# BIOLOGICAL TOOLS TO IMPROVE BIOGAS PRODUCTION FROM MICROALGAE BIOMASS

PhD Thesis


Presented by

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# CERTIFICADO

**Dr. Cristina González Fernández**, Investigador Titular - Procesos Biotecnológicos en IMDEA Energía y **Dr. Mercedes Ballesteros Perdices**, Jefe de la Unidad de Procesos Biotecnológicos en IMDEA Energía y Jefe de la Unidad de Biocarburantes del CIEMAT

**Certifica**

Que **Ahmed Abdelmohsen Mahdy Mohamed** ha realizado bajo su dirección y en las instalaciones de IMDEA Energía la presente tesis doctoral titulada:

*¨BIOLOGICAL TOOLS TO IMPROVE BIOGAS PRODUCTION FROM MICROALGAE BIOMASS¨*

La presente tesis ha cumplido con los objetivos planteados y dado resultados innovadores y originales. Por lo tanto, expresa su conformidad para proceder a la defensa pública de la tesis para optar al grado de Doctor.

Dr. Cristina González Fernández | Dr. Mercedes Ballesteros Perdices

Madrid, 29 de septiembre de 2016

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## PREAMBLE

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I. **Mahdy, A.**, Mendez, L., Ballesteros, M., González-Fernández, C., **2014**. Autohydrolysis and alkaline pretreatment effect on *Chlorella vulgaris* and *Scenedesmus* sp. methane production. Energy 78, 48-52.

II. **Mahdy, A.**, Mendez, L., Ballesteros, M., González-Fernández, C., **2014.** Enhanced methane production of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* by hydrolytic enzymes addition. Energy Conversion Management 85, 551-557.

III. **Mahdy, A.**, Mendez, L., Blanco, S., Ballesteros, M., González-Fernández, C., **2014**. Protease cell wall degradation of *Chlorella vulgaris*: effect on methane production. Bioresource Technology 171, 421-427.

IV. **Mahdy, A.**, Mendez, L., Ballesteros, M., González-Fernández, C., **2015**. Protease pretreated *Chlorella vulgaris* biomass bioconversion to methane via semicontinuous anaerobic digestion. Fuel 158, 35-41.

V. Demuez, M., **Mahdy, A.**, Tomas-Pejó, E., González-Fernández, C., Ballesteros, M., **2015**. Enzymatic cell disruption of microalgae biomass in biorefinery processes. Biotechnology and Bioengineering 112, 1955-1966.

VI. **Mahdy, A.**, Mendez, L., Tomas-Pejó, E., Morales, M.M., Ballesteros, M., González-Fernández, C., **2016**. Influence of the enzymatic hydrolysis on the biochemical methane production of *Chlorella vulgaris* and *Scenedesmus* sp. Journal of Chemical Technology and Biotechnology 91(5),1299-1305.

VII. **Mahdy, A.**, Ballesteros, M., González-Fernández, C., **2016**. Enzymatic pretreatment of *Chlorella vulgaris* for biogas production influence of urban wastewater as a sole nutrient source on macromolecular profile and biocatalyst efficiency. Bioresource Technology 199, 319-325.

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VIII. **Mahdy, A.,** Mendez, L., Demuez, M., Ballesteros, M., González-Fernández, C., **2013**. Microalgae carbohydrates profile: the effect of nutrients limitation. 2-CIAB 2nd Iberoamerican Congress on Biorefineries. Jaen- Spain. (Poster). http://www.ifeja.org/ciab/ingles/contenidos.php?secc=1039

IX. **Mahdy, A.**, Mendez, L., Ballesteros, M., González-Fernández, C., **2014.** Enzymatic pre-treatment: a promising procedure to enhance *Chlorella vulgaris* biomethane production. International Congress on Green Chemistry and Sustainable Engineering. Barcelona-Spain. (Oral presentation)

**ABSTRACT**

Great efforts are undertaken to develop biofuel-based microalgae biomass as replacements to non-renewable fossil fuels. According to the European directive on Renewable Energy (2009/28/EC) (RED, 2009), special emphasis should be placed on the production of renewable energy by stating that 10% of energy in transport should be renewable by 2020. Out of the possible energy forms, biogas production is envisaged as a feasible route to sustainable energy production. Biogas production has strong potential over other energy forms since its needs less concentrated biomass and no macromolecular extraction. However, due to the hard cell wall and low C/N ratio of microalgae biomass, its practical application is prevented by the low biogas yields obtained. Several possibilities for overcoming those limitations were investigated in this Doctoral Thesis in order to enhance microalgae anaerobic biodegradability. Even though some strains exhibit no cell wall, strains with hard cell wall prevail in outdoor culture conditions. Thus, strains possessing rigid cell walls are resistant to anaerobic digestion and the application of suitable pretreatments are highly required.

In this Doctoral Thesis, autohydrolysis and biological pretreatments have been applied in different microalgae biomass (namely, *Chlorella vulgaris*, *Scenedesmus* sp. and *Chlamydomonas reinhardtii*). The pretreatments were evaluated with regard to organic matter, protein, carbohydrate solubilisation as well as methane production enhancement. Autohydrolysis induced by low temperature application resulted in low organic matter solubilisation (6-16%) and low carbohydrates solubilisation (15-31%) and accordingly, methane production was enhanced to minor extent with *C. vulgaris* biomass (only 10% higher compared to raw biomass, 127 mL $CH_4$ g COD $in^{-1}$) or even negligible in the case of *Scenedesmus* biomass. In this context, it was concluded that autohydrolysis was unable to break down efficiently the complex cell wall of microalgae biomass and alternative approaches were studied to maximize biomass hydrolysis efficiency and justify the pretreatments costs.

Since carbohydrates have been traditionally recognized as the polymers responsible for the low microalgae digestibility and microalgae biomass exhibit typically a high protein content, microalgae biomass was subjected to two groups of biocatalysts, namely carbohydrases and proteases for microalgae hydrolysis prior to anaerobic digestion. Out of the several carbohydrases tested (Pectinase, Viscozyme and Celluclast), Viscozyme addition resulted in the highest carbohydrates solubilisation (85%, 96% and 36% with *C. vulgaris*, *C. reinhardtii* and *Scenedesmus* sp., respectively). Despite of the high carbohydrates solubisation, the increase on methane yield was low (14-16% with *C. vulgaris* and *Scenedesmus* sp. and negligible with *C. reinhardtii*).

In the case of *C. reinhardtii* biomass, the application of biocatalysts did not report significant enhancement since the raw biomass was already easily digestible (263 mL $CH_4$ g COD $in^{-1}$). Even though different microalgae biomass used herein exhibited different cell wall composition and/or different macromolecular distribution, protease addition resulted in highest organic matter solubilisation (47-57%) and highest methane yield (1.5-1.7-fold higher compared to raw biomass) for two of the most robust microalgae biomass, namely *C. vulgaris* and *Scenedesmus* sp. Carbohydrases and protease were also combined to verify potential synergetic effect on biomass hydrolysis but the results showed methane yield enhancement similar to that attained with protease pretreated biomass.

Different experiments were conducted in order to optimize protease hydrolysis of *C. vulgaris* biomass. The attempt of decreasing the enzymatic dosages (from 0.585 down to 0.146 AU g $DW^{-1}$) diminished hydrolysis efficiency (from 50 to 41% in COD terms) concomitantly with a decreased methane yield enhancement (from 256 down to 224 mL $CH_4$ g COD $in^{-1}$). The best result was achieved with protease dosage at 0.585 AU g $DW^{-1}$ by enhancing methane yield 1.73-fold. Additionally, increasing biomass loads from 16 up to 65 g $L^{-1}$ did not affect markedly the hydrolysis efficiency (51%) nor methane yield enhancement (1.55-fold) when using protease dosage of 0.585 AU g $DW^{-1}$.

Since the proteolytic enzyme gave promising results in batch mode anaerobic digestion, further investigation using this biocatalyst as pretreatment of *C. vulgaris* was assessed in semicontinuously fed reactors (CSTR). The CSTR was operated at organic loading rate (OLR) of 1.5 g COD $L^{-1}$ $d^{-1}$ and hydraulic retention time (HRT) of 20 days. In contrast to that attained in batch anaerobic digestion, feeding the CSTR with protease pretreated biomass showed volatile fatty acids (VFA) accumulation as a result of ammonium inhibition (1.9 g $N\text{-}NH_4^+$ $L^{-1}$). Therefore, a stepwise reduction in methane production rate and yield was observed throughout the digestion time. To overcome this issue, the protein content of microalgae biomass was reduced by cultivating the microalgae under nutrients limited conditions (wastewater was used a sole nutrient source). More specifically, *C. vulgaris* protein content decreased from 65% to 33% (VSS basis). In response to that macromolecular profile change, no VFAs accumulation nor ammonium inhibition were registered in the CSTR fed with carbohydrate-rich *C. vulgaris* and a steady methane production rate was displayed along digestion. In addition, this carbohydrate rich biomass was also pretreated by using carbohydrases and fed to the CSTR, nevertheless, the results evidenced an increase in methane yield (3-fold compared to raw biomass 25 mL $CH_4$ g COD $in^{-1}$) but still lower than the enhancement attained with protease pretreated biomass (5-fold). Once circumvented the high ammonium concentration in the digesters and attained a stable methane production with protease pretreated carbohydrate-rich *C. vulgaris* biomass, the OLR was doubled (3 Kg COD $m^{-3}$ $day^{-1}$) and the HRT shortened (15 days) to verify anaerobic digestion stability. The data obtained in the later experimental conditions denoted not only the same stability but even higher methane production was achieved (6.3- fold higher methane yield compared to raw biomass).

Overall, this Thesis highlights the crucial role of microalgae proteins on their anaerobic digestion. Biomass protein content in *C. vulgaris* and *Scenedesmus* sp. hampered the anaerobic digestion in two ways, firstly as a polymer embedded in microalgae cell wall decreasing the access of anaerobes for degradation and secondly, by causing methanogens inhibition due to the high

ammonium concentration reached in the anaerobic digester when feeding protease pretreated biomass. The possible solutions (pretreatment with proteases and enrichment of biomass in carbohydrate fraction) to overcome the negative effects of high protein content of this microalgae biomass were deeply discussed and evaluated throughout the Thesis.

# RESUMEN

En los últimos años, se han dedicado grandes esfuerzos al desarrollo de biocombustibles basados en biomasa algal como reemplazo a los derivados de combustibles fósiles. De acuerdo con la Directiva de la Unión Europea relativa al fomento y uso de energía procedente de fuentes renovables, (2009/28/EC), al menos el 10% de la energía consumida en el sector transporte ha de ser de origen renovable en 2020. En ese contexto, la producción de biogás a partir de biomasa algal se enmarca como una de las rutas más factible para producir energía de una forma sostenible. La producción de biogás no necesita que la biomasa esté altamente concentrada y tampoco requiere la extracción de ninguna macromolécula, lo que resulta ventajoso frente a la generación de otras formas energéticas. Sin embargo, la dura pared celular de algunas microalgas y la baja relacion C/N hace que los rendimientos de biogás sean relativamente bajos, lo que limita su aplicación. A pesar de que algunas especies de microalgas no tienen pared celular, las especies cultivadas al aire libre presentan paredes celulares muy robustas. Las microalgas con este tipo de paredes celulares duras son altamente resistentes y es necesario aplicar un pretratamiento apropiado previo a la digestión anaerobia. En este estudio, se han investigado diferentes posibilidades para superar dichas limitaciones y aumentar así la biodegradabilidad anaerobia de las microalgas.

En la presente Tesis Doctoral, se han estudiado tanto la autohidrólisis como los pretratamientos mediante la adición de biocatalizadores a diferentes biomasas de microalgas (*Chlorella vulgaris*, *Scenedesmus* sp. y *Chlamydomonas reinhardtii*). La eficiencia de los pretratamientos fue evaluada en términos de solubilización de materia orgánica, proteínas y carbohidratos, al igual que por medio del aumento de producción de metano. La autohidrólisis inducida por la aplicación de baja temperatura resultó en una baja solubilización de materia orgánica (6-16%) y carbohidratos (15-31%) y por tanto, la producción de metano fue sólo un 10% mayor que en la biomasa fresca en el caso de la biomasa de *C. vulgaris* (127 mL $CH_4$ g COD $in^{-1}$) y despreciable en el caso de *Scenedesmus* sp. En este contexto, se concluyó que la autohidrólisis no era un método efectivo para romper la pared celular

de la biomasa algal y se estudiaron métodos alternativos que permitieran maximizar la cantidad de biogás producido y poder así justificar su coste.

Puesto que los carbohidratos han sido tradicionalmente reconocidos como los polímeros que confieren una baja digestibilidad a las microalgas y esta biomasa presenta típicamente un alto contenido proteico, se sometió la biomasa algal a la acción de dos biocatalizadores (i.e. carbohidrolasas y proteasas). De las diferentes carbohidrolasas estudiadas (Pectinase, Viscozyme y Celluclast), la adición de Viscozyme resulto en una alta solubilización de carbohidratos (85%, 96% y 36% para *C. vulgaris*, *C. reinhardtii* y *Scenedesmus* sp., respectivamente). No obstante, a pesar de la alta solubilización de carbohidratos, el aumento en términos de rendimiento metanogénico fue de 14-16% para *C. vulgaris* y *Scenedesmus* sp. y prácticamente despreciable para *C. reinhardtii*.

En el caso de *C. reinhardtii*, la biomasa fresca ya era fácilmente degradable (263 mL $CH_4$ g DQO $in^{-1}$) y por lo tanto, la adición de biocatalizadores no produjo ningún incremento significativo al potencial metanogénico. A pesar de que en esta Tesis Doctoral se han estudiado diferentes biomasas algales con diferentes perfiles macromoleculares, la adición de proteasa resultó en todos los casos en una mayor solubilización de materia orgánica (47-57%) y mayor rendimiento metanogénico (1.5-1.7 veces mayor que la biomasa fresca) para dos de las algas con paredes celulares más duras, *Chlorella vulgaris* y *Scenedesmus* sp. En este trabajo se combinaron también carbohidrasas y proteasas para verificar si existía un efecto sinérgico entre ambas enzimas pero los resultados indicaron que el rendimiento metanogénico era similar al que se obtenía con la biomasa pretratada exclusivamente con proteasa.

Una vez concluido el importante efecto de las proteasas sobre la digestibilidad, se llevaron a cabo una serie de experimentos para optimizar el tratamiento con proteasa sobre la biomasa de *C. vulgaris*. La disminución de la dosis enzimática (de 0.585 a 0.146 AU g $PS^{-1}$), disminuyó también la eficiencia de hidrólisis (de 50% a 41% en términos de DQO) y resultó en un menor incremento del rendimiento metanogénico (de 256 a 224 mL $CH_4$ g COD $in^{-1}$). El mejor resultado se obtuvo con una dosis de proteasa de 0.585

AU g $PS^{-1}$, que aumentó el rendimiento metanogénico 1.73 veces en comparación con la biomasa fresca. Además, empleando una dosis de proteasa fija de 0.585 AU g $PS^{-1}$, se incrementó la carga de biomasa de 16 a 65 g $L^{-1}$ sin verse afectada la eficiencia hidrolítica (51%) ni el aumento del rendimiento metanogénico (1.55-veces mayor que la biomasa fresca).

Puesto que el cóctel proteolítico proporcionó los mejores resultados en la digestión anaerobia llevada a cabo en modo discontinuo, este trabajo de investigación se centró también en el uso de este pretratamiento en la digestión anaerobia de *C. vulgaris* alimentándose reactores CSTR en modo semicontinuo. Los CSTR fueron operados a una carga orgánica (OLR) de 1.5 g DQO $L^{-1}$ $d^{-1}$ y un tiempo hidráulico de residencia (HTR) de 20 días. Al contrario de lo que se obtuvo en los reactores en discontinuo, en el CSTR alimentado con biomasa algal pretratada con proteasa se observó una acumulación de ácidos grasos volátiles (AGVs) como resultado de una inhibición por alta concentración de amonio (1.9 g $N\text{-}NH_4^+$ $L^{-1}$). Como consecuencia de esta inhibición, se produjo una reducción de la productividad y del rendimiento de metano durante la digestión. Para solventar este problema, se redujo la fracción proteica de la biomasa algal mediante su cultivo en condiciones limitantes de nutrientes (empleando únicamente agua residual como fuente de nutrientes). Más concretamente, el contenido proteico de *C. vulgaris* se redujo de 65% a 33% (en SSV). Como respuesta a este cambio de perfil macromolecular, no se observó ni acumulación de AGVs ni inhibición por amonio en el CSTR alimentado con la biomasa de *C. vulgaris* rica en proteínas y por lo tanto, se obtuvo una productividad de metano constante a lo largo de la digestión. Por otro lado, la biomasa rica en carbohidratos fue pretratada mediante la adición de carbohidrolasa y alimentada también a un CSTR. En este caso se observó un aumento en el rendimiento metanogénico (3 veces mayor comparado con la biomasa fresca 25 mL $CH_4$ g COD $in^{-1}$) pero menor que el obtenido con la biomasa pretratada con proteasas (5 veces mayor comparado con la biomasa fresca). Una vez evitada la alta concentración de amonio en el digestor y estabilizada la producción de metano a partir de *C. vulgaris* rica en carbohidratos y pretratada con proteasas, se aumentó la OLR hasta 3 kg COD $m^{-3}$ $día^{-1}$ y se

disminuyó la HRT a 15 días para verificar la estabilidad de la digestión anaerobia. Los resultados obtenidos bajo estas condiciones experimentales demostraron no solo la misma estabilidad si no 6.3 veces mayor producción de metano.

Este trabajo de Tesis destaca el papel crucial de las proteínas presentes en la microalgas en su digestión anaerobia. El contenido proteico de *C. vulgaris* y *Scenedesmus* sp. obstaculiza su digestión anaerobia de dos posibles maneras. En primer lugar como polímero de proteínas embebido en la pared celular disminuyendo la accesibilidad a las bacterias anaerobias para su degradación. En segundo lugar, la fracción proteica puede causar la inhibición de los microorganismos metanogénicos debido a la alta concentración de amonio alcanzada en el digestor cuando se alimenta con biomasa pretratado con proteasa. Las posibles soluciones (pretratamiento con proteasas y enriquecimiento de la biomasa algal en carbohidratos) para superar los efectos negativos producidos por el alto contenido proteico de estas especies de microalgas han sido ampliamente discutidas y evaluadas a lo largo de esta Tesis.

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# RESUMEN EN CASTELLANO

## Resumen en castellano

### 1. Introducción

El constante agotamiento de los combustibles fósiles ha impulsado la búsqueda de fuentes alternativas de energía renovable para producir biocombustibles (biodiesel, bioetanol y biogás, entre otros). A lo largo de cinco décadas, las microalgas han sido consideradas como materia prima prometedora para la producción de biocombustibles no basada en cultivos alimentarios. A diferencia del biodiesel y bioetanol, que solo transforman en energía una parte de los componentes de las microalgas, la producción de biogás es un proceso más directo donde todas las macromoléculas pueden ser transformadas en biocombustible. La digestión anaerobia es un proceso biológico bien establecido; sin embargo, la utilización de biomasas nuevas como las microalgas hace necesario seguir avanzando en la investigación de este proceso.

Se sabe que la mayoría de las microalgas tienen una pared celular rígida que dificulta su eficiente biodegradabilidad (González-Fernández et al., 2012). De las fases implicadas en la digestión anaerobia, la primera etapa de hidrólisis es la limitante cuando se utiliza como materia prima material particulado como las microalgas. Esta limitación conlleva conversiones de biomasa en biogás muy lentas. La alteración de la pared celular supone un paso imprescindible para alcanzar buenos rendimientos de biogás en el proceso de digestión anaerobia a parir de microalgas. Por ello, se debe realizar un pretratamiento de las microalgas antes de la digestión anaerobia con el fin de alterar la pared celular y solubilizar la materia orgánica que hay en su interior. El pretratamiento permite una mayor accesibilidad de los microorganismos anaeróbicos a la biomasa algal, mejorando así el rendimiento de la conversión en metano de la materia orgánica presente en las microalgas. El factor clave en estos pretratamientos es su viabilidad económica, ya que su coste tiene que justificarse por el aumento de los rendimientos del proceso. Los pretratamientos térmicos han sido los más estudiados pero tienen la limitación de que se generan algunas sustancias tóxicas que pueden dificultar los posteriores procesos de bioconversión. Actualmente han cobrado interés

los pretratamientos a baja temperatura ya que mejoran la biodisponibilidad de la materia orgánica al ataque bacteriano. La autohidrólisis es una respuesta biológica de los microorganismos inducida por el calor que cumple con estos requisitos. A temperaturas moderadas (<55ºC) se favorece la secreción de enzimas hidrolíticas al medio que ayudan a romper la pared celular. Provocar la hidrólisis enzimática de la pared celular de las microalgas mediante la adición de enzimas exógenas también es una prometedora alternativa a los pretatamientos térmicos y mecánicos de alto consumo energético y a las hidrólisis con catalizadores químicos que generan subproductos no deseables. El pretratamiento enzimático no genera sustancias inhibidoras, es respetuoso con el medio ambiente y altamente selectivo. En este trabajo se han estudiado comparado diferentes pretratamientos para aumentar la biodisponibilidad de la biomasa algal durante el proceso de digestión anaerobia.

