present research was to determine the ability of microalgal-bacterial consortia for treating agroindustrial wastewaters and compare the obtained biomass in terms of lipid, protein and carbohydrate content.

Materials and Methods

The microalgae *Chlorella sorokiniana* was obtained from the culture collection of the University of Goettingen (Germany) and the aerobic bacteria were obtained from an activated sludge reactor of the municipal wastewater treatment plant of Valladolid (Spain).

Aerobic effluent (TE) was collected from a pig manure treatment plant. Potato processing wastewater (PP) was produced during processing operations in a potato industry. Chemical characterization of TE and PP wastewater is shown in Table 1.

Two 5 L photobioreactors were employed in this study being constantly illuminated with four fluorescent lamps at 6,000 lux (Phillips 50W) and the temperature was 24.1±2.7ºC.Reactors RTE and RPP were fed with TE and PP wastewater respectively at an HRT of 10 days.

Table 1. *Characterization of TE and PP wastewater. Standard derivation is shown in brackets.*

	TE	PP
pH	7.5 (0.3)	5.8 (0.2)
TS (mg L^{-1})	3319 (147.9)	1603 (388.2)
TCOD (mg L^{-1})	616 (44.8)	1536 (529.1)
SCOD (mg L^{-1})	465 (38.5)	745 (227.2)
BOD_5 (mg L^{-1})	63.0	917
NH_4^+-N (mg L^{-1})	12.3 (1.7)	12.1 (1.7)
NO_3^--N (mg L^{-1})	53.8 (6.1)	n.d.
NO_2^--N (mg L^{-1})	132 (5.7)	n.d.
SP (mg L^{-1})	47.5 (4.4)	3.4 (0.6)

n.d.: not detected

Results and discussion

SCOD removal efficiency was approximately 48% for RTE and 71% for RPP (Table 2). The higher SCOD removal efficiency achieved in photobiorreactor RPP could be due to the higher BOD_5/TCOD ratio for PP wastewater (0.59) than for TE wastewater (0.10).

High NH_4^+-N removal efficiencies (up to 90%) were achieved in both photobioreactors. Ammonia volatilization accounted for 25% in RTE, and 3% in RPP. This difference could be attributed to the higher pH achieved in reactor RTE (8.8) in comparison to RPP (8.0), as a consequence of the higher bacterial activity in photobioreactor RPP due to higher SCOD removal in that reactor. SP removal efficiency differences (Table 2) could be attributed to the lower SP loading rate (4.75 and 0.34 mg L^{-1} d^{-1}) in RTE and RPP respectively.

Significant differences were detected in protein content in biomass produced in RTE (8.6%) and in RPP (26.6 %), as shown in Table 3. These differences could be attributed to higher ammonia volatilization in RTE, therefore lower ammonia assimilation by biomass occurred in this reactor. Significant differences were obtained in lipid content between biomass produced in RTE and RPP (4.3 and 30.2 %, respectively). These differences could be likely due to higher SP concentration in TE than in PP wastewater. In conclussion, microalgae-bacteria consortia presented high organic matter and nutrient removal efficiencies and biomass composition was highly influenced by nutrient concentration in photobioreactors.

Table 2. *Different parameter removal efficiencies (%) in photobioreactor RTE and RPP. Standard deviation is shown in brackets.*

	RTE	RPP
TCOD reduction (%)	46.6 (6.6)	51.4 (13.5)
SCOD reduction (%)	48.1 (8.1)	70.6 (11.4)
$NH4^+$-N reduction (%)	90.1 (15.2)	> 99
$NH4^+$-N stripped (%)	25.4 (8)	2.9 (1.3)
Soluble Phosphorus (%)	48.4 (8.3)	71.9 (6.3)

Table 3. *Biomass composition in protein, lipid, carbohydrates and ashes content in photobioreactor RTE and RPP.*

Composition	RTE	RPP
Proteins (%)	8.60	26.60
Lipids (%)	4.30	30.20
Carbohydrates (%)	86.0	40.70
Ashes (%)	1.10	2.60

Acknowledgments

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Physical pre-treatments of three microalgal species as a previous step to produce bioethanol

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Introduction

Liquid biofuels are expected to provide the main contribution to decrease fossil fuels dependence, being bioethanol which has assumed a leading position among biofuels mostly from first generation technologies. Bioethanol is produced by fermenting simple sugars from biomass in the presence of microorganisms, mainly yeasts.