Además de su rígida pared celular, la composición de las microalgas también se ha identificado como factor que limita la eficacia de la digestión anaerobia. La composición típica de las microalgas puede variar entre el 10-30% de carbohidratos y 2-20% de lípidos (Becker, 2004), prevaleciendo las proteínas como macromolécula predominante. El alto contenido en nitrógeno de las microalgas las convierte en un sustrato problemático para la digestión anaerobia. Durante el proceso de digestión anaerobia los compuestos nitrogenados se transforman finalmente en amoníaco. Altas concentraciones de amoniaco provocan un efecto negativo tanto en la estabilidad del proceso de digestión anaerobia como en su rendimiento, lo que puede conducir a su total inhibición. Una de las opciones para reducir el contenido en proteínas de las microalgas, y aumentar la de otros componentes de interés para el proceso de digestión anaerobia como los lípidos o los carbohidratos, es actuar sobre su metabolismo. En condiciones de limitación de nutrientes las microalgas son capaces de modular su metabolismo y acumular diferentes compuestos. Asimismo, las condiciones operacionales, tales como las características del medio de cultivo, la luz y la temperatura también actúan sobre el metabolismo de las microalgas (González-Fernández y Ballesteros, 2012). Una alternativa muy interesante para reducir el contenido de proteínas

de las microalgas creciéndolas en condiciones limitantes en nutrientes es la utilización de aguas residuales urbanas o industriales como medios de cultivo. Además de permitir la obtención de biomasa algal con bajo contenido en proteínas, combina de manera interesante la depuración de aguas residuales con la producción de biocombustibles. De esta manera la producción de biocombustibles pueden llevarse a cabo junto con el biotratamiento de aguas residuales, lo que añade valor a todo el proceso, reduciendo costes y suministrando biocombustible de manera sostenible.

En este trabajo también se ha estudiado la utilización de aguas residuales como medio de cultivo con el objetivo de reducir el contenido en proteínas de las microalgas y mejorar los rendimientos en metano cuando se utilizan microalgas pretratadas con proteasas como sustrato en el proceso de digestión anaerobia.

El método habitual de determinar la capacidad de producción de metano y la biodegradabilidad anaerobia de un sustrato orgánico es mediante ensayos a escala de laboratorio en reactores discontinuos en los que se denomina Potencial Bioquímico de Metano (PBM), por ello en este trabajo se ha evaluado el efecto del pretratamiento en la mejora de los rendimientos de metano de las microalgas pretratadas en ensayos en discontinuo. Sin embargo, para evaluar los beneficios de un determinado pretratamiento sobre el proceso de digestión anaerobia es indispensable estudiar en profundidad la actuación de los microorganismos anaeróbicos en un reactor en continuo (CSTR) para determinar posibles efectos de inhibición. De hecho, los ensayos en CSTR puede dar lugar a rendimientos de metano más bajos que las observados en los ensayos de discontinuo ya que estos resultados solo pueden extrapolarse a un CSTR cuando no se produce la inhibición. Por ello, en este trabajo se han realizado ensayos de digestión anaerobia con las microalgas pretratadas con proteasas tanto en reactores discontinuos como en CSTR para evaluar los potenciales mecanismos inhibidores que pueden producirse en estos últimos ensayos.

## 2. Objetivos

El objetivo principal de la tesis es optimizar la utilización de la biomasa de microalgas como materia prima para la producción de biometano. Esta optimización se llevó a cabo mediante el estudio de diversos pretratamientos de la biomasa antes de la digestión anaeróbica para romper su pared celular y aumentar su biodegradbilidad y la optimización del proceso de pretratamiento. Una vez determinado el mejor proceso de pretratamiento se estudiaron diferentes alternativas para reducir el efecto de los inhibidores que afectan el proceso de digestión anaerobia.

Los objetivos parciales de este trabajo son:

- Evaluar el efecto de la autohidrólisis y el pretratamiento enzimático (carbohidrasas y proteasas) como agentes disruptores de la pared celular de la biomasa de microalga y su efecto en la mejora de la producción de metano.
- Optimización de pretratamientos enzimáticos con el fin de maximizar su efectividad en la solubilización de la materia orgánica y la mejora de la producción de metano
- Valorar los beneficios del pretratamiento en reactores en continuo (CSTR).
- Utilización de medios de cultivo bajos en nutrientes (aguas residuales) para reducir el contenido en proteínas de las microalgas.

## 3. Material y métodos

### 3.1. Sustrato y inóculo

En este estudio se han utilizado las siguientes microalgas: *Chlorella vulgaris*, *Scenedesmus* sp., y *Chlamydomonas reinhardtii*.

Las microalgas, dependiendo de los ensayos realizados, se crecieron bajo iluminación continua, en medio sintético elemental de Bold modificado o en aguas residuales urbanas (proveniente de la planta de tratamiento de aguas residuales de la Universidad Rey Juan Carlos, Madrid, España).

Como inóculo en los ensayos de digestión anaerobia se utilizaron lodos anaeróbicos amablemente cedidos por la planta de tratamiento de aguas residuales de Valladolid (España).

### 3.2. Pretratamiento de las microalgas

#### 3.2.1. Autohidrólisis

Las biomasas de las microalgas se sometieron a un pretratamiento de baja temperatura: autohidrólisis. El pretratamiento de autohidrólisis se realizó a 50ºC durante 24 y 48 h. La efectividad del pretratamiento se evaluó determinando la solubilización de la materia orgánica y la producción de metano.

#### 3.2.2. Pretratamiento enzimático

##### 3.2.2.1. Biocatalizadores empleados

Se estudió el efecto de diversas carbohidrasas (Celluclast 1.5 L, Viscozyme L y Pectinex-Ultra SP-L) y proteasas (Alcalase 2.5 L). Todas estas enzimas están disponibles comercialmente y fueron gentilmente suministradas por Novozymes (Dinamarca).

##### 3.2.2.2. Tratamiento enzimático

Las dosis de enzimas y las condiciones de hidrólisis (temperatura y pH) se establecieron de acuerdo a las recomendaciones del proveedor. En este sentido, se utilizaron las siguientes condiciones:

| Enzimas | Dosis (mL $g^{-1}$ sustrato) | pH |
|---|---|---|
| Celluclast | 0.25 | 5 |
| Viscozyme | 0.20 | 5.5 |
| Pectinase | 0.25 | 4.8 |
| Alcalase | 0.30 | 8 |

La hidrólisis se realizó incubando la mezcla microalga-enzima a 50ºC y 130 rpm durante 3 h. A lo largo de la hidrólisis enzimática, el pH se ajustó cuando fue necesario con HCl o NaOH. Para detener la hidrólisis las enzimas fueron desactivadas elevando la temperatura a 75ºC durante 30 min. Para evaluar el efecto de la temperatura sobre la hidrólisis de las microalgas sin biocatalizador se realizó un ensayo control a 50ºC.

#### 3.2.2.3. Optimización del pretratamiento enzimático

##### 3.2.2.3.1. Tratamientotérmico previo a la hidrólisis enzimática

Con el fin de evaluar si existen impedimentos para que las enzimas accedan a la pared celular de las microalgas que pueden afectar el rendimientode la enzmas, se estudió el efecto de aplicar un tratamiento térmico a temperaturas media (75ºC durante 30 min) previo a la hidrólisis enzimática. Tras el tratamiento térmico las muestras se enfriaron a temperatura ambiente y se realizaron los ensayos de hidrólisis enzimática como se ha descrito anteriormente.

##### 3.2.2.3.2. Efecto de la combinación de carbohidrasas y proteasas

Con el fin de comprobar si existen sinergias entre ambas enzimas y su acción conjunta podría aportar beneficios adicionales en la hidrólisis en comparación con la acción de las enzimas de forma independiente, también se estudió su combinación. La hidrólisis se realizó en dos etapas, debido a los diferentes pHs óptimos en de ambas enzima. De este modo, el primer paso consistió en el tratamiento con las carbohidrasas durante 3 h, en las condiciones de ensayo descritas anteriormente. En la segunda etapa, el pH se aumentó a 8,0 añadiendo NaOH (4 M) y después se añadieron las proteasas.

##### 3.2.2.3.3. Efecto de la dosis de enzima

La microalga (16 g $L^{-1}$) fue hidrolizada con diferentes concentraciones de Alcalase (es decir, 0.144, 0.293 y 0.585 AU g $ms^{-1}$). La concentración de enzima fue calculada según la actividad declarada por el proveedor. La mayor dosis de proteasa estudiada corresponde a la que arrojó el mejor resultado en Sección 4.2.

#### 3.2.2.3.4. Efecto de la carga de biomasa

Después de determinar la dosis enzimática óptima, se estudió el efecto de diferentes cargas de biomasa. De esta manera, la concentración inicial de biomasa algal se aumentó 2 y 4 veces con respecto a la concentración utilizada anteriormente (32 g $L^{-1}$ y 65 g $L^{-1}$) y fueron hidrolizadas con la dosis óptima de enzima seleccionada previamente.

## 3.3. Eficiencia de hidrólisis

La eficiencia de la hidrólisis fue definida de acuerdo con la siguiente ecuación:

$$Eficiencia\ de\ la\ hidrolisis\ (\%) = \frac{sDQO\ concentración\ después\ del\ tratamiento - sDQO\ concentración\ en\ biomasa\ sin\ tratar}{concentración\ tDQO\ - Concentración\ de\ biomasa\ sin\ tratar\ sDQO} * 100$$

Donde sDQO es la demanda de oxígeno químico soluble y tDQO es la demanda de oxígeno químico total. La eficiencia de los carbohidratos liberados durante el pretratamiento biológico fue también medido utilizando la misma fórmula, pero considerando la fracción soluble y total de carbohidratos en vez de la DQO.

## 3.4. Digestion anaerobia

### 3.4.1. Potencial bioquímico de metano (BMP)

Para determinar el efecto del pretratamiento sobre los rendimientos en metano, se realizaron ensayos de biodegradabilidad anaerobia en discontinuo con la biomasa microalgal pretratada y sin tratar. Las digestiones anaerobias se realizaron en biorreactores de 120 mL con una relación sustrato/inóculo (S/I) de 0.5 g DQO g $VS^{-1}$. El volumen del medio de fermentación se calculó para obtener un volumen final de 70 mL de la mezcla líquida, permitiendo 50 mL como espacio superior para la producción de biogás. Para mantener el pH se añadió 1 g $L^{-1}$ de carbonato de calcio. Para eliminar el oxígeno del espacio en cabeza de los reactores se inyectó helio en los reactores para desplazar el aire y posteriormente sellados con tapones de impermeables. La producción de biogás acumulado fue determinada

considerando el aumento de presión en el espacio en cabeza del reactor. Para cuantificar la cantidad de metano producida por la respiración endógena del lodo anaerobio usado como inoculo (sin añadir sustrato). La producción de biogás neto fue calculada restando la producción de metano endogena a la proporcionada por cada muestra. El periodo de incubación a 35ºC fue de aproximadamente 3-5 semanas.

### 3.4.2. Ensayos en reactores de mezcla completa (CSTR)

Los experimentos de CSTR se realizaron en biorreactores de 1 L alimentados con *C. vulgaris* sin tratar y pretratada. Con el fin de evitar cualquier actividad fotosintética, los digestores se cubrieron con papel de aluminio. Los digestores fueron mantenidos en un rango de temperaturas mesofílico (35ºC) mediante la circulación de agua en un reactor encaminado. La mezcla contenida en el reactor se agitó a 250 rpm mediante un agitador magnético. El pH (7-7.5) fue monitorizado pero no controlado. La carga orgánica (OLR) empleada fue de 1.5 kg DQO $m^{-3}$ $d^{-1}$ y el tiempo de residencia hidráulico (RHT) fue fijado en 15 y 20 días, puesto que estudios previos realizados en discontinuo demostraron que casi toda la producción de biogás se llevaba a cabo durante los primeros 20 días (Méndez et al., 2013). Diariamente se retiró un volumen conocido del digestor que fue remplazado por el mismo volumen de sustrato a la carga orgánica especificada anterioramente utilizando jeringas de plástico.

El volumen de biogás generado en los CSTRs se midió por mediante desplazamiento de agua. El contenido de metano fue analizado dos veces por semana. El estado estacionario se alcanzó al obtener una producción estable de gas y una concentración de demanda de oxígeno químico (DQO) de los efluentes (3xHRT).

Después de determinar el pretratamiento óptimo para aumentar la producción de biogás, se llevó a cabo un experimento adicional con el fin de examinar la posibilidad de duplicar la carga orgánica alimentada en el reactor y disminuir el THRa 15 días.

### 3.5. Medidas analíticas

Los sólidos totales (TS), los sólidos volátiles (VS) y el nitrógeno Kjeldahl total (TKN) se analizaron según los métodos estándar (Eaton et al., 2005). Las demandas de oxígeno químico soluble y total (tDQO, sDQO) se midieron utilizando kits de prueba (Merck, ISO 15705). El contenido de amonio se determinó utilizando kits comerciales (Merch, ISO 000683). Los parámetros solubles fueron determinados después de la centrifugación de las muestras a 14.600 rpm durante 5 min (Mini-spin Eppendorf 5424).

Los carbohidratos totales se determinaron según el método de fenol/ácido sulfúrico a una longitud de onda de 490 nm utilizando D-glucosa como estándar (Dubois et al., 1965). El almidón se determinó según el método de ácido perclórico modificado (Brányiková et al., 2011). El contenido de proteína se calculó sobre la base de la medida de la TKN, utilizando el factor de corrección de 5,95. Los lípidos fueron estimados como la fracción restante de los sólidos totales después de la determinación de proteínas, carbohidratos y cenizas.

Los nitratos, nitritos y fosfatos fueron medidos por cromatografía iónica en un aparato 930 Compact IC Flex (Metrohm) equipado con una columna Metro Sep A sup 5-250/4.0. Los ácidos grasos volátiles (VFAs) fueron analizados en la fase soluble por HPLC una cromatografía líquida de alta rendimiento en un cromatógrafo 1260 Agilent equipado con una columna Aminex HPX-87H y un detector de UV-Vis. La composición de biogás fue analizada utilizando un cromatógrafo de gases (Agilent 7820A) con un detector de conductividad térmica y equipado con una columna HP-PLOT Q. La temperatura del puerto de inyección se fijó a 250ºC, la estufa a 40ºC y la temperatura del detector a 275ºC.

## 4. Resultados y discusión

### 4.1. Pretratamiendo térmico

#### 4.1.1. Efecto del pretratamiento sobre la solubilización de la materia orgánica y los carbohidratos

El tratamiento térmico (autohidrólisis) de la biomasa de *Chlorella vulgaris* y *Scenedesmus* sp produjo un aumento en la materia orgánica soluble del 16% y el 6%, respectivamente (Tabla 4.2). Estos resultados concuerdan con los obtenido por Passos et al. (2011). La mayor rigidez de la pared celular de *Scenedesmus* podría ser la responsable de la menor solubilización obtenida con este pretratamiento en comparación con *C. vulgaris*. En ninguno de los experimentos realizados se observó mejora de la solubilización de materia orgánica al incrementar el tiempo de incubación.

Con respecto a la solubilización de los carbohidratos en los ensayos con *C. vulgaris*, si se observó un aumento con el tiempo de incubación. Se produjo un 10.8 y 15% de solubilización de carbohidratos tras 24 y 48 h de incubación, respectivamente. En los ensayos con *Scenedesmus* se obtuvieron solubilizaciones de carbohidratos del 30% sin importar el tiempo de incubación.

La solubilización de carbohidratos provocada por el autohidrólisis es dependiente del tipo de microalga utilizada. En general, los resultados muestran que la solubilización de los carbohidratos fue notablemente mayor en *Scenedesmus* que los obtenidos cuando se utilizó biomasa de *C. vulgaris*.

#### 4.1.2. Efecto del pretratamiento térmico sobre la producción de metano

El tratamiento térmico aplicado a la biomasa de *C. vulgars* aumento ligeramente (10%) la producción de metano. Estos resultados están en concordancia con los obtenidos por Passos et al. (2011) que obtuvieron un aumento de la producción de metano del 13% tras pretratar la biomasa algal a 55ºC durante 15 h. En los experimentos con *Scenedesmus*, no se obtuvo ninguna mejora con el tratamiento de autohidrólisis con respecto a la biomasa sin tratar (Figura 4.2).

En general, la mejora del rendimiento del metano fue bastante baja en comparación con otros estudios (Méndez et al., 2013; 2014). En las mejores condiciones sólo se obtuvo un aumento de la producción de metano un 10% en los ensayos con *C. vulgaris*. La escasa mejora en la producción de metano puede explicarse por el hecho de que la solubilización de materia orgánica favorecida por el pretratamiento fue probablemente mediada por la liberación de exopolímeros pero no fue un pretratamiento eficiente para romper la pared celular que permita liberar al medio los componentes intracelulares.

A la vista de los resultados obtenidos puede concluirse que el tratamiento térmico suave aplicado en estos experimentos no tiene un efecto significativo en el incremento de la producción de metano y, por lo tanto, deben estudiarse otros pretratamientos con el fin de mejorar la producción de metano de manera sustancial e integrar el pretratamiento en el proceso global de obtención de metano mediante digestión anaerobia.

### 4.2. Pretratamiento enzimático

#### 4.2.1. Efecto del pretratamiento enzimático sobre la solubilización de la materia orgánica y los carbohidratos

La biomasa de las microalgas se sometió a hidrólisis enzimática utilizando varios biocatalizadores comerciales (Celluclast, Viscozyme, Pectinase and Alcalase). En todos los ensayos tratados con enzimas aumentó la materia orgánica soluble con respecto al ensayo control incubado a la misma temperatura sin biocatalizador (18%). En los experimentos con *C. vulgaris,* la solubilización de materia orgánica provocada por las carbohidrasas (25-29%) fue menor que la observada en los experimentos en los que la biomasa algal fue tratada con proteasas. La máxima solubilización se observó después de 1 h de tratamiento y luego se mantuvo prácticamente constante en todos los ensayos excepto en los que se utilizaron pectinasas en los que la solubilización fue incrementándose durante todo el experimento.

A diferencia de lo que se observó en los experimentos con *C. vulgaris*, la solubilización de materia orgánica con el tratamiento enzimático con la biomasa de *Scenedesmus* mostró una tendencia diferente. La solubilización

de materia orgánica durante la hidrólisis de *Scenedesmus* fue continua a lo largo del tiempo y, por lo tanto, el ensayo con proteasas se prolongó por más tiempo (como se describe más adelante). En estos ensayos, los tratados con Viscozyme son los que presentan menos materia orgánica soluble (8%) y esta permanececonstante después de 1 h de hidrólisis. La eficiencia de hidrólisis registrada para la celulasa y para la pectinasa fue de alrededor del 20% después de 3 h de hidrólisis. Al contrario de lo observado en los ensayos con *C. vulgaris*, la hidrólisis con proteasas de la biomasa de *Scenedesmus* sp. no produjo un aumento significativo de la materia orgánica soluble. En este caso, se estimó que solo el 15% de la de la materia orgánica estaba en forma soluble tras el tratamiento con proteasas después de 3 h. Este e valor se incrementó a un 30% después de 8 h de hidrólisis. Los resultados obtenidos muestran que la eficiencia de la hidrólisis obtenidas cuando se utilizan proteasas en *Scenedesmus* sp. fueron 1.5-2.8 más bajas que las obtenidas con *C. vulgaris* mostrando una mayor reticiencia a la hidrólisis. De hecho, varios estudios han indicado que *Scenedesmus* posee una pared célular más gruesa que *C. vulgaris* (González-Fernández et al., 2012; Mussgnug et al., 2010).

En cuanto a la solubilización de los carbohidratos, independientemente del género de las microalgas estudiadas, los mejores resultados se obtuvieron con Viscozyme. El 84% y el 36% de los carbohidratos totales de *C. vulgaris* y *Scenedesmus* sp. Se encontraron en la fase soluble después de 3 h de hidrólisis. El tratamiento con Celluclast solubilizó el 48% del total de los carbohidratos presentes en *C. vulgaris*, mientras que las otras carbohidrasas utilizadas y la proteasa solubilizaron el 30-40% de los carbohidratos, respectivamente.

En el ensayos con *Scendesmus,* la solubilización de carbohidratos osciló entre el 15% y el 20% con todos los biocatalizadores estudiados (excepto con Viscozyme, tal y como se ha explicado anteriormente). De hecho, la adición de enzimas no produjo un aumentó en la solubilización de carbohidratos solubilizados en comparación con el ensayo control en el que únicamente se incubaron las muestras a 50ºC sin adicionarles ningún catalizados. Los resultados obtenidos con el tratamiento con proteasas indican que estas

enzimas actúan hidrolizando las glicoproteínas presentes en la pared de las células de las microalgas (Popper y Tuohy 2010), alterando su estructura y permitiendo la liberación de los carbohidratos internos. Puesto que se obtuvieron mejores resultados de solubilización en los experimentos con *C. vulgaris,* puede concluirse que este biocatalizador es más adecuado para hidrolizar la pared de esta microalga, mientras que la composición de la pared de *Scenedesmus* es más recalcitrante a la hidrólisis.

**4.2.2. Efecto del pretratamiento enzimático sobre la producción de metano**

A pesar de la diferente composición macromolecular, ambas biomasas de microalgas sin pretratar presentaron rendimientos bastante similares de metano: 142 mL $CH_4$ g de DQO $en^{-1}$. Sin embargo, las productividades fueron significativamente diferentes en los dos microalgas. En el caso de *C. vulgaris,* tras 10 días de digestión se alcanzó el 90% del rendimiento total de metano., mientras que *Scenedesmus* sp. requirió 17 días. De los resultados se puede concluir por tanto que la biomasa de *Scenedesmus* sp. requiere para su conversión eficiente mayores tiempos de retención. La producción de metano utilizando la biomasa hidrolizada enzimáticamente fue considerablemente mayor que la de la biomasa no tratada (Figura 4.6). De las carbohidrasas estudiadas, los cócteles enzimáticos Celluclast y Viscozyme fueron los que menor aumento de la producción de metano de la biomasa de *C. vulgaris produjeron (*170 mL $CH_4$ $g^{-1}$ de DQO $en^{-1}$). En contraste con ligero incremento en la rpdicción de metano (1.2 veces) obtenido con estos dos cócteles en *C. vulgaris*, el efecto de la pectinasa produjo una notable mejora (1.54 veces). Este resultado parece indicar que es necesario hidrolizar los ácidos urónicos presentes en la pared celular para producir buenas solubilizaciones de materia orgánica y aumentar la eficiencia de la digestión anaerobia de *C. vulgaris.* Estos resultados están en concordancia con los obtenidos en otros estudios que también demostraron que el pretratamiento de las microlagas con celulasass, lisozima, xilanasas y amilasas no tuvieron efecto significativo en la hidrólisis de la pared celular de las microalgas (Kim et al., 2014; Gerken et al., 2013).

La mejora registrada en la producción de metano cuando se utilizaban como materia prima biomasas hidrolizadas con carbohidrasas fue sin embargo baja en comparación con los valores obtenidos para *C. vulgaris* pretratadas con proteasas. Los rendimientos de metano obtenidos en estos ensayos fueron de 248.1 mL $CH_4$ g de DQO $en^{-1}$ (Figura 4.6a). Con respecto a la biomasa sin pretratar, el uso de este biocatalizador mejoró 1.72 veces el rendimiento de metano. En los ensayos con *Scenedesmus* sp. utilizando carbohidrasas los mejores resultados se obtuvieron con con pectinasa (1,3 veces en comparación con la materia prima) (Figura 4.6b). Los incrementos en metano obtenidos con Celluclast and Viscozyme fueron 1.2-1.3 veces el control, respectivamente. En el caso de la biomasa hidrolizada con proteasas, el rendimiento de metano alcanzó los 216 mL $CH_4$ g de DQO $en^{-1}$, que corresponden al 62% de biodegradabilidad anaeróbica.

Aunque los carbohidratos solubilizados durante la hidrólisis enzimática fueron considerablemente inferiores a los valores registrados para *C. vulgaris*, el aumento en la producción de metano fue similar en ambas microalgas. Una vez más, este hecho sugiere que los carbohidratos no fueron directamente los responsables de la optimización de la digestión anaerobia de estos sustratos.

### 4.2.3. Optimización del pretratamiento

#### 4.2.3.1. Efecto del pretratameinto térmico previo al enzimático y la combinación de enzimas sobre la solubilización de materia orgánica

Este estudio se realizó con el fin de evaluar la influencia del pretratamiento térmico previo a la hidrólisis enzimática, la posibilidad de aumento o disminución del tiempo de pretratamiento y el beneficio adicional de combinar las enzimas de carbohidrasa y proteasas y su efecto sobre la solubilización de materia orgánica y la mejora de la producción de metano. La hidrólisis enzimática se realizó en dos diferentes biomasas de microalgas, *C. vulgaris* y *C. reinhardtii* yse estudiaron tres condiciones diferentes: la adicción de Viscozyme L (carbohidrasa), la de Alcalase 2.5 L (proteasa) y la combinación de Viscozyme y Alcalase.

#### 4.2.3.2. Solubilización de la materia orgánica

Los resultados demostraron que, independientemente de la biomasa de microalgas, el mayor impacto sobre la solubilización de materia orgánica se obtuvo en la hidrólisis realizada con la proteasa. Sin embargo, la hidrólisis enzimática siempre fue menos eficiente en *C. reinhardtii* que en *C. vulgaris*.

Más concretamente, en el caso de *C. reinhardtii*, la adicción de Viscozyme y Alcalase provocó un aumento de la materia orgánica soluble de 1.6 y 2.4 veces (Figura 4.7), mientras que en *C. vulgaris* fue de 2 y 4 veces, respectivamente. Los menores rendimientos de hidrólisis enzimática obtenidos en los experimentos con *C. reinhardtii* pueden ser atribuidos al hecho de que en esta especie ya la biomasa sin tratar presenta un contendio alto de materia orgánica soluble. Se ha descrito que esta microalga es capaz de secretar grandes cantidades de exopolisacáridos (Bafana 2013) y, por tanto, el alto porcentaje de materia orgánica soluble registrado en los experimentos con estabiomasa puede ser debido a la presencia de estos exopolímeros.

Con el fin de confirmar si el pretratamiento térmico podía favorecer la acción de las enzimas hidrolíticas se realizó un tratamento térmico previo al enzimático a 75ºC durante 30 min utilizando biomasa de *C. vulgaris* y *C. reinhardtii*. No se obtuvieron diferencias significativas en ninguno de los ensayos realizados (Figura 4.7). Por lo tanto, se puede concluir que las enzimas no tuvieron problemas de accesibilidad a los componentes que tienen que hidrolizar que pudieran ser mejorados con el tratamiento térmico. Para conocer concretamente qué componente se solubilizó preferentemente se realizó un seguimiento del contenido en carbohidratos y proteínas de la fracción soluble durante la hidrólisis enzimática. Ambas enzimas (carbohidrolasa y proteasa) mostraron alto niveles de eficiencia de hidrólisis, ya que casi el 100% de los carbohidratos y proteínas presentes en la biomasa se encontraron en la fase soluble. Este hecho es importante, ya que hasta ahora los pretratamientos estudiados con el mismo objetivo, han registrado que la solubilización de los carbohidratos prevalece sobre el de las proteínas (Méndez et al., 2013).

Con el fin de comprobar si Viscozyme y alcalasa podían tener un efecto sinérgico sobre la solubilización de materia orgánica en comparación con la hidrólisis realizada con las enzimas de forma independiente, también fue estudiada su combinación. En una primera etapa se añadió Viscozyme bajo las condiciones de recomendadas por el proveedor (50 °C, pH 5.5). Se observa una hidrólisis de los carbohidratos y el material soluble aumentó en 2.2 veces después de 2 h de hidrólisis. Después de este tiempo, el grado de solubilización se mantuvo constante hasta que se añadió la Alcalasa. Después de 5 h de hidrólisis, la combinación de Viscozyme y Alcalase produjo una solubilización de materia orgánica ligeramente superior a la obtenida con la Alcalasa sola.

El tiempo de hidrólisis óptimo para ambas enzimas fue de 2 h. Incubaciones más prolongadas no proporcionaron mejoras apreciable en el materia soluble. Estos resultadosestán en concordancia con los obtenidos por otros autores (Romero-Garcia et al., 2012; Lee et al., 2013). Estos estudios mostraron un grado constante de hidrólisis después de 2 h de tratamiento cuando se utilizó Alcalasa con biomasa de *Scenedesmus almeriensis* (Romero-Garcia et al., 2012) y Viscozyme con *Dunaliella tertiolecta* (Lee et al., 2013).

**4.2.3.3. Efecto del pretratameinto térmico previo al enzimático y la combinación de enzimas sobre la producción de metano**

En los ensayos con *C. vulgaris* tratadacon Viscozyme, la producción de metano se incrementó en un 14% (Figura 4.10a). El tratamiento térmico previo a la hidrólisis enzimática no mejoró la producción de metano. Estos resultados, junto con los obtenidos de solubilizaciónn de carbohidratos durante la hidrólisis enzimática realizada con lacarbohidrolasa sugieren que los carbohidratos de la pared celular o no son los responsables de obstaculizar el acceso de la materia orgánica a bacterias hidrolíticas y limitar la producción de metano. Aunque la solubilización de los carbohidratos en estos ensayos alcanzó el 86%, la producción de metano no se incrementó significativamente.

La hidrólisis con proteasa fue mucho más eficiente en incrementar la producción de metano. Más específicamente, la adición de Alcalasa mejoró

un 51% la producción de metano (287.0±1.3 mL $CH_4$ g de DQO $en^{-1}$, Figura 4.11b) en comparación con la biomasa sin tratar. La combinación de Viscozyme y Alcalasa produjo resultados similares no observándose ningún efecto sinérgico.

El tratamiento térmico previo a la adicción de la enzima disminuyó la producción de metano, 270.8±5.7 mL $CH_4$ g de DQO $en^{-1}$ indicando que durante el tratamiento a 75ºC podrían producirse algunas sustancias de degradación de las proteínas y los cabohidratos que afectarían negativamente a la hidrólisis enzimática.

Los resultados de la producción de metano muestran claramente la ventaja de utilizar proteasas como pretratamiento de manera previa a la digestión anaerobia.

E contenido de metano en el biogás producido en los ensayos de digestión anaerobia de *C. reinhardtii* fueron similares a los obtenidos con *C. vulgaris*. La bionmasa de *C. reinhardtii* sin tratar produjo 263.1±0.1 mL $CH_4$ g de DQO $en^{-1}$, que corresponde al 75% de biodegradabilidad anaeróbica. Este valor está en concordancia con los obtenidos por Mussgnug et al. (2010) y es más elevado que el registrado por Passos et al. (2013).

Mientras que en los ensayos con *C. vulgaris*, la adicción de Viscozyme previa a la digestión anaerobia proporcionó un incremento en la producción de metano, el aumento fue insignificante en la biomasa de *C. reinhardtii*. La hidrólisis con Viscozyme y el tratamiento térmico previo antes de la adicción de Viscozyme produjo un 255.7±9.4 mL $CH_4$ g de DQO $en^{-1}$ y 279.4±1.1 mL $CH_4$ g de DQO $en^{-1}$, respectivamente (Figura 4.10b). De este modo, aunque la adición de Visozyme produjo un aumento en la solubilización de materia orgánica y más específicamente en la solubilización de los carbohidratos, puede concluirse que esta solubilización no se tradujo en un aumento en la producción de metano porque estos compuestos ya estaba fácilmente biodisponibles en la biomasa sin tratar. No se han encontrado referencias en la literatura en los que *C. reinhardtii* fuera hidrolizada con proteasas para mejorar la producción de metano.

#### 4.2.4. Efecto de la dosis de enzima y la concentración de sustrato sobre la solubilización de materia orgánica y la producción de metano.

Los resultados obtenidos en los experimentos anteriores utilizando distintas especies de microalgas con distinta composición macromolecular muestran que de las actividades enzimáticas ensayadas (carbohidrasas y proteasa) la actividad proteasa es la más efectiva para alterar la pared celular, aumentar la solubilización de la materia orgánica e incrementar la producción de metano, independientemente a la cepa de las microalgas. Por lo tanto, para los siguientes ensayos se utilizó la proteasa para estudiar el efecto de la dosis de enzima y de sustrato sobre la eficiencia de hidrólisis de la biomasa de *C. vulgaris*.

##### 4.2.4.1. Efecto de la dosis de enzima y la concentración de sustrato sobre la solubilización de la materia orgánica

Para evaluar el efecto de la carga enzimática en la producción final de biogás se utilizaron tres dosis de proteasa (0.144, 0.293 y 0.585 AU g $DW^{-1}$). La mayor solubilización de materia orgánica (49.3%) se obtuvo en los ensayos en los que la biomasa de *C. vulgaris* fue pretratada con 0.585 AU g $DW^{-1}$ de Alcalasa durante 3 h. La mayor parte de la materia orgánica se solubilizó en la primera hora de tratamiento. La prolongación de la incubación a 3 h solo incrementó la solubilización del 5 al 8%. Este resultado está en concordancia con los obtenidos en los ensayos anteriores (Sección 4.2.3.1.) en los que el tratamiento con Alcalasa de la biomasa de *C. vulgaris* provocó un 52.2% de solubilización de la materia orgánica tras 5 h de tratamiento. La solubiliación de la materia orgánica se redujo ligeramente en los ensayos con menos cantidad de enzima, siendo la eficiencia de hidrólisis de 45.2% y 41.2% para las dosis de enzimas de 0.293 y 0.144 AU g $DW^{-1}$, respectivamente (Tabla 4.7). Los resultados obtenidos muestran que existe un efecto positivo sobre la solubilización de materia orgánica a mayores carga de enzima debido al alto contenido en proteínas de la biomasa de *C. vulgaris*, No es posible comparar los resultados obtenidos con otros trabajos previos, ya que este es el primer estudio que se realiza sobre el efecto de la carga de proteasa en el pretratamiento de la biomasa de microalgas.

Una vez establecida la dosis óptima de enzimase estudió el efecto de la concentración inicial de biomasa. Para ello se utilizaron tres cargas de biomasa (16, 32 y 65 g $L^{-1}$) que fueron hidrolizadas con la dosis óptima de proteasa (0.585 AU g $DW^{-1}$) determinadaen los ensayos anteriores. Independientemente de la concentración de biomasa empleada, se obtuvo una eficiencia de hidrólisis en torno al 50% (Tabla 4.8). De este modo, se puede concluir que el aumento de la viscosidad de los medios que se produce como consecuencia de la mayor concentración de sustrato no afectó la hidrólisis enzimática. Estos resultados son diferentes a los obtenidos porpor Romero-Garcia et al. (2012). Estos autores observaron una reducción gradual del rendimiento de hidrólisis al incrementar las cargas de biomasa. Más concretamente, la eficiencia de la hidrólisis disminuyó de 55% a 20% al incrementar la carga inicial dede biomasa de *Scenedesmus almeriensis* de 200 g $L^{-1}$ a 350 g $L^{-1}$, respectivamente. Los autores atribuyeron este comportamiento a una limitada transferencia de masa debido al aumento de la viscosidad de la solución. En el presente estudio, las cargas de biomasa testadas fueron significativamente inferiores a las estudiadas por Romero-Garcia et al. (2012). De esta manera, puede concluirse que hasta 65 g $L^{-1}$, el sustrato es biodisponible para las enzimas y no fue detectada ninguna disminución en el nivel de eficacia de la hidrólisis.

#### 4.2.4.2. Efecto de la dosis de enzima en el proceso de digestión anaerobia

##### 4.2.4.2.1. Producción de metano

El rendimiento de metano del proceso de digestión anaerobia de la biomasa de *C. vulgaris* sin tratamiento osciló entre 147-160 mL $CH_4$ g de $DQO^{-1}$, lo que corresponde a una biodegradabilidad anaerobia de 42-45%. Este resultado está en el rango de los obtenidos en otros trabajos de producción de biogás utilizando biomasa de *C. vulgaris* (Ras et al., 2011 ; Mendez et al., 2013).

La producción de metano varía ligeramente con la dosis de enzima. En los ensayos con ladosis de proteasa más alta, 0.585 AU g $DW^{-1}$, se obtuvieron 256 mL $CH_4$ g de DQO $en^{-1}$, que corresponde a un 73% de biodegradabilidad (Tabla 4.7). El rendimiento de este ensayo en comparación con los obtenidos

con la la biomasa no tratada, fue un 60% superior. Estos resultados son los similares por los obtenido anteriormente (Sección 4.2.3.2.). Las otras dos dosis de enzimas, 0.293 y 0.146 AU g $DW^{-1}$ resultaron en producciones de metano de 232.5 y 224 mL $CH_4$ g de DQO $en^{-1}$, respectivamente (Tabla 4.7).A la vista de estos resultados, se puede concluir que el aumento de la producción de metano estaba directamente relacionado con la eficiencia de la hidrólisis. Lamentablemente, no es posible comparar los resultados con otros en la bibliografía ya que el pretratamiento de la biomasa de microalgas con proteasas para aumentar la producción de metano en el proceso de digestión anaerobia no ha sido investigado previamente.

En los ensayos con diferentes concentraciones iniciales de sustrato no se observaron diferencias en la producción de metano (Tabla. 4.8). Los resultados obtenidos demostraron que la aplicación de un tratamiento con proteasas de la biomasa algal, produce un aumento significativo de la materia orgánica soluble, asociada a la rotura de las paredes celulares, que se traduce en un aumento en la capacidad de producción de metano...

#### 4.2.4.2.2. Mineralización del nitrógeno e inhibición de amoniaco

Dado el alto contenido en proteínas de la biomasa de *C. vulgaris* y que el biocatalizador utilizado para el pretratamiento son proteasas, es de gran interés conocer la evolución del contenido de nitrógenodurante el proceso de digestión anaerobia. En los ensayos con la biomasa de *C. vulgaris* sin tratamiento y en el ensayo control incubado a 50ºC sin adición de enzimas, la mineralización del nitrógeno varió entre el 85-88%. Estos valores son bastante más altos que los registrados en la literatura. En el proceso de digestión anaerobia utilizando como sustrato biomasa de *S. obliquus* sin tratar y sometida a un pretratamiento térmico se obtuvo una mineralización de nitrógeno del 40-43% (González-Fernández et al., 2011 ; 2013).

A diferencia de lo que se observó en los experimentos de producción de biogás, las diferentes concentraciones de sustrato (biomsa algal pretatada a 0.585 AU g $DW^{-1}$) produjeron diferentes mineralizaciones de nitrógeno. En los ensayos realizados con diferentes concentraciones iniciales de sustrato pretratados con la misma dosis de proteasa, se observa un descenso de la

mineralización de nitrógeno al aumentar la carga desustrato. Más concretamente, el nitrógeno orgánico convertido en amonio fue del 51,5% cuando se utilizó una concentración de sustrato de 32 g $L^{-1}$y un 32% cuando se aumentó hasta 65 g $L^{-1}$.

Teniendo en cuenta la concentración de amonio medido al final de la digestión, el pH del medio y la constante de acidificación del amonio/amoniaco, se calculó la concentración de amoníaco en los ensayos de potencial bioquímico de metano. Como era de esperar por lo valores de pH al final del proceso de digestión (cercano a la neutralidad) (Tabla 4.7 y la Tabla 4.8), la concentración de amoníaco fue insignificante. Más concretamente, los valores oscilaron entre 2 y 3% del total de amonio registrado en la solución. De esta manera, la concentración de amoníaco/amonio estaba lejos del umbral de toxicidad. En este punto cabe destacar que estos ensayos se realizaron en ensayos discontinuos y esta ausencia de inhibición también debería confirmarse en ensayos en reactores en continuo en los l que el amonio puede acumularse llegando a concentraciones tóxicas.

## 4.3. Producción de biogás en el proceso de digestión anaerobia semi-continua (CSTR) de *C. vulgaris*

### 4.3.1. Producción de metano en CSTR dutilizando como sustrato la biomasa de *C. vulgaris* sin tratar

La producción diaria de biogás en el CSTR alimentado con *C. vulgaris* sin tratar y un HRT de 15 días se muestra en la Figura 4.17. Puesto que el contenido de metano en el biogás estaba alrededor del 73%, el rendimiento de metano calculado fue de 50 mL $CH_4$ g de DQO $en^{-1}$. Dado la escasa producción de metano obtenida en estos ensayos, no se incluye en este trabajo una descripción detallada de estos experimentos. El HRT del CSTR se fijó en 15 días, de acuerdo con los valores obtenidos en la digestión anaerobia en modo discontinuos. En el BMP de *C. vulgaris* sin tratar en los primeros 15 días ó se obtuvo más del 90% del rendimiento total de metano (McMillan et al., 2013). En el caso particular de *C. vulgaris*, Ras et al. (2011) observaron una mayor de remoción de DQO cuando se alarga el HRT de 16

a 28 días. En este trabajo, y con el fin de mejorar la producción de biogás en el proceso de digestión anaerobia de *C. vulgaris,* se sometió a la biomasa a un pretratamiento enzimático utilizando proteasa como biocatalizador de manera previa a la digestión anaeróbica y se alargó en HRT a 20 días.

### 4.3.2. Producción de metano en el CSTR alimentado con biomasa de *C. vulgaris* tratadas enzimáticamente

En comparación con el CSTR de *C. vulgaris* sin pretratamiento, la alimentación con biomasa pretratada aumentó el rendimiento de metano 2,6 veces (128.4 mL $CH_4$ g de DQO $en^{-1}$, Figura 4.17). Este incremento fue similar al obtenido en otros CSTRs alimentados con *Scenedesmus* sp. pretratados térmicamente (2.9 veces); (González-Fernández et al., 2013) y superior a la obtenida con biomasa de microalgas pretratada con microondas (1.6 veces); (Passos et al., 2014). Aunque el incremento en el rendimiento del metano obtenido en el CSTR (2.6 veces) alimentado con biomasa pretratada enzimáticamente fue superior al obtenido en el BMP (1.72 veces); (Sección 4.2.2.), los valores absolutos del rendimiento de metano fueron menores. El rendimiento de metano obtenido en los ensayos en discontinuo con la biomasa de *C. vulgaris* pretratada fue de 224.5 mL $CH_4$ g de DQO $en^{-1}$, superior en un 41% al obtenido en el CSTR. Con el fin de establecer los fenómenos responsables de esta reducción de los rendimientos de metano observados en el CSTR se analizaron los inhibidores que comúnmente se producen en la digestión anaerobia [ácidos orgánicos volátiles (VFAs) y concentración de amoníaco].

### 4.3.3. Concentración de VFAs y $NH_3$

Como se muestra en la Figura 4.19, la concentración VFAs en el efluente del CSTR alimentado con biomasa pretratada eran prácticamente despreciables en el segundo tiempo de retención. Durante el tercer tiempo de retención (últimos 20 días de la digestión), los VFAs empezaron a acumularse gradualmente y aumentaron desde 57.1 mg $L^{-1}$ hasta los 615.1 mg $L^{-1}$ alcanzados al final del ensayo. En las últimas muestras analizadas se alcanzaron valores de 274 y 700 mg $L^{-1}$ en los días 55 y 58, respectivamente. Se puede concluir por tanto que casi todos la DQO solubles al final del

experimento corresponde a la presencia de VFA. El pH se mantuvo en 7.5 durante todo el tiempo del experimento. La acumulación VFAs es un síntoma de la inhibición de la actividad metanogénica debido a la alta concentración de amonio (Yenigün y Demirel, 2013) Los resultados parecen indicar que, aunque la materia orgánica soluble (VFAs) estaba disponible, los microorganismos metanogénicos estaban inhibidos y no eran lo suficientemente rápidos para convertir el sustrato en biogás. Por otro lado, la concentración de amonio en el efluente fue 1895.9±76,5 mg $L^{-1}$. Este resultado es mucho mayor que el aportado en otros estudios con otros CSTRs alimentados con biomasa de microalgas (González-Fernández et al., 2013) y (Ras et al., 2011). Aunque el límite del umbral de toxicidad del amonio depende del sustrato, el inóculo, el tiempo de retención y las condiciones ambientales, concentraciones de amonio de alrededor de 1700-1800 mg $L^{-1}$ se consideran inhibitorias para inóculos no aclimatados (Yenigün y Demirel, 2013).