Most of the carbohydrates present in microalgae are contained within the cell wall; thus, previous to fermentation an efficient pre-treatment must be performed in order to release fermentable sugars. This work presents a comparative study of the efficiency of several physical pre-treatments using *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus almeriensis,* which have different cell wall structure and sugar composition.

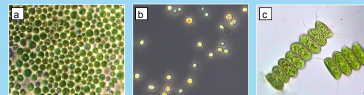

Figure 1. *Microalgal species used: a)* Chlorella sorokiniana *(50X),* *b)* Nannochloropsis gaditana *(100X oil-immersion objective) and* *c)* Scenedesmus almeriensis *(50X).*

Materials and Methods

The microalgae *C. sorokiniana* was obtained from the culture collection of the University of Goettingen (Germany) and the microalgae *N. gaditana* and *S. almeriensis* were obtained in lyophilized form from the Food Innovation and Sustainability Center in Almería, Spain (**Figure 1**).

Three physical pre-treatments (microwave, sonication and autoclaving) were performed for microalgal cell wall breakdown. i) The microwave pre-treatment was carried out with microalgal biomass in a concentration of 200 g volatile suspended solids L^{-1} and then microwaved for 40 seconds and 150 W, followed by an ice bath for 10 min. This procedure was repeated three times. ii) The sonication experiments were performed with biomass at a concentration of 30 g VSS L^{-1}. In the first experiment biomass was sonicated with an amplitude of 90%, for 1 min and 400 W for three times. In the second experiment biomass was sonicated once for 6 min. iii) The autoclaving assays were carried out with microalgal biomass at a concentration of 30 g VSS L^{-1}. The effect of temperature on sugar extraction was evaluated by autoclaving the biomass at 121 ºC for 30, 45, 60 and 90 min.

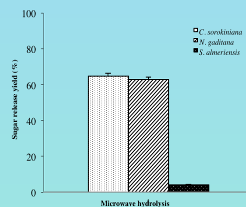

Figure 2. *Effect of microwave hydrolysis on* C. sorokiniana, N. gaditana and S. almeriensis.

Results and discussion

The efficiency of microwave pre-treatment in sugar release yield (SRY) varied considerably among the three-studied microalgae (**Figure 2**). SRY was 64.6% for *C. sorokiniana*, approximately 62.7% for *N. gaditana*, and less than 4.1% for *S. almeriensis*. Differences in SRY could be attributed to the thinner cell wall of *C. sorokiniana* and *N. gaditana* compared to *S. almeriensis*.

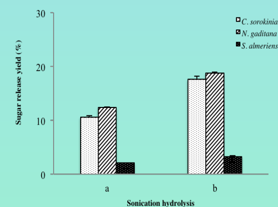

Figure 3. *Effect of sonication hydrolysis on microalgal biomass for 1 min during three consecutive times (a), and once for 6 min (b).*

Figure 3 shows remarkable SRY differences in the efficiency of sonication process between the three-studied microalgae. Independently of the biomass species, a higher cell disruption was achieved when microalgal biomass was submitted to sonication for 6 min. Probably, it could be attributed to the combined effect of sonication and high temperature (about 75 ºC) reached in the case of 6 min of sonication time, compared to assays performed three times for 1 min (37 ºC); thus, temperature seems to play an important role in SRY in these three microalgal species. Inter-specific differences in SRY may be attributed to cell wall thickness and carbohydrates composition; being *S. almeriensis* SRY lower.