El amoníaco es otra de las formas de nitrógeno inorgánico que se puede encontrar en el proceso de digestión anaerobia. La concentración de amoníaco se calculó en base al pH y a la concentración de amonio medido en el efluente del CSTR. Como era de esperar de los valores de pH medido a lo largo de todo el experimento, la forma predominante de nitrógeno inorgánico fue el amonio. La concentración de amoníaco fue de 69.7 mg $L^{-1}$. La concentración del umbral de toxicidad del amoniaco es mucho menor que el amonio. De hecho, valores de alrededor de 300 mg $L^{-1}$ han sido registrados como inhibidores para la actividad metanogénica (Braun et al., 1981). En los ensayos realizados, la concentración de amoníaco no era demasiado alta, pero se observó un claro efecto inhibidor debido probablemente a la alta concentración de amonio medido en los efluentes.

### 4.4. Producción de biogás en CSTR alimentado con *C. vulgaris* pretratadas con proteasas cultivadas en aguas residuales urbanas como única fuente de nutrientes

Como se ha mostrado anteriormente en los ensayos de digestión anaerobia de CSTR, la combinación del pretratamiento junto con el alto contenido de proteína de la microalga da lugar a una alta concentración de amonio durante

el proceso. La alta concentración de amonio inhibe los microorganismos metanogénicos y causa una acumulación gradual de ácidos grasos volátiles que redujeron de manera concomitante la producción de biogás. Para evitar este problema, se contemplaron dos opciones: (i) reducir el contenido de proteínas de la biomasa o (ii) utilizar para el pretratamiento biocatalizadores distintos a las proteasas que actúen sobre otras macromoléculas (carbohidratos o lípidos) en lugar de proteínas. En este estudio se evaluaron las dos opciones. En la primera, las microalgas fueron cultivadas en aguas residuales con el fin de reducir el contenido de proteína de la biomasa y evaluar la viabilidad de combianr la biorremediación de aguas residuales con la producción de un biocombustible como el biogás. En segundo lugar, se estudió el efecto de la producción de biogás del pretratamiento con proteasas y carbohidrasas.

#### 4.4.1. Caracterización de biomasa de *C. vulgaris* cultivada en aguas residuales

El perfil macromolecular de la biomasa de *C. vulgaris* cuando fue cultivada en aguas residuales fue marcadamente diferente que cuando esta microalga creció en un medio sintético. Los resultados muestras que cuando se cultiva en medios sintéticos, la materia orgánica de esta microalga estuvo formada principalmente por proteínas, mientras que cuando se cultivó en aguas residuales urbanas, el perfil macromoleculares se desplazó hacia la acumulación de carbohidratos. Más concretamente, el perfil macromolecular correspondía a un 64% de proteínas y un 22% de carbohidratos en la biomasa de *C. vulgaris crecida* en un medio sintético (Sección 4.3.). Por el contrario, cuando se cultivó la misma microalga en aguas residuales urbanas, la fracción de carbohidrato se duplicó (40% en base VSS) mientras que el contenido de proteínas se redujo a la mitad (33,3%). Esta notable disminución en el contenido de proteína observada en la biomasa cultivada en aguas residuales urbanas se atribuyó al bajo contenido de amonio de este medio de cultivo.

En estas condiciones de limitación de nitrógeno, investigaciones anteriores han demostrado un comportamiento similar de las microalgas. La limitación de nitrógeno incrementó el contenido de lípidos o carbohidratos dependiendo,

no sólo de las condiciones operativas durante el cultivo, sino también de la cepa de microalgas utilizada (Dragone et al., 2011 , Ho et al., 2012 y Siaut et al., 2011). En el caso particular de *C. vulgaris*, diversos autores han demostrado que la limitación de aumenta el contenido de carbohidratos de esta biomasa (Ho et al., 2013 y Dragone et al., 2011).

### 4.4.2. Efectos del pretratamiento enzimático sobre la solubilización de la materia orgánica

Estudios previos han demostrado que la eficiencia de la hidrólisis es mayor cuando *C. vulgaris* cultivadas en medios sintéticos se pretrataron con proteasas que cuando esta biomasa fue tratada con carbohidrasas (Sección 4.2. and 4.2.3.). Sin embargo, estos resultados no pueden extrapolarse a estos experimentos ya que la biomasa cultivada en aguas residuales urbanas es rica en carbohidratos y no en proteínas, como en el caso de los estudios anterioes. Por lo tanto, con el fin de comprobar si la composición macromolecular de la microalga afecta la eficacia de hidrólisis de los distintos biocatalizadores, la biomasa de *C. vulgaris* crecida en aguas residuales se sometió a la acción de la proteasa y carbohidrasa. Ambos biocatalizadores mostraron un gran efecto en la solubilización de la materia orgánica. En comparación con la *C. vulgaris* sin pretratar, en la qu sólo el 2% de la tDQO fue soluble, la biomasa hidrolizada con ambas enzimas muestra una eficiencia de la hidrólisis de la materia orgánica del 28.4%±3,9 y 54.7%±5,6 para las muestras pretratadas con Viscozyme (carbohidrasa) y Alcalase (proteasa), respectivamente. Los resultados muestran que, a pesar del menos contenido en proteína, las proteasas siguen siendo las enzimas más eficientes en alterar la pared celular y aumentar la cantidad de materia orgánica solubilizada.

### 4.4.3. Digestión anaerobia semi-continua de biomasa de *C. vulgaris* sin pretratar y pretratada enzimáticamente

La producción diaria de biogás obtenida con la biomasa pretratada con carbohidrasas fue de 150 mL $L^{-1}$ $día^{-1}$ (3 veces mayor que el CSTR alimentado con biomasa sin refinar, Figura 4.23). Este aumento en la producción de metano fue resultado de la hidrólisis de la materia orgánica

(aprox. 30%) registrada en el tratamiento de *C. vulgaris* con carbohidrasas. A pesar de esta mejora, la biodegradabilidad anaeróbica fue baja y sólo el 40% de tDQO fue retirado, mientras que el rendimiento de metano osciló en un 65 mL $CH_4$ g de DQO en$^{-1}$. Por otra parte, en el CSTR alimentado con biomasa pretratada con proteasas la eliminación total de DQO fue del 52% y el rendimiento de metano alcanzó los mL $CH_4$ g de DQO en$^{-1}$. En este caso, el rendimiento del metano fue 6 veces mayor que el CSTR alimentado con biomasa sin pretratar. Es importante destacar que los resultados obtenidos durante la digestión en el CSTRs alimentados con biomasa pretratada con proteasas son similares independientemente de que la biomasa algal tenga o no alto contenido en proteínas. Sin embargo, a diferencia del CSTR alimentado con biomasa rica en proteínas (Sección 4.3), el CSTR alimentado con *C. vulgaris* rica en carbohidratos (crecida en agua residual) y pretratada con proteasas mostró un nivel de producción de metano constante a lo largo de la digestión (Figura 4.23) no observándose fenómenos de inhibición.

En vista de los buenos resultados obtenidos en el CSTR alimentado con biomasa crecida en agua residual y pretratada con proteasa, para verificar la estabilidad y la reproducibilidad de los resultados, se duplicó el OLR (3 kg DQO m$^{-3}$ días$^{-1}$) y se redujo el HRT a 15 días. Sorprendentemente, el CSTR alimentado con *C. vulgaris* pretratada con proteasa se comportó incluso mejor utilizando estas últimas condiciones operacionales. Cuando las microalgas pretatadas con proteasas fueron digeridas en el sistema semi-continuo a un OLR más alto, la producción de metano aumentó de 136.9±19,2 mL $CH_4$ g de DQO en$^{-1}$ registrado en 1.5 kg tDQO m$^{-3}$ día$^{-1}$ a 173.2±10,7 mL $CH_4$ g de DQO en$^{-1}$ a 3 kg tDQO m$^{-3}$ día$^{-1}$ (Figura 4.23).

#### 4.4.4. Evolución de los ácidos orgánicos volátiles (VFAs) y el nitróneno en los CSTRs.

La inhibición de los microorganismos anaerobios causada por la alta concentración de amonio en el digestor de sustratos ricos en proteínas está directamente relacionada con la mineralización del nitrógeno. La mineralización del nitrógeno obtenida en el CSTR alimentado con *C. vulgaris* pretratada con carbohidrasas fue de 39.6±5.5%. Este resultado es similar al obtenido por González-Fernández et al. (2013), en el que se obtuvo un 43.5%

de mineralización del nitrógeno cuando se ejecutó un CSTR alimentado con *Scenedesmus* sp. pretratado térmicamente. Por otro lado, la mineralización del nitrógeno obtenido por CSTR alimentado con biomasa pretratada con proteasa fue claramente superior (68.6%±14.4). A pesar de la diferencia en el contenido de proteína del sustrato utilizado en los ensayos anteriores (microlagas crecidas en medio sintético), no hubo diferencia en la mineralización del nitrógeno. En esta línea, en los experimentos anteriores (Sección 4.3.3.) se obtuvo un 77% de mineralización del nitrógeno en el CSTR alimentado con biomasa rica en proteínas (63% proteína DW) pretatada con proteasa.

Aunque los valores obtenidos para la mineralización del nitrógeno fueron similares a los obtenidos en los ensayos con la biomasa crecida en medio sintético (Sección 4.3.3.) , la concentración de amonio del efluente fue bastante diferente. Más concretamente, la concentración de amonio en los ensayos con la biomasa crecida en aguas residuales fue de 856±103 mg $L^{-1}$ mientras que, en las crecidas en medio sintético, la concentración de amonio fue 1895±77 mg $L^{-1}$. La concentración de amoníaco en el CSTR alimentado con *C. vulgaris* pretratada con proteasa fue como promedio de 28.2±4,6 mg $L^{-1}$ y aún menor (6.3±0.8 mg $L^{-1}$) en el CSTR alimentado con biomasa pretratada con carbohidrasa.

Se midieron los VFAs con el fin de evaluar si el proceso de digestión anaerobia estaba sometido a sobrecarga orgánica. Los niveles VFA fueron insignificantes a lo largo de los experimentos. La ausencia de VFA y los niveles de pH (7.1-7.5, Tabla 4.13) indicaron que no llegó a producirse sobrecarga orgánica.

En resumen, el seguimiento de los compuestos inhibidores más comunes en el proceso de digestión anaerobia reveló que la inhibición de amonio/amoniaco puede evitarse mediante el uso de biomasa de *C. vulgaris* rica en carbohidratos. Frente a lo que se observó con anterioridad con la *C. vulgaris* rica en proteína, el pretratamiento con proteasas no se tradujo en una concentración elevada de amonio en el efluente y, por tanto, la acumulación de VFAs se evitó. Como consecuencia, la producción de biogás se mantuvo estable a lo largo del periodo de digestión.

## 5. Conclusiones

En este trabajo de Tesis Doctoral se ha estudiado la optimización del pretratamiento (térmico y enzimático) de la biomasa de microalgas con el objetivo de incrementar la solubilización de materia orgánica y mejorar la producción de metano. De los resultados obtenidos pueden inferirse las siguientes conclusiones:

- Aunque el tratamiento térmico a 50ºC produjo un aumento en la solubilización de materia orgánica y carbohidratos, no se observó incremento en la producción de metano en *Scenedesmus* y solo aumentó un 10% en los ensayos con *Chlorella vulgaris.* Este ligero incremento puede atribuirse a la solubilización de exopolímeros más que a la rotura de la pared celular y liberación de los componentes intracelulares. Por lo tanto, el tratamiento térmico a 50ºC no aumenta significativamente los rendimientos de metano del proceso de digestión anaerobia.
- Independientemente de la especie de microalga, de su composición macromolecular y del medio utilizado para su cultivo, los mejores resultados de solubilización de materia orgánica y producción de metano se obtuvieron utilizando proteasas (Alcalasa 2.5L). A pesar de que es generalmente aceptado que los carbohidratos de la pared celular son los responsables de la baja digestibilidad de las microalgas, este estudio muestra que las proteínas son el principal polímero que limita la digestión anaerobia. El tratamiento con proteasas previo a la digestión anaerobia es una estrategia muy prometedora para reducir los costes energéticos asociados al pretratamiento necesario para romper la pared celular de las microalgas y aumentar los rendimientos en metano. En concreto, el pretratamiento con proteasas de la biomasa de *C. vulgaris* y *Scenedesmus* sp aumenta la eficiencia de hidrólisis un 47% y un 30% y la producción de metano un 71% y un 62%, respectivamente.
- Independientemente de la concentración inicial de sustrato el mayor incremento en la producción de metano (64%) se observó a una dosis de proteasa de 0.585 AU g $DW^{-1}$. La disminución de la dosis de enzima redujo de manera concomitante el nivel de eficiencia y la producción de metano.

- De las carbohidrasas estudiadas, la más efectiva en solubilizar los carbohidratos fue Viscozyme. El pretratamiento con este enzima, aunque permite solubilizar casi todos los carbohidratos presentes en la materia prima, sólo incrementa el rendimiento de hidrólisis en un 14% con *C. vulgaris,* un 16% con *Scenedesmus* sp. y es prácticamente inapreciable en *C. reinhardtii.*. Estos resultados indican que no son los carbohidratos los responsables de los bajos rendimientos de metano obtenidos cuando se utiliza como sustrato la biomasa de microalgas.
- En general, 2-3 h de pretratamiento son suficientes para que actúen las enzimas. Tiempos de incubación más altos no mejoran los resultados de solubilización de materia orgánica y producción de metano, a excepción de los ensayos con Sc*enedesmus* sp. pretratado con proteasas en los que los rendimientos de hidrólisis más altos se obtuvieron a las 8 h.
- El tratamiento combinado de carbohidrasas y proteasas sólo produce un ligero aumento de los rendimientos de hidrólisis y la producción de metano comparado con los obtenidos únicamente con proteasas, por lo que no se justifica el coste económico asociado con la utilización de dos biocatalizadores y los mayores tiempos de proceso.
- La aplicación de un tratamiento térmico (75ºC, 30 min) previo a la hidrólisis enzimática no supuso un incremento en la eficiencia de hidrólisis, mostrando que no hay problemas de accesibilidad de las enzimas a la biomasa algal.
- No se observaron diferencias en la eficiencia de solubilización de materia orgánica o del rendimiento en metano al pretratar *C. vulgaris* con 0.585 AU g $DW^{-1}$ de proteasas a distintas de concentraciones iniciales de sustrato (16, 32 and 65 g $L^{-1}$). Estos resultados demuestran que el aumento de la viscosidad en los ensayos a mayor concentración de sustrato no afecta la eficiencia de hidrólisis.
- La solubilización de materia orgánica es dependiente de la dosis de enzima. El tratamiento de la biomasa de *C. vulgaris* con la dosis más baja de enzima reduce un 10% la solubilización de materia orgánica y la producción de metano en comparación con los resultados obtenidos a la carga más alta.

- Durante el proceso de digestión anaerobia de la biomasa de *C. vulgaris* pretratada con proteasas en reactores en discontinuo se alcanza una mineralización total del nitrógeno orgánico. A diferencia de lo que ocurría con la solubilización de materia orgánica, la mineralización de nitrógeno no es dependiente de la dosis de proteasa utilizada en el pretratamiento.
- A pesar de la menor mineralización de nitrógeno (77%) alcanzado en el reactor semicontinuo (CSTR), la alta concentración de amonio (≈ 1900 mg $L^{-1}$) provoca inhibición de los microrganismos metanogénicos que conduce a una acumulación de ácidos orgánicos volátiles que reducen el rendimiento en metano.
- La utilización de aguas residuales con bajo contenido en nitrógeno como medio de cultivo de *C. vulgaris* condujo a la obtención de una biomasa enriquecida en carbohidratos.
- A pesar del alto contenido en carbohidratos de la biomasa, se obtuvieron mejores rendimientos cuando el pretratamiento se realizó con proteasas y no con carbohidrasas.
- En los ensayos de CSTR alimentados con la biomasa hidrolizada (15 días el HRT y 3 kg tDQO $m^{-3}$ $día^{-1}$) no se observa inhibición por amonio o por acumulación VFAs.

# CHAPTER 1

# THESIS JUSTIFICATION AND OBJECTIVES

## 1.1. Justification of the thesis

The steady depletion of fossil fuels has boosted the interest in finding alternative renewable energy sources to produce biofuels (biodiesel, bioethanol and biogas). Among the various options, anaerobic digestion seems an attractive route for permitting maximum conversion of all organic matter (carbohydrate, protein and lipid) contained in the feedstocks. Anaerobic digestion is a well-established biological process suited for biomass residues containing high levels of organic matter, however, the use of new biomass feedstocks deserve further investigation. Microalgae, for example, hold great promise as raw material for anaerobic digestion as they possess numerous advantages including high growth rate, they do not compete with arable land and they are able to grow in saline and wastewater media. Nevertheless, there are a number of fundamental challenges affecting their suitability as possible substrates for anaerobic digestion.

As a matter of fact, the hard cell wall of microalgae is recognized as a rate-limiting factor that may hinder the attack of the anaerobic microorganisms during digestion. Additionally, their complex organic matter reduces the conversion yield of microalgae biomass into methane. For instance, the low carbon to nitrogen ratio of microalgae biomass can mediate anaerobic microorganism's inhibition by high ammonium/ammonia concentration levels. Both drawbacks might render the anaerobic digestion process too long to be efficient or even collapse.

Therefore, research is needed to overcome the above mentioned limitations and to provide (1) better understanding of microalgae cell wall composition and biomass hydrolysis before undergoing anaerobic digestion, (2) low cost alternatives for microalgae cultivation and (3) optimal digestion conditions of microalgae biomass (feeding mode, reactor configuration and operational parameters used during the digestion condition) with the goal of increasing biogas yield and avoiding inhibitors. As a result, microalgae biomass-based anaerobic digestion could be optimized to play a promising role in the future of clean and sustainable energy.

### 1.2 Main objectives

The general objective of this work is to optimize the utilization of microalgae biomass as feedstock for biomethane production. This optimization was carried out by applying different biomass pretreatment prior to anaerobic digestion. To accomplish the general objective, the following specific objectives were proposed:

**1.** Evaluating the effect of low temperature (50ºC) autohydrolysis on cell wall disruption of microalgae (*Chlorella vulgaris* and *Scenedesmus* sp.) and methane production enhancement.

**2.** Screening and optimization of different commercial enzymatic cocktails, including carbohydrases and proteases, to evaluate their hydrolytic efficiencies in terms of organic matter solubilisation and methane production enhancement. Those biocatalysts were tested on *Chlorella vulgaris, Scenedesmus* sp. and *Chlamydomonas reinhardtii* biomass. Special attention was given to:

- **A.** Determine the enzyme exhibiting the highest impact on both biomass hydrolysis and biodegradability efficiency, and hence gaining insights in which polymer (carbohydrates and proteins) of microalgae biomass is hampering anaerobic digestion.
- **B.** Optimization of the pretreatment process by controlling hydrolysis time, application of thermal pretreatment prior to enzymes addition, microalgae biomass loads, enzyme dosage and potential benefits of combined enzymatic activities (carbohydrases and proteases).

**3.** Strategies to overcome anaerobic digestion inhibition using protease pretreated microalgae biomass as substrate. Special attention was given to:

- **A.** Assess the main intermediates produced during organic matter degradation in order to discard the most common inhibitions related to protein rich substrates

**B.** Study the effects of those intermediates on the anaerobic process performance and the activity of anaerobic microorganisms involved in the process

**C.** Avoid ammonium inhibition during anaerobic digestion by feeding the reactors with carbohydrate-rich microalgae grown on urban wastewater.

**D.** Investigate the possibility of further optimize the anaerobic process by increasing the organic loading rate and shorten the hydraulic retention time

### 1.3 Development of the thesis

According to the objectives defined previously, this thesis is composed of five chapters structured as follows:

**Chapter 1** presents the main goals, context, and motivations for the development of this thesis. It consists in a guideline for the overall work presented in the further chapters.

**Chapter 2** provides a brief review of the literature. Microalgae definition, diversity, cultivation and biomass utilization are described. A detailed state of the art related to anaerobic digestion of microalgae was given in this chapter. Additionally, the expected problems for an efficient conversion to biogas and potential solutions are covered.

**Chapter 3** describes the experimental procedures and methods used for microalgae cultivation, pretreatments, anaerobic digestion process and analytical determinations.

**Chapter 4** shows the outcomes of all experimental work and presents and discusses the results of:

- The effect of low temperature autohydrolysis at different incubation time on the cell wall disruption of *Chlorella vulgaris* and *Scenedesmus* sp. with regard to organic matter and carbohydrates solubilisation as well

as methane production enhancement of pretreated biomass compared to raw biomass (Section 4.1.1).

- The screening of different commercial enzymatic cocktails, including carbohydrases and proteases, on *Chlorella vulgaris* and *Scenedesmus* sp. biomass in order to evaluate their hydrolytic efficiencies in terms of organic matter solubilisation and biogas production (Section 4.1.2).
- The optimization of enzymatic pretreatment of microalgae biomass (*Chlorella vulgaris* and *Chlamydomonas reinhardtii*) with regard to hydrolysis time, thermal application prior to enzymatic pretreatment and the combination of different enzymes. This optimization was evaluated in terms of biomass hydrolysis and biodegradability efficiencies (Section 4.2).
- Further optimization of the most appropriate enzyme (protease) pretreatment was conducted by elucidating the effect of enzyme dosages and microalgae biomass loads. Organic matter solubilisation after the enzymatic pretreatments and methane production under batch anaerobic digestion were also investigated in order to determine the optimum pretreatment conditions to be implemented in semi-continuous operated anaerobic digester (Section 4.2.6).
- Assess the benefits of the proteolytic pretreatment in semicontinuously fed reactors. Organic matter removal, biogas quality, methane yield, intermediate products and nitrogen mineralization achieved during anaerobic digestion were also evaluated (Section 4.3).
- Determine the feasibility of coupling wastewater bioremediation with energy production (biogas) by cultivating microalgae in wastewater as sole nutrient source with the aim at reducing biomass protein content. The effect of protease and carbohydrase hydrolysis of the harvested biomass was compared in terms of biogas production, organic matter removal, biogas production rate, methane yield, intermediate products and nitrogen mineralization achieved during anaerobic digestion of these substrates (Section 4.4).