In **Figure 4** are represented the effects of autoclaving time on SRY. Important differences were observed between not autoclaved (n.a.) microalgae and autoclaved algae for 30, 45, 60 and 90 min. The effect of time of autoclave increased SRY in all microalgal species, being specially evidenced in *C. sorokiniana.* In autoclave hydrolysis, differences between the three microalgal species resulted considerably lower than previous assays; thus, this pre-treatment was specially efficient in *S. almeriensis* SRY, obtaining higher than 75% of sugars when microalgae was autoclaved for 90 min.

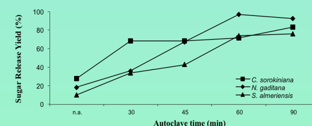

Figure 4. *Effect of autoclave hydrolysis on microalgal biomass for 30, 45, 60 and 90 min. n.a.: not autoclaved.*

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Alg'n' Chem 2014
Which future for algae in industry?
Montpellier, France

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14° EUROPEAN MEETING ON SUPERCRITICAL FLUIDS
MARSEILLE, 18-21 MAY 2014

MICROWAVE AS A PRE-TREATMENT TO FACILITATE THE SUPERCRITICAL EXTRACTION OF LIPIDS FROM MICROALGAE

Approach

Microalgae:
one of the most promising alternative sources for biodiesel [1]

Oil extraction:
Microwaving, which causes a rapid alignment and realignment of dipoles in a polar solvent, resulting in a heat generation, can alter and **break down the cell structures** [2]

In this study:
Microwave (MW) is studied as **a pre-treatment previous to $SCCO_2$**

Aim of the work

Find out the microalgae species whose supercritical extraction is positively affected by a microwave pre-treatment and to determine its effect on lipid content.

Experimental

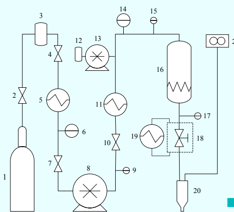
Pretreatment: lyophilized microalgae powder was grounded and sieved.

?

Microwave: 1 and 5 minutes, at 1.2 kW and 2.45 GHz.

$SCCO_2$ extraction:
Microalgae: 0.5 g
CO_2 flow rate: 0.4 kg/h
Co-solvent: 5% (v/v)
Time: 90 minutes
Pressure: 30 MPa
Temperature: 45°C

Schematic diagram of the supercritical extraction equipment: 1. CO_2 tank; 2, 4, 7, 10. Valves; 3. CO2 container; 5. Cooler; 6, 14. Pressure gauges; 8. High pressure pump; 9, 15, 17. Temperature indicators; 11, 19. Heater; 12. Co-solvent container; 13. Co-solvent pump; 16. Extraction cell; 18. Depressurization valve immersed in a water bath; 20. Collector; 21. Flow meter



Comparative extraction methods:
Soxhlet: methanol: chloroform 2:1 (v/v) At 105 °C during 18 hours.
Kochert: methanol: chloroform 1:2 (v/v). [3]

Analysis: gas chromatography.

Results

➢ ***Comparison between Kochert, Soxhlet and $SCCO_2$ extraction methods***

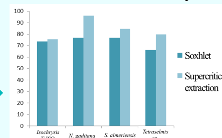
Lipid yield

Extract percentage[a]	Kochert	Soxhlet	$SCCO_2$
Isochrysis T-ISO	12.7	23.1	14.7
N. gaditana	19.1	17.7	12.9
S. almeriensis	15.7	22.4	13.2
Tetraselmis sp.	14.5	18.1	14.1

[a] Extract percentage= (mass of microalgae extract/mass of dried microalgae powder)×100

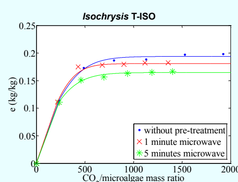
- *N. gaditana* was the only microalgae strain in which Kochert method yielded higher extract percentage than Soxhlet method.
- Comparing Soxhlet and $SCCO_2$ methods, a higher lipid yield was obtained by the first one from all microalgae strains tested.

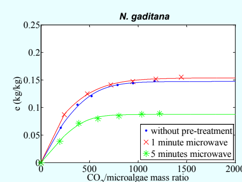
FFA content of the oil extracted by Soxhlet and by $SCCO_2$

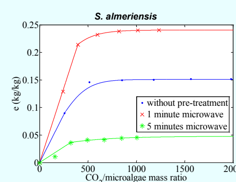


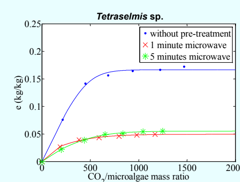
The FFA content of the oil extracted by Soxhlet was lower than the one obtained by $SCCO_2$ for all the microalgae species: **$SCCO_2$ is more selective**.

➢ ***$SCCO_2$: Effect of the microwave pre-treatment on the extraction yield***









Microwave pre-treatment slightly affects the supercritical extraction.

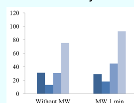
1 min MW: practically no differences were found on the yield.
5 min MW: the yield decreased considerably.

1 min MW: the yield increased considerably.
5 min MW: considerable decrease of lipid yield.

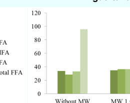
It was negatively affected by the microwave pre-treatment regardless of the time.

➢ ***$SCCO_2$ Effect of the microwave pre-treatment on the fatty acids content***

Isochrysis T-ISO



N. gaditana



Microwave pre-treatment for 1 minute:
total FFA is higher when the microwave pre-treatment during 1 minute is applied.

S. almeriensis

Tetraselmis sp.

Microwave pre-treatment for 5 minutes:
considerable decrease in FFA content.

Conclusions

- **Supercritical extraction on *S. almeriensis*, whose wall is described as one of the most resistant, is strongly improved by the microwave pre-treatment.**
- **The total content of fatty acids extracted is higher when the microwave pre-treatment is applied for one minute on the two microalgae strains investigated, *Isochrysis* T-ISO and *N. gaditana*.**
- **Exposing the microalgae to microwave during 5 minutes lowers lipids extraction by $SCCO_2$ regardless of the species.**

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Growth of microalgal biomass in raceways using agroindustrial wastewater: Biomass production and fatty acids accumulation

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Approach

- *Agroindustrial wastewater is an interesting source of nutriens to grow microalgae for extraction of fatty acids.*
- *Supercritical carbon dioxide has shown to be an efficient technique to extract fatty acids.*
- *Microalgae efficiently remove nutrients excess from agroindustrial wastewater*

Objective

This work evaluates the use of slaughterhouse wastewater (SW) as culture medium for the production of microalgal biomass. Furthermore, the influence of operational conditions, temperature and light supply in the accumulation and composition of fatty acids and in microalgal biomass production was evaluated.

Materials and methods

Microalgal biomass were collected form a lagoon containing aerobically treated swine manure located in Segovia. It was mainly composed by the genus *Rhizopoda* (35%), *Chlorella* (23%) and *Chlamydomonas* (22%) among others.

Two identical 75 L open-raceway ponds were inoculated with microalgae, performed at different operational conditions **(Table 1).** In one raceway (R1) temperature and light was controlled and the second raceway (R2) was performed under natural light/dark cycles inside a greenhouse.

Agroindustrial wastewater was collected from a slaughterhouse located in Segovia (Spain). Wastewater was diluted three times, homogenized and stored at 4 ºC for further use. Chemical characterization of SW is shown in **Table 2**.