**Chapter 5** presents the main conclusions and the perspectives for future research.

# CHAPTER 2

# INTRODUCTION

## 2. INTRODUCTION

Fossil energy/fuel replacement with sustainable alternative resources is increasingly imperative in the light of impending climate change and fossil energy/fuel depletion. To cope with those issues, a number of renewable energy/fuel options are under development, including wind, solar, hydroelectric and biomass energy/biofuel. Unlike biomass, almost all sustainable resources focus on electricity generation however the global electricity demand accounts for only 30% of the energy required and thus, a variety of fuels devoted to other applications than electricity is mandatory. Liquid and gaseous energy forms that can be stored and transported seem to be an attractive alternative (Parmar et al., 2011). Biomass, for instance, can be converted into liquid or gas fuel, so-called “biofuel”. The use of biofuels minimizes concerns associated to fossil fuel problems such as reducing emissions of particulate matter, CO, hydrocarbons, and $So_x$ (Mata et al., 2010).

Biomass refers to all organic matter that can be obtained from photosynthesis, most often it refers to plants, lignocellulosic materials and algae. Organic matter is used as a substrate that can be converted into energy forms (ethanol, biogas, biodiesel…) through biological or chemical processes. Biomass is a renewable, sustainable, biodegradable, and environmentally friendly resource. Biomass can remove $CO_2$ from industrial flue gases while growing (thus producing more biomass that can be further valorized).

Biofuels are classified based on the biomass used to produce them. In this sense, first generation biofuels include conventional crops (such as corn and soybean) while second generation biofuels are obtained from lignocellulosic material derived from agriculture or forest wastes. Microalgae, as a third generation biofuels, are an attractive alternative substrate for biofuel production since they could avoid many of the concerns encountered for first and second generations’ biofuel. Microalgae can be grown in un-arable lands using non-potable water and thus, reducing competition with food industry. Furthermore, microalgae exhibit higher growth rate than terrestrial plants

(Chen et al., 2015) and they are no seasonal dependent. However, microalgae give optimal growth in a narrow range of temperature and hence, they are climate dependent. All these features results in much higher biomass productivity with regard to conventional biomass. Biomass productivity of microalgae could be 50 times higher than that of switchgrass (fastest growing terrestrial plant, Li et al., 2008) and accordingly, microalgae also exhibit the highest $CO_2$ fixation efficiency (Wang et al., 2008).

Microalgae can utilize nitrogen and phosphorous from a variety of wastewater sources such as domestic and livestock wastewater (piggery, poultry and cattle), which concomitantly provides the additional benefit of wastewater bioremediation. Although plants are able to uptake nutrients form wastewater, plants require more space for their maintenance and growth and thus, plant-based technologies are difficult to scale up at industrial levels (Renuka et al., 2015). The advantages of microalgae biomass as feedstock for biofuel production are summarized in Figure 2.1.

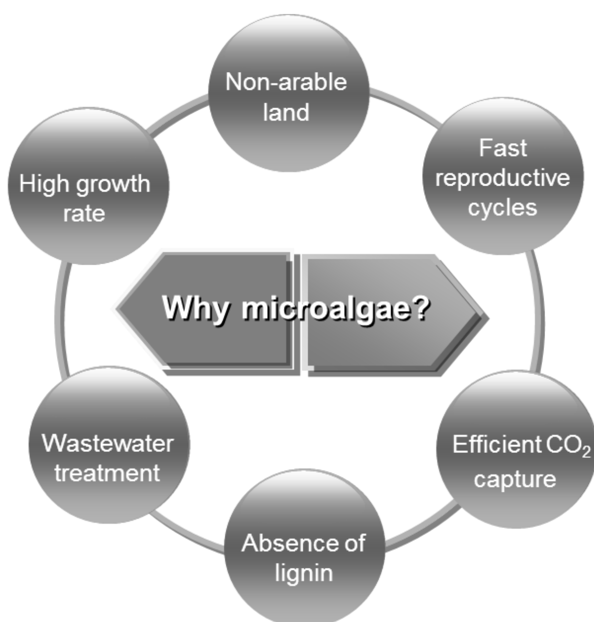



**Figure 2.1.** Advantages of microalgae as feedstock for biofuel production

Despite all the positive aspects mentioned above, there are many challenges to be overcome when using microalgae as a precursor for biofuel production. The overall costs of biofuels production from microalgae biomass including cultivation, harvesting and downstream processing must remain as low as possible for cost competitiveness with current conventional fuel production. Therefore, new strategies should be investigated in these three stages in order to produce large-scale sustainable products cost-effectively.

### 2.1. Microalgae definition and diversity

Algae are photosynthetic organisms able to convert light energy to chemical energy through biomass production. Based on their morphology and size, algae are grouped in two categories, macroalgae and microalgae. Macroalgae are composed of multiple cells organized as structures resembling roots, stems, and leaves of higher plants (Chen et al., 2009). On the contrary, microalgae are microscopic (2–200 μm) (Ratha and Prasanna, 2012) and unicellular or exhibiting simple multicellular structures (Figure 2.2.). Microalgae can double their mass in few hours (Chisti, 2007) and thus, cell populations might change drastically in a short period of time.

From a practical point of view, microalgae are easy to cultivate using non-potable water and thus, they can easily have access to nutrients from different cheap resources such as wastewater. From an application point of view, microalgae can be described as a single cell factory, as their biomass can be used for the obtaining of different products such as biofuel or high value products such as pigments and pharmaceuticals (Priyadarshani and Rath 2012).

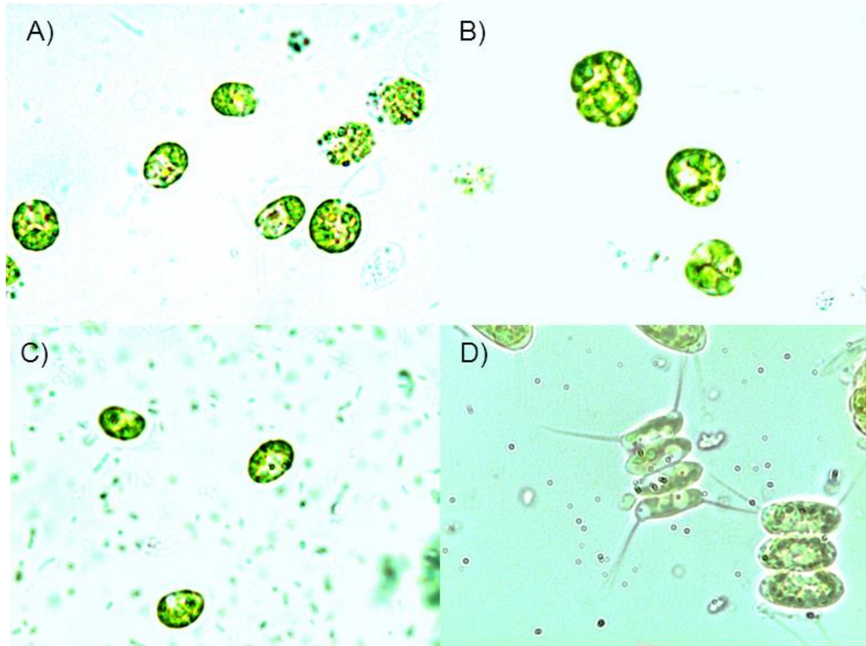



**Figure 2.2.** Optic microscopic images of *Chlamydomonas reinhardtii* (A), *Chlorella vulgaris* (B) and *Scenedesmus* sp. (C and D) used in this thesis.

Microalgae are grouped into 11 divisions involving two prokaryotic divisions namely, cyanophyta and prochlorophyta. The nine divisions of eukaryotic microalgae include Glaucophyta, Rhodophyta, Heterokontophyta (Bacillariophyta), Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta and Chlorophyta (Graham and Wilcox 2000). The main criteria for categorizing microalgae are based on some parameters like morphologic characteristics, pigment composition, storage products, and a variety of ultra-structural features. The most abundant microalgae found in water environments are cyanobacteria (Cyanophyta), green algae (Chlorophyta), diatoms and golden microalgae (Heterokontophyta) (Khan et al., 2009). The biodiversity of microalgae is enormous. As a matter of fact, even if only about 35,000 species have been identified, it has been estimated that 200,000 to 800,000 species exist and still need to be explored (Ratha and Prasanna, 2012).

## 2.2. Microalgae biomass production

### 2.2.1. Microalgae growth phases and growth modes

#### 2.2.1.1 Growth phases

Microalgae growth refers to the increase in microalgae biomass over time. Dry weight and cell count are common methods for growth determination. Microalgae have five growth phases (namely, lag phase, exponential phase, decline relative growth phase, stationary phase and death phase, Figure 2.3.). Lag phase is attributed to the physiological adaptation of cell metabolism to the cultivation media and through it, a little increase in cell numbers occurs. The second phase, exponential phase, entails a rapid increase in cells number as a function of time, according to a logarithmic function:

$$C_t = C_0.e^{\mu t}$$

where $C_t$ and $C_0$ are the cell concentrations at time (t) and (0), respectively and μ is the specific growth rate. Table 2.1. shows specific growth rate under different condition for different microalgae species.

In the declining growth rate phase, cell division slows down as a result of physical and chemical factors reduction (such as nutrients, light, etc.). In stationary phase, growth factors (such as nutrients and light) becomes limiting and the cell number becomes relatively constant. In this phase, a balance between growth and death takes place. During death phase, the cells number decrease rapidly and the culture eventually collapses as a function of nutrient depletion.

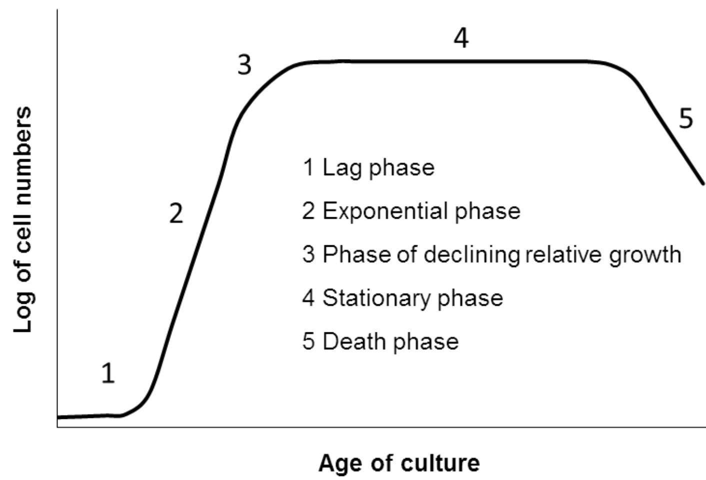



**Figure 2.3.** Growth phases of microalgae culture (Levens and Sorgeloos,1996).

**Table 2.1.** Specific growth rate of different microalgae species under different cultivation conditions.

| Microalgae species | Cultivation condition | Specific growth rate (μ) ($day^{-1}$) | References |
|---|---|---|---|
| *Chlorella vulgaris* | Basal medium, light intensity 60 μmol $m^{-2}$ $S^{-1}$, $CO_2$ (2%), 28ºC, pH 6.2 | 0.99 | Ho et al., 2013a |
| *Chlamydomonas orbicularis* | Basal medium, light intensity 60 μmol $m^{-2}$ $S^{-1}$, $CO_2$ feeding concentration (2%), 28ºC, pH 6.2 | 0.41 | Ho et al., 2013a |
| *Scenedesmus obliquus* | Detmer medium, light intensity 210-230 μmol $m^{-2}$ $S^{-1}$, CO2 (2.5%), 24ºC, pH 6.2 | 1.60 | Ho et al., 2013b |
| *Scenedesmus obliquus* | Basal medium, light intensity 210-230 μmol $m^{-2}$ $S^{-1}$, $CO_2$ (2.5%), 33ºC, pH 6.2 | 1.40 | Ho et al., 2013b |
| *Scenedesmus sp.* | Modified BG11, light intensity 55-60 μmol $m^{-2}$ $S^{-1}$, Light/dark 14/10h, 25ºC | 0.77 | Xin et al., 2011 |
| *Scenedesmus obtusus* | Modified Bold basal medium, light intensity 30 μmol $m^{-2}$ $S^{-1}$, $CO_2$ 10% 25ºC | 0.15 | Chandra et al., 2016 |
| *Chlorella sorokiniana* | Kuhl medium light intensity 100 μmol $m^{-2}$ $S^{-1}$, CO2 1%,25°C | 1.24 | Li et al., 2016 |
| *Chlorella sorokiniana* | Kuhl medium, light intensity 100 μmol $m^{-2}$ $S^{-1}$, air, 25°C | 0.60 | Li et al., 2016 |
| *Chlorella vulgaris* | Municipal wastewater, light intensity 45 μmol $m^{-2}$ $S^{-1}$, CO2 15%, 27°C | 1.37 | Ji et al., 2013 |
| *Ourococcus multisporus* | Municipal wastewater, light intensity 45 μmol $m^{-2}$ $S^{-1}$, CO2 15%, 27°C | 1.00 | Ji et al., 2013 |
| *Chlorella zofingiensis* | Autoclaved wastewater (piggery), light intensity 230 μmol $m^{-2}$ $S^{-1}$, $CO_2$ 5-6% 25ºC, pH 6.2 | 0.34 | Zhu et al., 2013 |
| *Monoraphidium* sp. | Wastewater, light intensity 90 μmol $m^{-2}S^{-1}$, air, 25 ºC | 0.29 | Jiang et al., 2016 |
| *Botryococcus braunii* | Wastewater, light intensity 3500 lux, 25ºC, pH 7.2 | 0.11-0.2 | Sydney et al., 2011 |
| *Chlorella vulgaris* | Wastwater, Light intensity 6000 lux, 22-32ºC | 0.25-0.32 | Mendez et al., 2016 |

Although biomass productivity of microalgae is strain-dependent, the growth medium and operational conditions applied for microalgae cultivation could also affect relatively biomass production (Table 2.2.). In this context, microalgae growth in synthetic media could result in greater biomass productivity than in wastewater. For instance, biomass productivity of *Chlorella vulgaris* and *Scenedesmus obliquus* ranged 0.16-0.19 g $L^{-1}day^{-1}$ (Ho et al., 2013a) and 0.35-0.44 g $L^{-1}day^{-1}$ (Ho et al., 2012) when grown in synthetic media while the productivity value diminished to 0.05 g $L^{-1}day^{-1}$ and 0.22 g $L^{-1}day^{-1}$, respectively when grown in wastewater (Gouveia et al., 2016). In a well-designed production system, microalgae biomass productivity can reach values of around 1.54 g $L^{-1}$ $day^{-1}$ (Chisti, 2008).

**Table 2.2.** Growth productivity of different microalgae species grown in different cultivation media.

| Microalgae species | Nutrient media | Scale | Growth productivity g $L^{-1}d^{-1}$ | References |
|---|---|---|---|---|
| *Scenedesmus obliquus* | Synthetic media/Detmer | Lab scale | 0.35-0.44 | Ho et al., 2012 |
| *Chlorella vulgaris* | Synthetic media/BG11 | Lab scale | 0.16-0.19 | Ho et al., 2013a |
| *Chlorella minutissima* | Wastewater/poultry | Lab scale | 0.07-0.08 | Singh et al., 2011 |
| *Chlorella sorokiniana* | Wastewater/poultry | Lab scale | 0.06-0.07 | Singh et al., 2011 |
| *Scenedesmus bijuga* | Wastewater/poultry | Lab scale | 0.07-0.08 | Singh et al., 2011 |
| *Chlorella zofingiensis* | Wastewater/piggery | Lab scale | 0.20- 0.30 | Zhu et al., 2013 |
| *Scenedesmus obliquus* | Wastewater/urban | Up scale | 0.22 | Gouveia et al., 2016 |
| *Chlorella vulgaris* | Wastewater/urban | Up scale | 0.05 | Gouveia et al., 2016 |
| *Scenedesmus* sp. | Wastewater/municipal | Up scale | 0.13 | McGinn et al., 2012 |

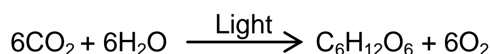
#### 2.2.1.2 Growth modes

Different types of metabolisms can be distinguished for microalgae growth based on the energy and carbon sources (Table 2.3.).

**Table 2.3.** Summary of different metabolisms found in microalgae (Chojnacka and Marquez-Rocha, 2004).

| Metabolism | Energy source | Carbon source |
|---|---|---|
| **Phototroph** | Light | Inorganic |
| **Heterotroph** | Organic | Organic |
| **Photoheterotroph** | Light | Organic |
| **Mixotroph** | Light and organic | Inorganic and organic |

Microalgae that are able to fix $CO_2$ in the presence of light to produce organic compound during photosynthesis are known as phototrophs. Photosynthesis is a simple reaction that can be represented by the following equation:



$$6CO_2 + 6H_2O \xrightarrow{\text{Light}} C_6H_{12}O_6 + 6O_2$$

Although the majority of microalgae are phototrophic organism able to use light as a sole energy source (phototrophic metabolism), some microalgae are able to thrive in the dark using organic compounds as carbon and energy source. This later growth mode is called heterotrophic metabolism. Photoheterotrophic metabolism is a combination of phototrophic and heterotrophic growth in which microalgae get their metabolic energy from light and the carbon source from organic matter.

As a third option, some microalgae can also exhibit a mixotrophic metabolism. During mixotrophic growth, microalgae are able to obtain energy from both

light and organic matter. Additionally, they are able to use both inorganic and organic carbon sources.

Some microalgae can use only one mode (obligate phototrophs such as *Phaeodactylum tricornutum and Volvox carteri* (Zaslavskaia et al., 2001; Perez-Garcia et al. 2011) or obligate heterotrophs such as *Cryptothecodinium cohnii* (Mendez et al., 2009)) whereas some other microalgae are capable of metabolic shifts as a response to changes in environmental conditions. For example, some organisms, known as amphitrophy (subtype of mixotrophy), are able to live either autotrophically or heterotrophically depending on the concentration of organic compounds and light intensity available (Chojnacka and Marquez-Rocha 2004).

### 2.2.2. Cultivation conditions

In order to obtain sufficient microalgae biomass, it is important to provide appropriate amounts of light, water, carbon, nitrogen, phosphorous and a variety of mineral **nutrients** to microalgae. Given the estimated molecular formula of the microalgae biomass ($CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$, Chisti, 2007), nutritional requirements can be identified. Carbon represents approximately 50% of microalgae dry weight while nitrogen and phosphorous account approximately for 7-10% and 1-3% of the dry weight, respectively (Hu 2004). Nitrogen is required for protein synthesis and it can be provided in many forms (nitrate, ammonia, urea, and nitrite). Nitrate, nitrite and urea have to be reduced or hydrolyzed to ammonium before assimilation and thus, ammonium is considered the easiest nitrogenous form for microalgae assimilation. Phosphorous is needed for nucleic acid synthesis, energy transfer and cell division. Inorganic phosphate, mainly in the form of $H_2PO_4^-$ and $HPO_4^{-2}$, is uptaken by microalgae and incorporated into organic compounds via different phosphorylation processes (Martinez et al., 1999; Cai et al., 2013). Besides, sulphur, potassium, magnesium and calcium as well as some trace elements, manganese, zinc, cobalt, molybdenum and EDTA (Ethylenediaminetetraacetic acid) are important nutrients for microalgae growth (Becker, 2004; Garrett and Grisham, 2005).

Environmental factors, such as light, temperature and pH values, have significant effect on microalgae biomass production. **Light** is one of the principal requirement for photosynthesis and both light intensity, a measure of the number of photons available for photosynthesis, and photoperiod are critical parameters in microalgae biomass production. Microalgae, as plants, have two photosystems: photosystem I and II with peak absorption at 680 nm and 700 nm, respectively, however, microalgae can absorb light within the range of 400-740 nm (Zhu et al., 2008). While outdoors conducted experiments depend on sun light, supply of light intensities in the range of 60-230 µmol $m^{-2}$ $s^{-1}$ are employed in most of the studies conducted at lab scale (Table 2.1.). This parameter should be carefully controlled since excessive intensity or low light levels may lead to photoinhibition or growth-limitation, respectively (Carvalho et al., 2011).

With regard to **temperature**, this parameter influences the rate of all chemical reactions involved in growth and metabolism of microalgae (Sandnes et al., 2005). Different species have different tolerance to temperatures ranges. The optimal temperature for freshwater microalgae production ranges 20-30ºC; however, they are able to adapt to temperature in the range of 10-35ºC (Singh and Singh, 2015). In this context, Ho et al., (2013b) elucidated that despite the ability of microalgae to grow at wide range of temperature, temperature over 33ºC or under 18ºC affected negatively microalgae growth. For example, the biomass productivity and $CO_2$ consumption rate were decreased from 0.57 g $L^{-1}$ $d^{-1}$ and 1.0 g $L^{-1}$ $d^{-1}$ down to 0.09 g $L^{-1}$ $d^{-1}$ and 0.16 g $L^{-1}$ $d^{-1}$ when growing *S. obliquus* at 24ºC and 33ºC, respectively (Ho et al., 2013b). Opposite, Mendez et al., (2016) found negligible differences in the growth of *C. vulgaris* at different temperature (22, 27 and 32 ºC) when cultivated in wastewater. Once again, this fact shows the high dependence on microalgae strain and cultivation conditions. No general rules can be set *a priori*.

**pH** is also one of the important factors for microalgae growth as it can affect the activity of different cellular enzymes. More specifically, metabolic pathways of microalgae involve enzymes that have determined pHs at which their activity is optimum. Any changes in internal pH may affect the rate of metabolic processes. For instance, the activity of glutamine synthetase, an important enzyme in the metabolic pathway of glutamine and glutamate

synthesis, decreased from 0.79 U at pH 7 down to 0.1 at pH 9.3 in *Skeletonema costatum* (Taraldsvik and Myklestad, 2000).