***Table 1.** Operational conditions.*
Standard deviation is shown in brackets.

	R1	R2
HRT Period I (days)	10	10
HRT Period II (days)	15	15
Temp (ºC)	25 (2.2)	19 (5.8)
Temp max (ºC)	27.4	34.0
Temp min (ºC)	21.2	3.5
Time illumination (h)	12	13.2 (1.1)
Light (W/m²)	123	266

***Table 2.** Characterization of SW.*
Standard deviation is shown in brackets.

	SW
pH	7.31 (0.3)
TS (mg/L)	330 (110)
VS (mg/L)	320 (95)
TCOD (mg/L)	629 (164)
TKN (mg/L)	53 (21)
NH4+ (mg/L)	7.14 (3.84)
SP (mg/L)	1.32 (0.60)

Results and discussion

The results showed a high influence of operational conditions in the biomass growth (**Fig. 1**). Biomass productivity increased up to 80% for R1 and up to 60% for R2 when HRT decreased. Furthermore, differences in biomass production between both raceway ponds resulted higher than 35%, that probably may be attributed to remarkable differences in light supply between R1 compared to R2. Then, lipids were extracted from microalgae by supercritical carbon dioxide extraction method. Fatty acids (FFA) content in produced biomass was similar in both raceway ponds, regardless the HRT applied. Saturated FFA supposed more than 50% of total FFA in all cases (**Fig. 2**). Small differences were observed in monounsaturated and polyunsaturated FFA concentration (less than 6%) in all operational conditions. Finally, the wastewater was depurated when using microalgal-based systems, obtaining an organic matter removal higher than 80% and 70% in R1 and R2, respectively. R2 showed higher ammonium and soluble phosphorous removal than R1 under the same HRT, with maximum removals of 76% for ammonium and 81% for soluble phosphorous. Therefore, microalgae growth using wastewater could reduce cost production for further valorisation for the biomass as biofuel, as well as treating a high-contaminated wastewater.

***Figure 1.** Daily harvested biomass production in R1 and R2.*

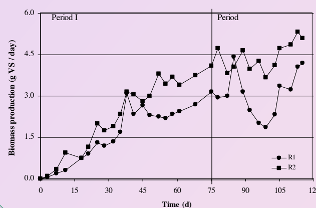
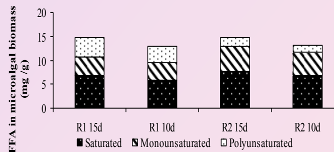


***Figure 2.** Composition of saturated, monounsaturated and polyunsaturated fatty acids extracted from microalgae in R1 and R2 at 15 and 10 days HRT.*



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Anaerobic digestion of microalgal-bacterial biomass growth in fish processing wastewater: impact of substrate/inoculum ratio

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BACKGROUND

Microalgal-based systems are currently receiving important attention due to their ability to treat wastewater, whilst also providing a high-added value product in the form of biomass. When the lipid content of the biomass produced in these systems does not exceed 40%, anaerobic digestion appears to be the optimal strategy for the energetic recovery of cell biomass on an energy balance basis (Sialve et al., 2009).

Objective

To evaluate the influence of substrate/inoculum ratio on anaerobic digestion of biomass produced in microalgal-based systems for fish processing wastewater treatment.

MATERIALS AND METHODS

Anaerobic biodegradability experimental set up

- Batch assays at mesophilic 35±2ºC.

-Microalgal-bacterial biomass grown in a photobioreactor treating fish processing wastewater was used.

- Anaerobic sludge was used as inoculum.

- Blanks (only inoculum) to determine the endogenous methane production.

- Substrate/inoculum ratio: between 0.5 and 2.0 g COD/g VS

Table 1. *Characterization of microalgal biomass used as substrate*

TCOD (g L^{-1})	5.5±0.5
% Lipids	18.9
% Proteins	30.8
% Carbohydrates	45.9

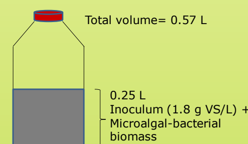

Figure 1. *Bottles used for batch experiments.*

RESULTS AND CONCLUSIONS

✓ Methane yield decreased from 321.8 to 191.5 mL CH_4 g COD^{-1} added when COD/VS ratio increased from 0.5 to 2.0 g COD/g VS (Fig.1). Thus, the COD/VS ratio highly influenced on methane yield.