Secondly, the pH of the culture broth can also affect the bioavailability of nutrients. During microalgae cultivation, pH increases as a response to carbon dioxide consumption during photosynthesis. The pH needs to be maintained within a certain range (7-9) to prevent depletion of carbon in the medium and thus, achieving optimal microalgae growth (Becker, 1994). Likewise, pH fluctuations can affect the nutrient solubility of ammonium and phosphate. At increasing pH, ammonia might be stripped out and phosphate precipited (Molinuevo-Salces et al., 2016, Pratt et al., 2012).

Lastly, **mixing** is also necessary to avoid sedimentation, provide light homogenously and prevent concentration gradients. Mixing is provided by magnetic stirring (in small vessels, erlenmeyers), aeration (in photobioreactors) or using paddle wheels (in raceways). In turn, mixing could be avoided in attached cultivation system as microalgae culture grows in thin-layers immobilized onto a support. Even though those systems exhibit the benefit of an easy biomass harvesting, light and nutrient gradients cannot be avoided in these systems and hence, biomass productivity may be compromised (de Godos et al., 2009).

### 2.2.3. Alternative sources for nutrients (N and P): Wastewater

Microalgae cultivation consumes large amounts of fresh water. Murphy and Allen (2011) estimated that microalgae cultivation in open systems consumes nearly 1 $m^3$ of water per kg of biomass produced. Some recent studies have shown the large quantity of water losses that can take place in these systems due to evaporation (Posadas et al., 2015a). In this manner, since microalgae cultivation at large scale will demand enormous amounts of water and nutrients, it seems imperative to find alternative fresh water sources to cultivate microalgae.

Microalgae can be grown in wastewaters (industrial, agricultural and domestic, González-Fernández et al., 2011a;b; Pittman et al., 2011; Chen et al., 2015; Mendez et al., 2016). This cultivation media entails water availability

and the presence of nutrients required for microalgae growth. Additionally, this growth media offers the additional benefit of bioremediation. Wastewater is considered water of poor quality since it contains pollutants (organic, inorganic, solids, toxins etc.) and microbes (bacteria and protozoa) that have to be treated prior to be discharged in water bodies. Microalgae are able to remove nutrients from wastewater such as organic matter, nitrogen and phosphorous and assimilate them into microalgae biomass. Therefore, the use of wastewater in microalgae-based technologies serves the dual purpose of fresh water and nutrient supply for microalgal biomass production whilst acting as a treatment for wastewater as well. This approach of using microalgae cultivation for nutrients removal was developed by Oswald et al., (1957). The integration of microalgae cultivation with wastewater treatment system is eco-friendly and decreases overall costs of both processes. Oswald (1995) demonstrated that mechanical aerated ponds required 0.8–6.4 kW h kg $BOD^{-1}$ removed (BOD: biological oxygen demand) while photosynthetically oxygenated ponds consumed 0–0.57 kW h kg $BOD^{-1}$.

Several studies have reported the efficiency of microalgae cultivation in wastewater in terms of biomass production and nutrient removal. González-Fernández et al. (2016) cultivated microalgae consortia in urban wastewater with an initial ammonium and phosphate concentration at 80 and 14 mg $L^{-1}$, respectively. Ammonium and phosphate were removed by 100% and 95% removal efficiency, respectively. Posadas et al., (2013) obtained removal efficiencies of 70% and 85% for nitrogen and phosphorous when a microalgae consortium was grown in domestic wastewater with initial concentration of nitrogen and phosphorous at 66 and 7 mg $L^{-1}$, respectively. Likewise, both *Chlorella kessleri* and *Chlorella vulgaris* removed nitrogen and phosphorous from urban wastewater (containing 39-65 mg $L^{-1}$ of ammonium and 3-5.4 of and phosphate) almost completely (>95%) (Caporgno et al., 2015). Those examples and many others available in scientific literature proved the efficiency of microalgae biomass for wastewater treatment.

Nevertheless, some wastewater characteristics such as nutrient concentration, turbidity and coloration, should be taken into consideration when using wastewater for microalgae cultivation. For instance, excessive

nutrients concentration can have a negative effect on microalgae growth. In this sense, Abeliovich and Azov (1976) have demonstrated that ammonium concentration higher than 1 g $L^{-1}$ could inhibit photosynthesis. A strong coloration and/or turbidity of wastewater has also a negative influence on microalgae growth by reducing transparency and thus, reducing light penetration in the culture (Marcilhac et al., 2014).

### 2.2.4. Cultivation systems

The optimum cultivation system depends ultimately on the purpose of biomass production. Advantages and disadvantages of each system would sometimes depend on the cost of the product generated out of the biomass cultivated. Together with the cultivation conditions, the cultivation system employed for the production of biomass also affects microalgae growth. In this manner, Abomohra et al., (2016) reported that closed systems supported over ten-fold higher volumetric productivity than open system (1.5 g $L^{-1}$ $d^{-1}$ vs. 0.12 g $L^{-1}$ $d^{-1}$). Most common types of open systems are raceways and high rate algal ponds. Both systems have low water depth, large surface area and are open to the environment. Closed systems are commonly called photobioreactors (PBR). PBRs have different shapes including plastic bags, flat panels, and tubes. The review of Tan et al., (2015) summarized the advantages and disadvantages of each system. Briefly, in closed system, cultivation condition can be controlled, external contamination and evaporation losses can be avoided and thus, high growth rate could be achieved. Nevertheless, the negative points of these systems are their difficulties to be up-scaled due to the high building costs and operation of the system. For instance, closed PBRs normally require gas exchange equipment which concomitantly increases the cost of these cultivation systems. In some cases the high biomass productivity that the systems can achieve and/or the product extracted out of microalgae biomass may compensate the high costs of those bioreactors. Open systems, on the other hand, are cheaper and easier to maintain than closed systems. However, open systems are difficult to control since external contamination and evaporation losses can take place more easily.

Alternatively to suspended microalgae cultures, attached growth systems have been lately investigated. Those cultivation systems can decrease water consumption while obtaining a dense microalgae biomass easy to be harvested (Posadas et al., 2013). Microalgae get attached to supporting materials such as cotton, nylon, stainless steel, polycarbonate and polyethylene (Hoh et al., 2016). Many types of biofilm attached reactors have been reported in several studies, including horizontal, flow lane, vertical or rotating biofilm reactors (Ozkan et al., 2012; Irving and Allen, 2011; Liu et al., 2013; Blanken et al., 2014). Despite of the easier harvesting of the biomass grown in such PBRs, the main disadvantages of those reactors are the high cost of supporting material together with their structural weakness (Posadas et al., 2013) and the light and nutrients gradients taking place (de Godos et al., 2009).

### 2.3. Microalgae biomass utilization

#### 2.3.1 Microalgae for high value products

Microalgae do not only contain energy-rich carbon compounds but they are also rich in many specific high-value compounds that make them interesting for numerous applications. Microalgae can be used to produce a wide range of metabolites such as proteins, lipids, carbohydrates, food and feed additives, vitamins, carotenoids, cosmetics and pharmaceuticals (Cuellar-Bermudez et al., 2014; Koller et al., 2014).

Microalgae have been cultivated traditionally as a food source containing all 20 amino acids (Singh and Gu, 2010) and significant quantities of poly-unsaturated fatty acids such as omega-3 acids (Pulz and Gross, 2004). Likewise, pigments, such as β-carotene, used as food supplement and as colorant are produced from *Dunaliella* sp. (Chisti, 2006; Singh et al., 2005). Phycobiliproteins, phycocyanin and phycoerythin are produced from *Porphyridium cruentum* and *Galdieria sulphuraria* and are also used in food and cosmetics commercial sectors (Cuellar-Bermudez et al., 2014). For instance, *Crypthecodinium cohnii* produces docosahexaenoic acid (DHA) (Borowitzka, 2006; Spolaore et al., 2006) which is used as a dietary supplement and supplement in infant formulas while sulphated

polysaccharides from *Porphyridium* are used for cosmetic products (Arad and levy-Ontman, 2010). Microalgae derivatives have revealed a positive effect on animals physiology and are common additives in animal feed and aquaculture (Pulz and Gross, 2004).

Microalgae also contain sterols which could be used as building blocks for pharmaceuticals. Different compounds with antibacterial, antiviral and antifungicidal activity can be found in microalgae (Prasanna et al., 2008; Becker 2004). Besides, many vitamins and minerals like nicotinate, biotin, folic acid, pantothenic acid, niacin, iodine, potassium, iron, magnesium and calcium are abundantly found in microalgae (Koller et al., 2014; Markou and Nerantzis, 2013).

Additionally, after high value products extraction, microalgae residuals can also be used as fertilizers due to their high content of macro and micro nutrient that are essential for plant growth. This type of biomass degrades slowly, thereby it is considered as a slow release fertilizer which can improve nutrients plant uptake (Coppens et al., 2016). Furthermore, some microalgae and cyanobacteria are capable of promoting plant growth by excreting polysaccharides that stimulate plant growth and/or producing active compounds that inhibit the growth of pathogenic bacteria and fungi (Grzesik and Romanowska-Duda, 2015).

### 2.3.2. Microalgae for biofuels

Moving further into biofuel production using microalgae, this biomass can be subjected to a wide number of conversion processes. Microalgae biomass are renewable, ecofriendly, carbon neutral, non-toxic and biodegradable. Microalgae are of particular interest in the field of biofuels production due to their dual capacity to capture solar energy and convert it into carbohydrate, protein and lipid. Thus, this biomass can be further valorized for energy purposes. The chemical composition of various microalgae strains is shown in Table 2.4.

**Table 2.4.** Gross composition (percent dry weight) of several microalgae species.

| Microalgae strain | Protein | Carbohydrate | Lipid | References |
|---|---|---|---|---|
| *Nannochloropsis oculata* | 57 | 8 | 32 | Biller and Ross, 2011 |
| *Chlorella vulgaris* | 51–58 | 12–17 | 14–22 | Becker, 2004 |
| *Chlamydomonas reinhardtii* | 48 | 17 | 21 | Becker, 2004 |
| *Scenedesmus obliquus* | 50–56 | 10–17 | 12–14 | Becker, 2004 |
| *Chlorella vulgaris* | 55 | 9 | 25 | Biller and Ross 2011 |
| *Botryococcus braunii* | 39.6 | 2.4 | 33 | Sydney et al., 2010 |
| *Dunaliella tertiolecta* | 29.4 | 14 | 11.4 | Sydney et al., 2010 |
| *Scenedesmus almeriensis* | 41.8 | 38.7 | 11.2 | Romero-Garcia et al., 2012 |
| *Chlorella vulgaris* | 64.2 | 20.1 | 7.7 | Mendez et al., 2015 |
| *Chlorella vulgaris* | 63.4 | 18.6 | 9.8 | Mahdy et al., 2014a |

Biofuels production should consider the whole value chain: from microalgae cultivation to the obtaining of final product. Many different options for cultivating, harvesting and transforming microalgae into a wide range of biofuels exist. A summary of biofuel production routes from microalgae cultivation to final products is illustrated in Figure 2.4.

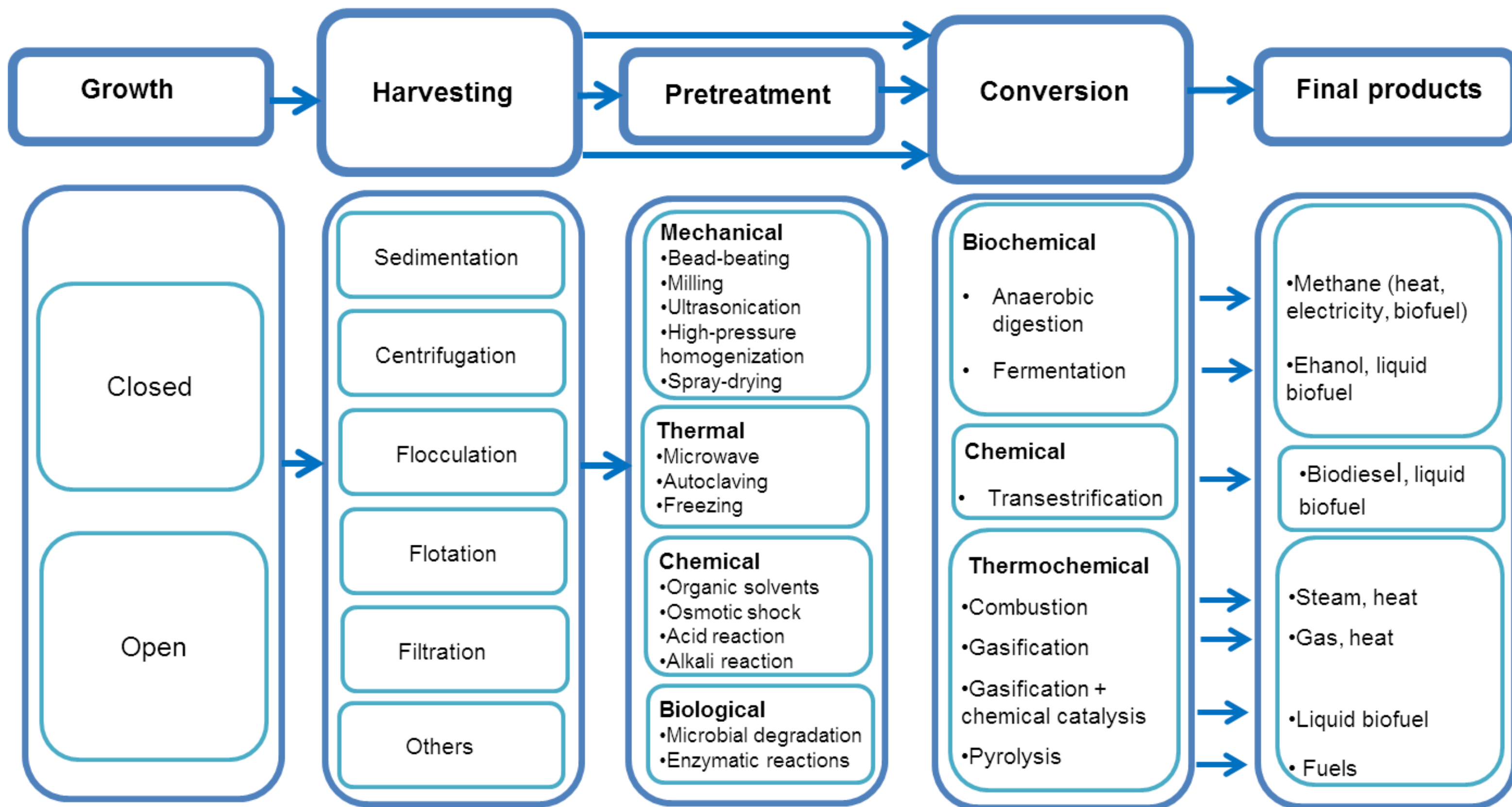



**Figure 2.4.** Summary of biofuel production routes from microalgae cultivation to final products

Microalgae can provide feedstock for several types of renewable fuels. Until now, the biofuels most investigated in this field include biodiesel, bioethanol and biogas.

#### 2.3.2.1. Biodiesel

Due to the high lipid content of some microalgae, many research efforts to obtain biofuels from microalgae have been focused on biodiesel production. Some species might accumulate 50% or more of their dry weight as lipids (Griffiths and Harrison, 2009; Hu et al., 2008; Patil et al., 2008). Nevertheless, this is not the case for all species and most commonly, this accumulation occurs under nutrient stress where the growth rate is reduced, limiting the global yield of lipids production. For instance, although *Botryococcus braunii* is able to accumulate high lipid content (45% DW), its growth rate is very low ($\mu$= 0.144 day$^{-1}$), reducing the volumetric lipid productivity (Nascimento et al., 2014).

In the process to produce biodiesel from microalgae, the lipid fraction of microalgae biomass is extracted, and then processed and refined to the final product. Various methods have been reported for microalgae lipids extraction; nonetheless, solvent extraction, expeller/oil press, supercritical fluid extraction and ultrasound techniques are the most common methods (Singh and Gu, 2010). Triacylglycerides (TAGs), once extracted, are converted to fatty acid methyl esters by a transesterification process through a chemical reaction with methanol (or another short chain alcohol) in the presence of a catalyst, such as sodium or potassium hydroxide (Figure 2.5.). TAGs vary considerably among individual species, strains and among taxonomic groups. However, in most microalgae, TAGs are composed mostly of saturated or mono-unsaturated C14–C18 fatty acids (Hu et al., 2008).

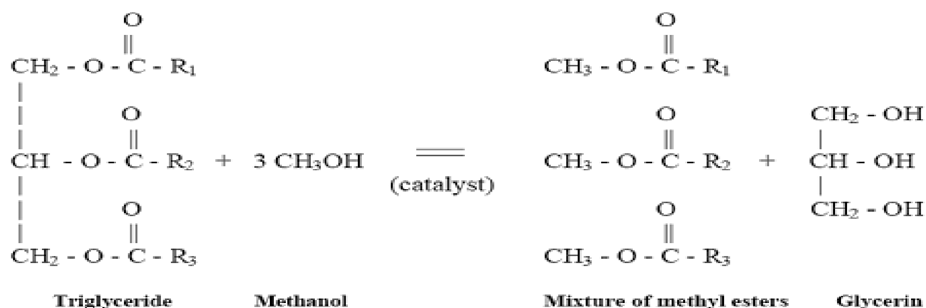



**Figure 2.5.** Chemical reaction for biodiesel production by transesterification (Salam et al., 2016).

Although for the success of biodiesel-based microalgae industry, high growth rates should be coupled with high lipid content of microalgae biomass, lipid content of naturally dominant robust species (*Scenedesmus* sp. and *Chlorella* sp.) often tends to be low (typical range of lipid content for wild species varies from 1.5 to 10.5% (Abubakar et al., 2012; Mendez et al., 2013; Mahdy et al., 2014b). Likewise, regarding the economic aspects of producing biodiesel from microalgae, the life cycle analysis revealed that biodiesel from microalgae is an energetically unbalanced process due to the high energy consumption of lipid extraction (70-90% of all energy consumption employed in the biodiesel production process, Lardon et al., 2009). This fact together with the low lipid content of wild microalgae species, as mentioned previously, make the production of biodiesel difficult.

#### 2.3.2.2. Bioethanol

Even though some microalgae such as *Chlorella* are able to produce ethanol through heterotrophic self-fermentation by consumption of its intracellular starch (Ferrell and Sarisky-Reed, 2010), the most common procedure is to use microalgae biomass as substrate. Ethanol production is based on the fermentation of microalgae carbohydrates. Several microalgae (*Chlorella, Scenedesmus, Chlamydomonas, Dunaliella* and *Tetraselmis* sp.) are able to accumulate large amount of carbohydrates (> of 40% DW, John et al., 2011). Yeast, bacteria or fungi are microorganisms used to ferment carbohydrates to produce ethanol. Bioethanol production through yeast fermentation process is a very well-established industrially. To achieve high ethanol yields, it is

important first to screen for high carbohydrate producing microalgae strains and then convert complex carbohydrates to simple sugars suitable for yeast fermentation via different pretreatments. Literature dealing with those pretreatments includes physical, biological and chemical methods devoted to microalgae biomass hydrolysis (Choi et al., 2010; Kim et al., 2014, Zhou et al., 2011). After the pretreatment, both hexose and pentose sugars might be produced via saccharification. Microalgae carbohydrates are in this way hydrolyzed to fermentable sugars. According to the following simplified reaction equation, stoichiometric yields are 0.51 kg ethanol and 0.49 kg $CO_2$ per kg of glucose:

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$$

However, the hurdle of using microalgae for bioethanol production is that ethanologenic yeasts can ferment only simple sugars while microalgae carbohydrates consist of complex mixtures. More specifically, those microalgae carbohydrates have evidenced different composition of, neutral sugar, uronic acid and amino sugar (Sui et al., 2012; Cheng et al., 2011). Therefore, not only the carbohydrate content of this biomass is relevant but the composition can decrease ethanol yields considerably. As it can be seen in Table 2.5., the ethanol yield of microalgae ranges 0.13- 0.29 g EtOH/g biomass depending on the type of pretreatment, carbohydrate content and fermenting microorganism. This yield is far below the yields that can be attained with other substrates such as rice straw (0.45 g EtOH/g) and wheat straw (0.41 g EtOH/g) (Sarkar et al., 2012). Additionally, as show in Table 2.4., most microalgae are not exhibiting high carbohydrate content under non-restrictive cultivation condition. Overall, the limitation for microalgal carbohydrates applied to bioethanol production is their low content and compositional complexity (yet to be elucidated).

**Table 2.5.** Bioethanol yield under various type of pretreatment and microalgae species.

| Microalgal strains | Pretreatment/ fermenting microorganisms | Carbohydrate content (% DW) | Ethanol yield (g EtOH/g biomass) | References |
|---|---|---|---|---|
| *Scenedesmus obliquus* | Chemical /*Zymomonas mobilis* | 49.4 | 0.195 | Ho et al., 2013b |
| *Chlamydomonas reinhardtii* | Hydrothermal acid/ *Saccharomyces cerevisiae* | 60.0 | 0.292 | Nguyen et al., 2009 |
| *Chlamydomonas reinhardtii* | Enzymatic/ *Saccharomyces cerevisiae* | 60.0 | 0.235 | Choi et al., 2010 |
| *Chlorella vulgaris* | Chemical/*Zymomonas mobilis* | 54.4 | 0.233 | Ho et al., 2013c |
| *Chlorella vulgaris* | Physical+enzymatic/ *Saccharomyces cerevisiae* | 22.3 | 0.131 | Kim et al., 2014 |

#### 2.3.2.3. Biogas

Biogas, a mixture of carbon dioxide and methane, is produced through anaerobic degradation of organic matter. Anaerobic digestion is a mature technology widely employed for wastes treatment in which both pollution control and energy recovery can be accomplished. Compared to bioethanol and biodiesel production, biogas production is the most flexible process since this biological process can use all the macromolecular components (lipids, carbohydrates and proteins) of microalgae biomass to produce energy/biofuel. In this process, the entire organic matter, not only lipids or carbohydrates, are digested and transformed into biogas. As a matter of fact, specific methane yields calculated for lipids resulted in 1.014 L $CH_4$ g VS $in^{-1}$ while for carbohydrates and proteins were 0.415, 0.496 L $CH_4$ g VS $in^{-1}$, respectively (Angelidaki and Sanders 2004). In this sense, the ideal situation would be to have a microalgal substrate with prevailing composition of lipids, however as shown in Table 2.4., this is not normally the case. Even though, lipids and carbohydrates content can be increased under stressful growth conditions (Brányiková et al., 2011; Cakmak et al., 2012; González-Fernández and Ballesteros, 2012), the inherent content of these two macromolecules is low under normal growth conditions (i.e., not repressed growth rate). All these features render anaerobic digestion the most straightforward process for energy production using microalgae biomass as substrates.