✓ Methane content in biogas ranged between 73.1 to 76.1% indicating a good conversion of microalgal biomass into methane. The stability of the process was also corroborated by pH values and ammonia concentrations at the end of the experiments (Table 2).

✓ Low methane yields could be attributed to the resistance of the microalgal cell wall to bacterial attack. Algal pretreatment would enhance the energetic and economical balance of the anaerobic process.

✓ Biochemical composition of the microalgal biomass is another key factor in methane production. Thus, even though lipids represented 18.9% of the dry matter, they accounted for a significant (35%) to the methane potential production since they present a relative high energy content compared with carbohydrates and proteins.

Table 2. *Intial and final pH and NH4+-N concentration in batch experiments for the different substrate/inoculum ratios.*

Ratio (g COD/ gVS)	pH		NH_4^+-N	
	Initial	Final	Initial	Final
0.5	7.4	7.0	27.3	58.1
1.0	7.2	7.1	37.0	89.5
1.5	6.6	7.0	45.1	116.8
2.0	6.9	7.1	46.1	135.3

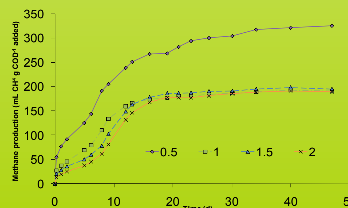

Figure 2. *Evolution of methane yields with time for the evaluated COD/VD ratios.*

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RECOVERY AND VALORIZATION OF NUTRIENTS FROM SLAUGHTERHOUSE WASTEWATER THROUGH MICROALGAE CULTIVATION IN HIGH RATE ALGAL PONDS

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Introduction

The European Commission estimated that European slaughterhouse industry produced approximately 150 millions m^3 of slaughterhouse wastewater (SW) per year. This wastewater must be treated previously to be discharged into rivers, aquifers or the municipal wastewater network. The use of microalgal-based systems may be a suitable technology to recover nutrients from wastewater and to valorise the resulting biomass by different processes.

The aim of this study was to evaluate the effect of light and hydraulic retention time, in the SW treatment efficiency and in the microalgal biomass productivity. To achieve this objective, the performance of two high rate algal ponds (HRAPs) were compared: one operated under greenhouse conditions of temperature and light supply, and another placed indoors under controlled conditions of temperature and light supply. Then, the produced biomass was characterized to determine its biochemical profile.

Materials and Methods

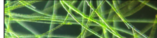
Microorganisms:

Chlamydomonas subcaudata

Anabaena sp.

Nitzschia sp.

Phormidium tergestinum



Aerobic sludge

The HRAPs were inoculated with 25 and 12 mg VSS/L of microalgae and aerobic sludge, respectively.

Experimental set up:

Two HRAPs with a surface of 0.43 m^2 and 75 L.

- *Controlled conditions* (4,500 lux; 25 ± 2 ºC) → R1
- *Greenhouse conditions* (40,000 lux; 20 ± 6 ºC) → R2

Period I (HRT = 15 days) lasted 75 days and Period II (HRT = 10 days) lasted 40 days. During both periods, organic loading rate (OLR), ammonium loading rate (ALR) and soluble phosphorous loading rate (SPLR) were modified.

Period I → OLR = 36 mg/L; ALR =0.2 mg/L; SPLR = 0.09 mg/L

Period II → OLR = 54 mg/L; ALR = 0.3 mg/L; SPLR =0.14 mg/L

Table 1. Characterization of slaughterhouse wastewater (SW). Standard deviation is shown in brackets.

Parameter	SW
pH	7.3 (0.4)
TS (mg/L)	753 (70)
VS (mg/L)	702 (66)
TCOD (mg/L)	1621 (105)
SCOD (mg/L)	1009 (123)
TKN (mg/L)	149.2 (11)
NH_4^+-N (mg/L)	9.2 (1.6)
NO_3^--N (mg/L)	0.44 (0.18)
NO_2^--N (mg/L)	0.03 (0.02)
TP (mg/L)	1.4 (0.1)
SP (mg/L)	1.3 (0.1)

Results and Discussion

Removal Efficiencies:

The removal efficiencies between R1 and R2 during period I and II were compared (**Figure 1**).

- Higher TCOD and SCOD removal efficiencies were obtained during period II than period I.
- Higher TCOD and SCOD removal efficiencies were obtained in R1 than in R2, which could be attributed to a microalgal change from autotrophic to heterotrophic behaviour (**Figure 2**), removing higher organic matter.
- The main mechanism for ammonium and nitrate removal was biomass assimilation as evidenced the daily TKN uptake (0.12 and 0.09g TKN/g TS in R1 and R2, respectively).
- Higher SP was removed in R2 than in R1.

Biomass productivity and theoretical Photosynthetic Efficiency (tPE):

The biomass growth was measured as VSS of produced biomass per day and square meter.

Controlled conditions (R1) → Period I = 4.2 g VSS /m^2; tPE = 5.5%; Period II = 9.7 g VSS /m^2; tPE = 8.9%

Greenhouse conditions (R2) → Period I = 7,2 g VSS /m^2; tPE = 2.5%; Period II = 10,6 g VSS /m^2; tPE = 3.9%

The differences in productivity observed between photobioreactors may be attributed to a higher light supply received in R2 (40,000 lux) than in R1 (4,500 lux).

The increase in biomass productivity observed in R1-PII probably may not be attributed to an improved photosynthetic efficiency, but it may be the result of a trophic switch in microalgal population, from autotrophic to heterotrophic behaviour. This change is clearly observed in **Figure 2**.

Characterization of microalgal biomass

Considerable variations in biochemical composition were observed when biomass from R1 and R2 was compared (**Figure 3**).

The differences in protein concentration between R1 and R2 were close to 10%, ranging from 58% (R1) to 46% (R2). Similar differences were also measured in carbohydrate content, being the average concentration in R1 14% and in R2 23%. The accumulation of carbohydrates in R2 may be a consequence of an excess of light supply (stress conditions), that would trigger an accumulation of carbohydrates.

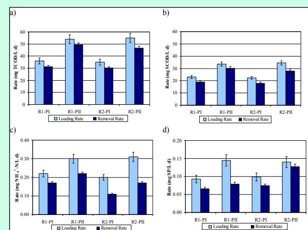


Figure 1. (a) TCOD, (b) SCOD, (c) ammonium loading rate and (d) soluble phosphorous loading rates and removal rates in R1 and R2 during period I and II.

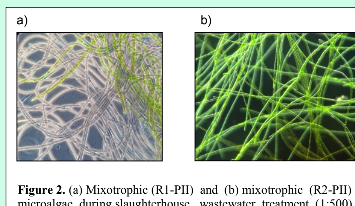


Figure 2. (a) Mixotrophic (R1-PII) and (b) mixotrophic (R2-PII) microalgae during slaughterhouse wastewater treatment (1:500)

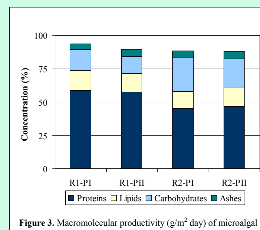


Figure 3. Macromolecular productivity (g/m^2 day) of microalgal Biomass from R1 and R2 during period I and II.

Conclusions

✓ **The slaughterhouse wastewater can be efficiently treated by microalgal-bacteria consortium. These microorganisms removed efficiently organic matter and nutrients from wastewater, achieving efficiencies higher than 84%, 70% and 90% for TCOD, ammonium and soluble phosphorous, respectively.**

✓ **Light limitation led a trophic change in microalgal biomass changing from autotrophic to heterotrophic microorganisms.**

✓ **Excess of light in R2 led to an accumulation of carbohydrates in biomass achieving values of 25%.**

✓ **The highest biomass production was achieved under greenhouse conditions with an HRT of 10 days.**

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