## 2.4. Microalgae and anaerobic digestion

Even though anaerobic digestion is a mature technology widely employed for wastes treatment, microalgae represent a new substrate for biogas production. During the first attempts undertaken using this technology, the achieved biogas yields were proven low (Golueke et al., 1957; Golueke and Oswald, 1959). In this sense, the anaerobic degradation of microalgae biomass was limited by their compositional and structural features. The conclusion withdrawn in early studies was that more efforts were required in this research field to turn the most feasible energy form (biogas) into an efficient bioprocess.

### 2.4.1. Anaerobic digestion process description

Anaerobic digestion is defined as the process that converts organic matter anaerobically into methane (energy-rich component) and carbon dioxide through several reactions. Based on the digested feedstock, methane content of biogas might range 50% -75% and carbon dioxide with a share between 25% and 50% (Table 2.6.). In addition, other gases are also produced in small amounts such as ammonia and hydrogen sulfide. Digestate is also produced during anaerobic digestion as the liquid fraction that could be used as a fertilizer or a nutrient source for microalgae growth (Tambone et al., 2010; Bjornsson et al., 2013).

**Table 2.6.** Methane content in the biogas produced over anaerobic digestion of different substrates**.**

| Substrate | Methane content (%) | References |
| --- | --- | --- |
| **Microalgae** | 67–70 | Mendez et al., 2013 |
| ***Opuntia maxima*** | 47 | Ramos-Suárez et al., 2014 |
| **Rice straw** | 35-54 | Chandra et al., 2012 |
| **Maize silage** | 54 | Mussgnug et al., 2010 |
| **Chicken manure** | 61 | Kafle and Chen 2016 |
| **Cattle manure** | 59 | Bah et al., 2014 |

Three major stages can be distinguished in anaerobic digestion: hydrolysis, fermentation (acidogenesis/acetogenesis) and methanogenesis (Figure 2.6.). Through the first stage, hydrolysis, complex organic matters (carbohydrates, proteins and lipids) are broken down by the activity of extracellular enzymes

(protease, lipase, amylase…) of hydrolytic bacteria to produce simple forms (sugars, amino acids, fatty acids). The second stage is carried out by acidogenic and acetogenic bacteria. Firstly, hydrolysis products are converted into volatile fatty acids, alcohol, hydrogen and carbon dioxide. Subsequently, further degradation takes place by acetogenic bacteria, converting long chain fatty acids and alcohol to acetate, hydrogen and carbon dioxide. The acetogenic bacteria are obligate anaerobes while acidogenic bacteria involves a very diverse group of bacteria both obligate and facultative anaerobes (Ryan et al., 2010; Amaya et al., 2013). The products of this reaction are used by methanogic archaea in the last stage of anaerobic digestion (methanogenesis). Two pathways can be followed to produce methane; namely acetoclastic and hydrogenotrophic pathways. The acetoclastic pathway (acetate is the precursor of methane production) has been considered the predominant pathway and normally represents 70% of the produced methane (Conrad et al., 2010; Angelidaki et al., 2011). In this pathway, acetate is split directly to methane and carbon dioxide. *Methanosarcina* spp. and *Methanosaeta* spp. are the main microorganisms involved in acetoclastic methanogenesis (González-Fernández et al., 2015). However, in presence of inhibitors such as ammonia, the main mechanism of acetate degradation shifts to syntrophic acetate oxidation. In this pathway, acetate is oxidized to hydrogen and carbon dioxide by acetate oxidizing bacteria (Karakashev et al., 2006). Alternatively, in the hydrogenotrophic pathway, the precursor of methane is carbon dioxide and hydrogen. Hydrogenotrophic methanogens belong to the orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobials*, and *Methanosarcinaceae* (Karakashev et al., 2006). Overall, anaerobic digestion is a multi-step process and depends on numerous microbial populations that operate in close interaction with each other for producing methane.

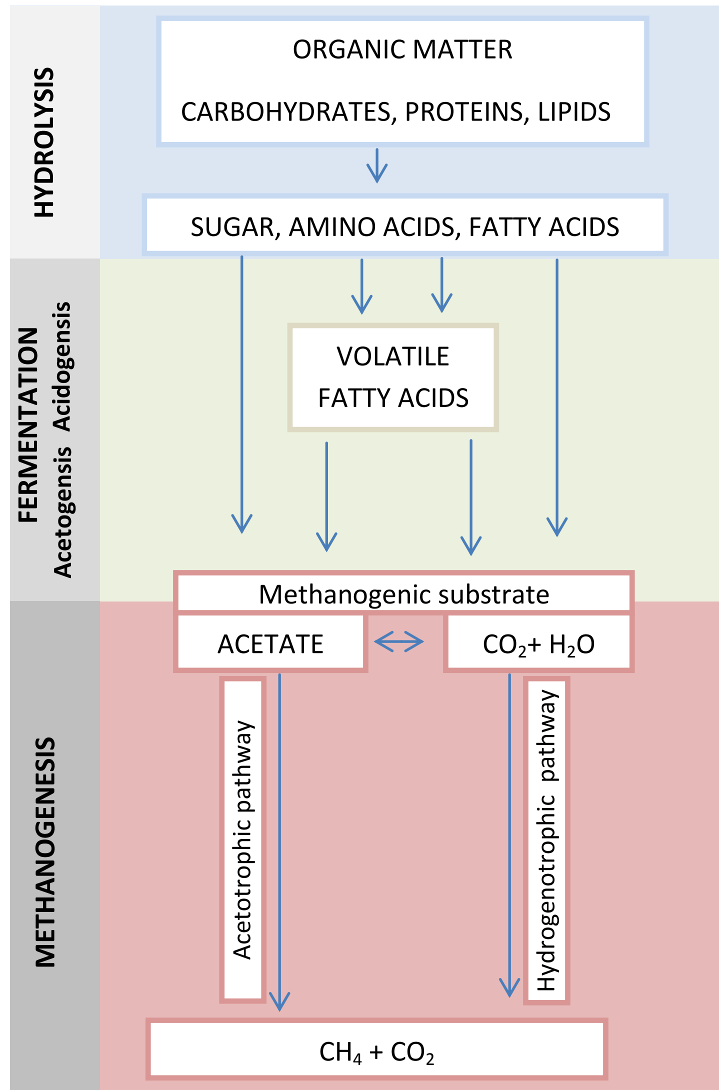
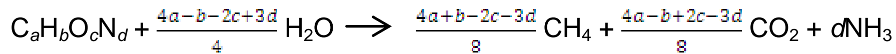



**Figure 2.6.** Schematic representation of the main conversion processes in anaerobic digestion.

### 2.4.2. Theoretical methane production

The theoretical methane yield of a known organic matter composition can be calculated by using the following formula (Symons and Buswell 1933):



$$C_aH_bO_cN_d + \frac{4a-b-2c+3d}{4} H_2O \rightarrow \frac{4a+b-2c-3d}{8} CH_4 + \frac{4a-b+2c-3d}{8} CO_2 + dNH_3$$

Thus, the specific methane yield as liters of methane per gram of volatile solids can be calculated as:

$$\text{Methane yield (L g VS in}^{-1}) = \frac{(4a-b-2c+3d)*Vm}{12a+b+16c+14d}$$

where Vm is the molar volume of methane or 22.4 L at standard temperature and pressure (0 °C and 1 atm).

Alternatively, theoretical methane yields can also be calculated based on the macromolecular composition of the digested substrate. Angelidaki and Sanders (2004) have calculated the theoretical methane yields for carbohydrates, proteins and lipids to be approximately 0.415, 0.496 and 1.014 L $CH_4$ g VS $in^{-1}$, respectively. Taking into consideration the theoretical methane yields of microalgae biomass, the biodegradability of microalgae biomass can be calculated by comparing experimental biochemical methane potential ([BMP] yield, $mLCH_4$ g VS $in^{-1}$) and theoretical ($BMP_{theo}$, $mLCH_4$ g VS $in^{-1}$) methane yields as follows:

$$\text{Biodegradability efficiency} = \frac{BMP}{BMP\ theo} * 100$$

In this manner, given the average macromolecular profile of *C. vulgaris* used in this thesis (63.7% for protein, 21.3% for carbohydrate, and 7% TS for lipid), the theoretical methane yield of microalgae was estimated to be approximately 0.440 L $CH_4$ g VS $in^{-1}$ (assuming an average VS/TS ratio of 93%). However, experimental methane production of microalgae achieved

until now is much lower than those theoretical values. As a matter of fact, several studies have reported methane production yields lower than 50% of that of the theoretical yield (Mahdy et al., 2015a; Mendez et al., 2013; González-Fernández et al., 2012a and Passos et al, 2013). One of the reasons for those low efficiency conversions is attributed to the microalgae macromolecular profile. The low carbon to nitrogen (C/N) ratio, characteristic of protein rich microalgae biomass (González-Fernández, et al, 2015; Mahdy et al., 2015a), can result in anaerobic microorganism's inhibition by high $NH_4^+/NH_3$ concentration that can be reached during digestion process. In addition, another main reason for the low conversion efficiency to methane is the resistance of microalgae cell wall to anaerobic bacteria attack (Section 2.4.4.1).

### 2.4.3. Parameters affecting anaerobic digestion

All aspects related to anaerobic digestion process, such as temperature, pH, digestion time and reactor configuration can affect the biodegradability and methane yields and, therefore, should be taken into consideration to describe and evaluate the performance of this biological transformation process.

**Temperature**

Temperature is one of the key parameters influencing anaerobic digestion. Anaerobic digestion can be conducted under a wide range of temperature (10-65ºC). Enzymes involved in anaerobic digestion process have limited temperature resistance and thus, temperature affects directly the growth of microorganisms and degradation kinetics. Psychrophilic processes are performed at <20ºC, mesophilic processes are performed at 25–40°C and thermophilic processes at 50–65°C. Mesophilic conditions render the anaerobic digestion less sensitive to environmental conditions, less vulnerable to ammonium toxicity and volatile acid accumulation, and thus this range of temperatures exhibits higher process stability compared to thermophilic conditions (Mao et al., 2015). These features make mesophilic conditions to be the most commonly used digestion temperature. Likewise, temperature can also affect ammonia /ammonium equilibrium during the digestion process as explained in section 2.4.4.3.

**pH**

Anaerobic digesters host a diverse group of microorganisms and each group needs different pH ranges for their growth and optimum activity. Fermentative bacteria, for instance, require pH range of 4.0-8.5, while most methane producers require neutral pH (Temudo et al., 2008; Kwiethiewska and Tys, 2014). Therefore, the ideal pH for anaerobic digestion is around 7.

pH values can be used as fast indicators to monitor the process and detect stress conditions in the system. Many intermediate products of the anaerobic digestion affect and change the pH. Ammonia, the final compound from protein degradation, can react with $CO_2$ and water to form ammonium carbonate, and thus provides alkalinity to the system. Opposite, volatile fatty acids (VFAs) can be accumulated at high loads (>1.5 g acetic $L^{-1}$, Angelidaki et al., 2005; Fotidis et al., 2014a) resulting in the decrease of pH values.

**Volatile fatty acids (VFAs)**

Long chain fatty acids (LCFAs) such as oleic acid, linoleic acid and palmitoleic acid are produced in the anaerobic digestion process through lipid degradation. Afterward, LCFAs are further degraded to VFAs. Due to the difference between lipids and LCFAs degradation rates, LCFA accumulation may take place in digestion process over time since LCFAs degradation is slower than lipid degradation (Ma et al., 2015). LCFAs accumulation has inhibitory effects on microbial activity of acidogens, acetogens and methanogens, nevertheless given the biomass studied in this thesis (poor in lipids content), this type of inhibition is not expected and therefore out out of scope of this thesis

Of the several intermediate products, VFAs are important intermediates in anaerobic digestion including acetate, propionate, butyrate, valerate and hexanoate. VFAs can be used as indicator of syntrophic interaction between acidogens/acetogens and methanogens microorganisms and also of the global performance of anaerobic digestion process (Mao et al, 2015; Yuan and Zhu 2016). High VFAs concentration decreases pH values and result in process failure. Wang et al., (2009) reported that acetic and butyric acid concentrations of 2400 and 1800 mg $L^{-1}$, respectively, did not cause

significant inhibition of methanogenic activity. However, propionic acid concentration of 900 mg $L^{-1}$ resulted in significant methanogenic bacteria inhibition. Similarly, Demirel et al., (2002) showed that increasing the propionic acid inside anaerobic process from 312 mg $L^{-1}$ to 1242 mg $L^{-1}$ resulted in reduction of organic matter removal efficiency from 85% down to 66%. Opposite to those authors pointing propionate as the main VFA responsible for inhibition, Pratap et al., (2001) found that propionic acid concentration at 2750 mg $L^{-1}$ did not affect methane production. Some other authors pointed out the relevance of acetate. This is the case for instance of Xu et al., (2014) who found that the acetic acid was the main inhibitor in methanogenesis, especially in high organic loads anaerobic digestion. Hill et al., (1987) demonstrated that to get a good performance and avoid process failure, the acetic acid concentration should not exceed 800 mg $L^{-1}$ and the propionic acid to acetic acid ratio should not exceed 1.4. Therefore, there are contradictory results in literature regarding which VFA is more important to be controlled in anaerobic digestion. Nevertheless, VFAs concentration (addition of all VFAs present in the digestion medium) threshold of 1500 mg $L^{-1}$ has been identified for optimum anaerobic digestion process performance (Angelidaki et al., 2005; Fotidis et al., 2014a). Factors that can lead to VFAs accumulation include substrate overloading or short HRT (Mao et al., 2015). In addition, VFAs accumulation may be a symptom of the effect of other intermediate products. High levels of ammonium can act as inhibitor for methanogens, leading to a reduced methanogenic activity compared to that of acidogens/acetogens activities. As a result, VFAs are accumulated and further degradation to methane is hampered (Mahdy et al., 2015b). It is worth to mention that VFAs concentration threshold to ensure an optimum digestion may vary between different systems depending on the seeding sludge and the substrate composition. In this manner, a VFAs concentration that may cause instability in one reactor does not necessarily affect equally to another digestion system (Angelidaki et al., 1993).

**Ammonia**

Due to the characteristics of the substrate evaluated in this PhD Thesis (microalgae), ammonia/ammonium is considered the main inhibitor in the anaerobic digestion of microalgae biomass and it will be deeply discussed in section 2.4.4.3.

**Metal elements**

Metal elements (light and heavy) are required for enzymes syntheses and activities of anaerobic microflora. It has been reported that the optimum concentrations of sodium and calcium for bacterial growth are less than 400 and 300 mg $L^{-1}$, respectively (Chen et al., 2008; Yu et al., 2001). Heavy metals such as Ni, Co and Mo are required at trace quantities for the correct functioning of many enzymes and coenzymes in anaerobic digestion; however, an excessive concentration of heavy metals can lead to inhibition or toxicity (Li and Fang, 2007). Inhibitory threshold levels depend on many factors such as metal concentration, chemical form of the metal, and pH.

**Reactor configuration**

Most of the studies on biogas production using microalgae have been conducted in batch mode. Reactor configuration and digestion mode can affect the performance of anaerobic digestion process and the final methane yield. With regard to digestion mode, batch assays results could be only extrapolated to continuous results when no inhibition occurs. It is worth to mention that the follow up of continuous mode can result in lower methane yields than observed on batch assays (Mendez et al., 2015; Schwede et al., 2013). In this sense, batch assays are an efficient tool to determine the potential biochemical methane potential of different substrates or preliminary assess the efficiency of pretreatments. Batch assays are relatively fast (20-30 days) compared to continuously fed reactors (several months). Batch assays should be further tested in semicontinuosly fed reactors to study in-depth the performance of anaerobic microorganisms fed with microalgae biomass. Scientific literature dealing with this digestion mode is rather scarce when compared to batch assays but nonetheless highly required to fully evaluate the potential of this substrate to produce energy.

In the context of semicontinuously fed reactors, there are many types of reactors configuration. More specifically, anaerobic digestion can be conducted into one-stage (such as completely mixed reactors and pug-flow reactors) or two-stage processes (for instance one reactor devoted to hydrolysis and acidogenesis and a second stage for methanogenesis with different HRT). Investigations conducted in two-stage reactors for the digestion of microalgae are scarce. One of the few studies using two stage reactor used an anaerobic baffled reactor (ABR) to digest cyanobacteria (Yu et al., 2014). ABR allows separation of hydrolysis and methanogensis stages and provides granule formation. These characteristics resulted in 80% COD removal when digesting this cyanobacteria at OLR of 1.5 g COD $L^{-1}d^{-1}$ and HRT of 5 days (Yu et al., 2014).  Among one-stage digestion configuration, CSTR and UASB (up-flow anaerobic sludge bed reactor) are the reactors frequently used. For instance, a UASB was used to digest *Scenedemus* sp. by Tartakovsky et al., (2015). This configuration is particularly suitable to be operated at high OLR (5-30 g COD $L^{-1}$ $d^{-1}$) and short HRT (7-72 h) (González-Fernández et al., 2015). In this manner, Tartakovsky et al., (2015) reported methane yield of 0.22 L $CH_4$ g VS $in^{-1}$ when a UASB was operated at HRT of 3.8 days and OLR of 2.25 g VS $L^{-1}d^{-1}$.

The most common used reactor for microalgae biomass digestion in continuous mode is the continuously stirred tank reactor (CSTR) (Schwede et al., 2013; González-Fernández et al., 2013; 2015, Passos et al., 2015). In CSTR, methane yield of 0.18 L $CH_4$ g VS $in^{-1}$ was obtained when digesting a microalgae biomass mixture of *Pediastrum* sp., *Micractinium* sp., and *Scenedesmus* sp.) at HRT of 14-16 days and OLR of 1 g VS $L^{-1}$ $d^{-1}$ (Kinnunen et al., 2014a). Similar methane yield (0.19 L $CH_4$ kg VS $in^{-1}$) was reported by Tartakovsky et al., (2013) who operated a CSTR fed with *Scenedesmus* sp. at HRT of 16 days and OLR of 0.64 g $L^{-1}$ $d^{-1}$. These yields were higher than that obtained by González-Fernández et al., (2013) who reported methane yield of 0.03 L $CH_4$ g COD in $^{-1}$ when digesting *Scenedesmus* sp. at HRT of 15 days and OLR of 1 g COD $L^{-1}d^{-1}$.

Until now, only few investigations have tested the performance of different reactor configurations probably due to the low methane yields attained. In this sense, a big research effort was focused on elucidating the most promising

pretreatment methods to increase these values. At this point, the performance of different reactor configuration should be reevaluated by feeding pretreated biomass. With the knowledge gained on biomass pretreatments, the options of using novel configurations rather than the traditional CSTRs should be reassessed.

In addition to the reactor configuration, an optimum digestion is highly dependent on other digestion parameters such as HRT and OLR. An optimum balance between OLR and HRT must be used to achieve maximum digestion efficiency. Out of the three stages involved in anaerobic digestion, methanogenesis can be rate limiting due to the slow growth rates of methanogenic archaea (Gerardi, 2003), and hence, appropriate retention times are required to ensure the growth of a large population of methanogens. More specifically, too short or too long HRT leads to incomplete substrate degradation or starving of bacterial community, respectively. HRT indicates the time needed for bacterial community to digest a specific amount of substrate (OLR). HRT can be calculated with the following equation:

$$HRT = \frac{V}{Q}$$

where HRT is the hydraulic retention time (d), V is the reactor volume (L) and Q is the influent flow rate (L $d^{-1}$).

OLR represents the amount of organic matter (VS or COD) fed into a digester volume per day and it can be calculated with the following equation:

$$OLR = \frac{VS\ (or\ COD) * Q}{V} = \frac{VS\ (or\ COD)}{HRT}$$

where OLR is the organic loading rate (g VS $L^{-1}$ $d^{-1}$), VS is volatile solids of the substrate (g VS $L^{-1}$), COD is chemical oxygen demand of the substrate (g COD $L^{-1}$), V is the reactor volume (L) and Q is the influent flow rate (L $d^{-1}$) and HRT is the hydraulic retention time (d).

Although both parameters, HRT and OLR, are mostly dependent on the characteristics of the substrate, typical HRT values are between 10-30 days with OLR ranging 1-6 g COD $L^{-1}$ $d^{-1}$ in the case of microalgae substrates (González-Fernández et al., 2015). González-Fernández et al., (2013) have reported a slight improvement in methane yield when OLR was increased from 1 g COD $L^{-1}$ $d^{-1}$ to 2.5 g COD $L^{-1}$ $d^{-1}$. The methane yield attained at the highest OLR was 0.111 L $CH_4$ g COD $in^{-1}$ compared to 0.097 L $CH_4$ g COD $in^{-1}$ registered at the lowest OLR. Thus, increasing the OLR up to 2.5 g COD $L^{-1}$ $d^{-1}$ did not cause organic overloading when the CSTR was fed with thermally pretreated *Scenedesumus* sp. at HRT of 15 days. The authors attributed the lower methane yield attainted at the lowest OLR to an underestimated activity of anaerobic microorganisms.

With regard to HRT, Ras et al., (2011) achieved better process performance using *C. vulgaris* at longer residence times. More specifically, those authors reported an increase in organic matter removal from 33% to 51% by increasing the HRT in CSTR from 16 to 28 days. This increase in HRT resulted in a methane yield enhancement of 1.6-fold. Additionally, it should be stressed that both parameters, HRT and OLR, are dependent on each other. For instance, Jegede (2012) studied the relation between OLR and methane production rates at fixed HRT (3 days). This investigation studied the methane potentials of cyanobacteria and *Chlorella* sp. in eight different CSTR at 25ºC and observed that the methane production rates increased concomitantly with OLR up to 7 g VS $L^{-1}$ $d^{-1}$ and declined at higher loading rates.

Due to the many factors that can affect the digestion, the optimal reactor configuration and operating parameters depend on a case-by-case assessment. In this context, Mendez et al., (2015) and Passos and Ferrer, (2015) have operated CSTRs at different HRT, OLR and using different microalgae species as substrates, they achieved similar methane yield (0.08 L $CH_4$ g COD $in^{-1}$). The CSTR of Mendez et al., (2015) was operated at HRT of 15 days with OLR of 1.5 g COD $L^{-1}d^{-1}$ and fed with *C. vulgaris* while Passos and Ferrer, (2015) fed the CSTR with *Oocystis* sp. at HRT of 20 days and OLR of 2.3 g COD $L^{-1}$ $d^{-1}$. Therefore, it can be suggested that the most suitable HRT and OLR depends on the microalgae strain to be digested.

Consequently, different biomass composition and cell wall structure among microalgae species together with the different reactor configurations and operational parameters employed (HRT and OLR) are required to be carefully selected to achieve optimum performance during the anaerobic digestion of these substrates.

### 2.4.4. Problems and potential solutions associated with the anaerobic digestion of microalgae

The early reports dealing with the anaerobic digestion of microalgae biomass (*Chlorella* and *Scenedesmus*) go back to the middle of last century (Golueke et al., 1957). Their calculations showed low conversion efficiency and pointed out ammonia-mediated inhibition or cell wall resistance to anaerobic bacterial attack as potential explanation. As mentioned above (Section 2.4.1), the first stage (hydrolysis) is the rate limiting step in the anaerobic digestion of complex particulate organic material such as microalgae biomass. Hydrolysis of particulate matter may result in a slow rate biological conversion of biomass into biogas. Complex organic matters are difficult to degrade by anaerobic microorganisms unless broken down in a previous stage into simpler polymers. Likewise, the low carbon to nitrogen (C/N) ratio, characteristic of microalgae biomass, cannot be neglected. This fact can result in anaerobic microorganism's inhibition by high $NH_4^+/NH_3$ concentration during the digestion process.

#### 2.4.4.1. Cell wall degradability

The composition and polymer arrangement of microalgae cell wall ensures its mechanical strength, shape and rigidity as necessary characteristics for cell protection against infection or predators. The composition, proportion and complexity of microalgae cell wall structure directly depend on phylogeny, development stage, cell type and cultivation conditions. For example, *Chlamydomonas reinhardtii* cell wall is arranged in six distinct layers and contains intricated cellulose-pectin complexes with others made of hydroxyproline rich glycoproteins (Imam et al., 1985). Microalgae cell walls consist of inner and outer cell wall layers. The composition of the outer cell

wall usually contains specific matrix polysaccharides such pectin, chitin agar, alginates or the aliphatic polymer algaenan (Scholz et al., 2014). The inner cell wall is usually composed of microfibrillar cellulose and other materials such as hemicellulose and glycoproteins (Domozych et al., 2012). For instance, cells of *Scenedesmus*, some species of *Chlorella*, *Nannochloropsis* sp., *Chlamydomonas*, *Haematococcus, Polytomella*, and *Botryococcus* colonies have been shown to contain the aliphatic polymer algaenan in the cell wall (Scholz et al., 2014; Zych et al., 2009). These components confer rigidity to microalgae cell wall and make microalgae highly resistant to microbial attack during anaerobic digestion which limits the efficiency of this bioprocess.

In the context of biogas production, the rigid cell walls have been identified as biological barriers that limit the efficiency of anaerobic digestion by preventing microbial attack (González-Fernández et al., 2012b). Therefore, cell wall disruption/hydrolysis is required to reach an optimum biogas conversion from microalgae. Depending on the microalgal species, the cell wall is different and thus, the pretreatment should be tailor-designed for targeted species. For instance, *Scenedesmus* biomass has been reported to display a harder cell wall than *Chlorella*; however, both of them belong to the group of microalgae most difficult to digest (Mussgnug et al., 2010; González-Fernández et al., 2012b). In contrast, the absence of cell wall in *Dunaliella salina* rendered this biomass as an easily degradable substrate (Mussgnug et al., 2010). Although the absence of cell wall in some saline microalgae, such as *D. salina,* can result in high methane yield, inhibition in anaerobic digestion process could take place at high salinity levels (Ward et al., 2014).

**Potential solution: pretreatment of microalgae biomass**

In order to disrupt/hydrolyze microalgae cell walls and make organic matter readily degradable for biogas production, a pretreatment prior to anaerobic digestion seems crucial (González-Fernández et al., 2012b; Passos et al., 2014). This pretreatment would result in a faster digestion and higher methane yields when compared to that of raw biomass. During the last years, a large number of studies have used different pretreatments to facilitate

microbial attack during anaerobic digestion of microalgae and thus enhancing biogas production (Mendez et al., 2013; González-Fernández et al., 2012a; Ometto et al., 2014).

Current cell disruption technologies can be grouped into four categories: mechanical (bead-beating, milling, ultrasonication, high-pressure homogenization, and spray-drying), thermal (microwave, autoclaving, and freezing), chemical (organic solvents, osmotic shock, and acid- alkali reactions) and biological processes (microbial degradation, enzymatic reactions) (Figure 2.4.). However, several studies have demonstrated that the energy consumption devoted to microalgae mechanical and thermal pretreatment is equal to, or higher, than the energy gained. For instance, Mendez et al., (2013) attained an enhancement of methane production 1.8-fold with thermally (at 120ºC for 40 min) pretreated microalgae biomass compared to raw biomass (which was 139 mL $CH_4$ g COD $in^{-1}$). Nevertheless, the calculated electricity that may be produced by this biomass resulted in 1657.4 kWh kg $TS^{-1}$ and, in turn, the heat required for this pretreatment was calculated to be around 6894 kWh Kg $TS^{-1}$. The same conclusion was highlighted by Lee et al., (2013) when using mechanical methods to disrupt microalgal cell walls. Mechanical methods display the highest specific energy consumption (de Boer et al., 2012; Günerken et al., 2015). For non-mechanical methods, energy consumption is mostly influenced by treatment time, temperature and stirring. Additionally, other problems that may occur with those pretreatments is the formation of recalcitrant compounds in the case of thermal pretreatments (Mendez et al., 2014a) and the need of re-adjusting the pH of substrates before anaerobic digestion in the case of chemical pretreatments.

The review of Günerken et al., (2015) focused on the comparison of conventional and emerging techniques devoted to microalgae cell disruption/hydrolysis. Their potential application for microalgae cell disruption in a biorefinery approach was evaluated in terms of disruption efficiency, product quality, process parameters, scalability and specific energy consumption. To avoid previous mentioned drawbacks of more traditional pretreatments, those authors emphasized the need to study non-mechanical

and mild temperature technologies such as pulse electric field (PEF) or biological cell wall hydrolysis.

PEF is conducted under mild temperature, using high electric field strengths to disrupt cell wall or cell membrane, and thus promoting the release of intracellular matter. PEF technology helps to reduce energy consumption and operation cost in pretreatment processes and therefore, it has a high potential for scale up. However, PEF technique has generally been used for the extraction of soluble compounds inside microalgae cell without solubilizing organic matter (Goettel et al., 2013) and in many cases, this technology needs to be combined with solvents (Joannes et al., 2015).

On the other hand, biological processes include the degradation of microalgae biomass using external addition of both purified or mixture of enzymes (Choi et al., 2010; Mahdy et al., 2016a). Biological cell hydrolysis can also be performed by microbial cell to cell interaction in co-culture or infection that induces the secretion of lytic enzymes or active molecules causing algae death (Chen et al., 2013; Cheng et al., 2013; Matsumoto et al., 2003; Muñoz et al., 2014). Advantages of enzymatic methods for microalgae cell disruption include mild reaction conditions, absence of inhibiting by-products and high reaction selectivity. An enzyme can selectively degrade a specific chemical linkage, whereas mechanical methods destroy almost every particle existing in the solution, and chemical methods usually induce side-reactions generating product damage. Due to the low temperature and neutral pH reaction conditions, enzymatic processes avoid devices corrosion that may occur during thermochemical processes; thereby lowering equipment costs. Therefore, enzymatic cell wall pretreatment is considered environmentally benign and less energy-consuming than mechanical and thermal pretreatments. Some existing studies have determined the feasibility of enzymatic processes to hydrolyze microalgae cell wall (Gerken et al., 2013; Harun and Danquah, 2011; Rodrigues and Bon, 2011). Compared with mechanical or chemical cell disruption methods, enzymatic methods have obtained very competitive results in terms of disruption efficiency and biofuel production (Ho et al., 2013c; Kim et al., 2014; Lee et al., 2013; Mahdy et al., 2014a; 2016a). The high cost of enzymes is the main hurdle of using this

pretreatment; however, producing enzymes *in situ* by different kind of bacteria and fungi is a possible solution to overcome this problem. In addition, a recent investigation postulated that the lytic enzymes that are involved in microalgae autolysis could be induced at demand and hence offering a promising alternative approach for development of innovative cost-effective disruption methods (Demuez et al., 2015). Enzymatic pretreatment has been widely used for saccharification of microalgae carbohydrates. For example, the studies of Harun and Danquah (2011), Fu et al., (2010), and Lee et al., (2011) reported the use of cellulases to hydrolyze microalgal biomass at different consistencies of 1, 2, and 5% (w/w dry weight (DW)) and they obtained saccharification yields of 64, 58, and 47% based on the total amount of carbohydrates contained in the biomass, respectively. Similarly, saccharification yield of 53-68% was achieved when subjecting *Chlorella* sp. to an enzyme mixture composed by amylase, cellulase and xylanase (Rodrigues and Bon, 2011). In addition, enzymatic pretreatment has been used for the production of L-amino-acids concentrates from microalgae biomass (Romero-García et al., 2012). The authors obtained hydrolysis yield close to 60% when subjecting *Scenedesmus almeriensis* to protease enzymes (Alcalase and Flavourzyme).

Recently, enzymatic pretreatment of microalgae biomass was evaluated prior to anaerobic digestion in order to enhance methane yields. In this manner, the effect of carbohydrases and proteases on biogas production enhancement was investigated. Regarding carbohydrases, despite the low carbohydrate content (13.4% DW) of *C. vulgaris,* subjecting this microalgae biomass to cellulase and hemicellulase prior to anaerobic digestion resulted in an increase in methane yield of 70% compared to raw biomass (0.25 L $CH_4$ g VS in$^{-1}$, Wieczorek et al., 2014). Lower methane yield enhancement was reported by Passos et al., (2016) when applying carbohydrases pretreatment, although the carbohydrate content of microalgae biomass was 1.6-fold higher than that reported by former author. When microalgae biomass was subjected to cellulase and enzyme mixture of cellulase, glucohydrolase and xylanase, the methane yield increased by 8% and 15%, respectively, compared to not treated biomass (0.19 L $CH_4$ g VS in$^{-1}$). Similarly, Ehimen et al., (2013)

achieved 20% enhancement in methane yield after pretreatment of *Rhizoclonium* sp. with cellulase.

Since the predominant macromolecules in microalgae biomass is protein (Table 2.4.), the effect of proteases on biogas production was also assessed. One of the first investigations working with protease pretreatments for enhancing methane yields was conducted by Mahdy et al., (2014a). Those authors achieved more than 85% proteins solubilisation when subjecting microalgae biomass to protease activity. The methane yield achieved with protease pretreated biomass was 51% higher than that attained with not treated biomass (0.19 and 0.14 L $CH_4$ g COD $in^{-1}$ for *C. vulgaris* and *Scenedesmus* sp., respectively, Mahdy et al., 2014a; 2016a). Likewise, *C. vulgaris* and *S. obliquus* were subjected to a mixture of esterase and protease to enhance methane production of raw biomass (0.27 L g VS $in^{-1}$, Ometto et al., 2014). The methane yield of *C. vulgaris* and *S. obliquus* was enhanced by 3.2-fold and 3.9-fold, respectively. Those authors attributed methane yield enhancement to the degradation of the cell wall constituents and the solubilisation of organic matter.

These investigations highlighted the efficiency of enzymatic pretreatments to hydrolyze microalgae biomass and hence increasing methane production. It is worth mentioning that cellulose/hemicellulose are the main responsible for microalgae cell wall hardness (Takeda 1991; 1996; Fu et al., 2010), nevertheless, recent investigations demonstrated that other fractions may be responsible for that hardness (Gerken et al., 2013, Kim et al., 2014, Mahdy et al., 2016a). In this sense, the high specificity of biocatalysts to degrade determined chemical linkages can provide crucial information regarding the polymeric composition of microalgae cell wall. This information is highly required and is missing in scientific literature. The knowledge of microalgae cell walls in terms of structure and composition can provide important information to optimize pretreatments devoted to increase methane yields. Therefore, biological pretreatments deserves further investigation in order to identify the most appropriate enzyme able to disrupt a broad range of microalgae strain, to optimize the pretreatment process and to fully elucidate their effect on anaerobic digestion.

#### 2.4.4.2. The carbon/nitrogen ratio and macromolecular distribution of microalgae biomass

Typically, protein is the predominant macromolecule of microalgae biomass. This feature results in low C/N ratio, often less than 7 (Mahdy et al., 2015a; Yen and Brune, 2007). This ratio is lower than the required C/N ratio for anaerobic microorganism metabolism. More specifically, microorganisms generally need C/N ratio of 20–30:1 (Mao et al., 2015) and hence, an imbalance between carbon and nitrogen requirements could be taking place when digesting microalgae biomass. This imbalance can lead to excessive ammonia concentration that may become inhibitory to methanogenic bacteria (as discussed in Section 2.4.4.3.).

One way to overcome this drawback is the possibility of cultivating microalgae in wastewater as a sole nutrient source. Although urban wastewater, for instance, contains organic and inorganic pollutants, their nitrogen concentrations are relatively low for microalgae cultivation and might induce nutrient limitation. For example, the nitrogen content in synthetic media used in this thesis was 320 mg N $L^{-1}$ compared to only 28±0.5 mg N $L^{-1}$ found in the used urban wastewater (Table 3.1. and 3.2. of material and methods section). Thus, wastewater characteristics might lead to shifting microalgae metabolisms towards non typical macromolecules. This different allocation of uptaken nutrients might result in accumulation of carbohydrates or lipids rather than proteins. Likewise, operational conditions, such as light and temperature employed during biomass production might also shift microalgae metabolisms (González-Fernández and Ballesteros, 2012). This capability can be tremendously useful when biodiesel or bioethanol production is aimed. In the case of biogas production, even though this bioprocess is not so dependent on the macromolecular profile of the substrate, the prevalence of carbohydrates or lipids can be useful to avoid ammonia/ammonium inhibition of protein-rich substrate. This is the case of the study conducted by González-Fernández et al., (2016) who cultivated microalgae consortia (namely *Chlorella vulgaris*, *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*) in urban wastewater. Due to low ammonium concentration measured in the wastewater (80 mg $NH_4^+$-N $L^{-1}$), the protein content decreased drastically from

60-65% (biomass grown in synthetic media, Mahdy et al., 2014a and 2015b) down to 29-37% (mg g $VSS^{-1}$). In turn, this study showed that carbohydrates content accumulated up to 53% compared to 25-35% that is the range generally reported in literature (Mendez et al., 2015; Mahdy et al., 2016a). Likewise, Mahdy et al., (2016b) demonstrated that the carbohydrate content of *Chlorella vulgaris* grown in urban wastewater (ammonium concentration of 36 mg $NH_4^+$-N $L^{-1}$) was increased by 1.8-fold when compared to the biomass grown in synthetic media (rich in nitrogen) exhibiting 22% carbohydrate content.

Another option to balance C/N would be codigestion approach in which different substrates are codigested with microalgae (Yen and Brune 2007; Mahdy et al., 2015a); however, this approach is out of this thesis scope.

#### 2.4.4.3. Inhibition of anaerobic digestion by ammonia/ammonium-nitrogen toxicity

As mentioned in Section 2.4.3, different factors might influence the performance of anaerobic digesters. Those factors include temperature regime, pH, substrate C/N ratio, OLR, ammonia/ammonium concentration and retention time (Mao et al., 2015). Accumulation of total ammonia nitrogen, produced from the degradation of protein-rich materials, is one of the most common detrimental factors affecting anaerobic digestion. Ammonia is the end product of nitrogen-containing biomass degradation which can exist in ionized form ($NH_4^+$) or unionized form ($NH_3$). The prevalence of one or other form is dependent on pH and temperature (Figure 2.7.).

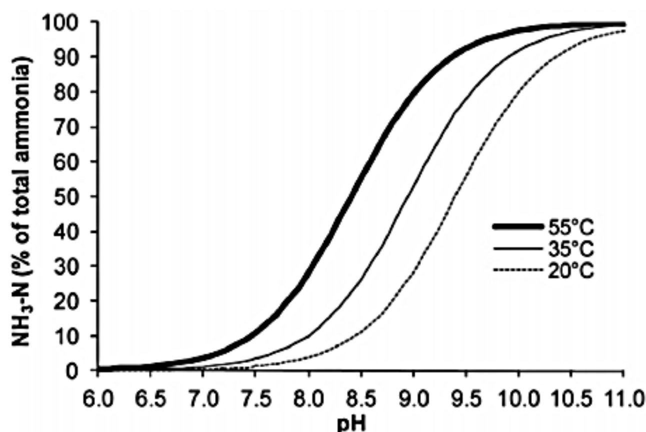
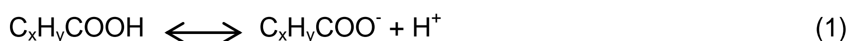



**Figure 2.7.** Free ammonia percentage in solution at 20, 35 and 55°C and varying pH (Fernandes et al., 2012).

Ammonium is essential for the growth of anaerobic flora involved in the anaerobic digestion process. Likewise, ammonium can also act as a buffering agent for the anaerobic process by neutralizing the produced VFAs as illustrated in following equations (Zhang et al., 2013):



$$C_xH_yCOOH \longleftrightarrow C_xH_yCOO^- + H^+ \quad (1)$$

$$NH_3 . H_2O \longleftrightarrow NH_4^+ + OH^- \quad (2)$$

$$C_xH_yCOOH + NH_3.H_2O \longrightarrow C_xH_yCOO- + NH_4^+ + H_2O \quad (3)$$

where $C_xH_yCOOH$ represents VFAs.

Despite the need of nitrogen for anaerobic digestion, the ammonium concentration for the metabolic role is low. It has been reported that ionized ammonium and unionized ammonia should be below 1500 mg $L^{-1}$ (Costa et al., 2012) and 50-100 mg $L^{-1}$ (Liu and Sung, 2002), respectively, to avoid anaerobic microorganisms inhibition. Nevertheless, those ranges can vary depending on the digester operating conditions and seeding sludge. The most common effect taking place during inhibition is VFAs accumulation (Shi et al., 2016; Mahdy et al., 2015b; Nie et al., 2015). In this manner, instead of only checking for a range of ammonia concentration, its effect can also evidence ammonia inhibition.

Three inhibition mechanisms of total ammonia nitrogen have been reported. In the first one, ammonia diffuses freely through the permeable membrane of methanogens cells causing changes in intracellular pH and, in response, potassium deficiency and/or proton imbalance may occur (second mechanism). The third mechanism concerns the ionized form (ammonium). Ammonium could inhibit enzymes that are involved in methane production as it is illustrated in Figure 2.8. (Sprott and Patel, 1986; Siles et al., 2010). As a result, the high concentration of total ammonia (ammonia/ammonium) can lead to VFA accumulation. Due to the imbalance occurring between the activities of fermentative and methanogenic microorganisms, the VFA accumulation leads to a pH drop, which in turn causes methanogenesis inhibition (Wang et al., 2009).

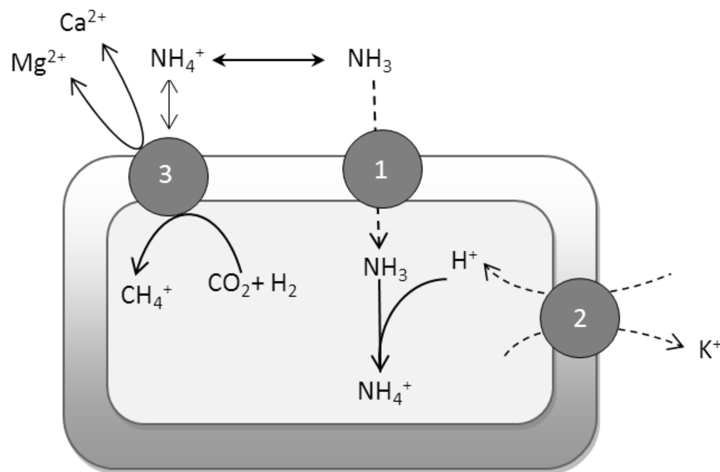



**Figure 2.8.** Mechanisms of ammonia inhibition in methanogens 1) ammonia diffusion, 2) potassium deficiency and/or proton imbalance 3) inhibition of methane synthesizing enzyme system (Adopted from Sprott and Patel 1986).

Many approaches have been investigated to alleviate ammonia inhibition in anaerobic digestion processes, including ammonia stripping at high pH (Zhang and Jahng, 2010) and by air addition (Wu et al., 2016), dilution of the reactor content (Kayhanian, 1999), struvite precipitation (Nelson et al., 2003),

acclimatization of microorganisms (Calli et al., 2005) and codigestion (Yen and Brune 2007). More recently, Fotidis et al., (2014b) highlighted the possibility of bioaugment the anaerobic sludge to alleviate ammonium inhibition effect as an innovative approach.

Bioaugmentation consists in the addition of specialized microorganisms to biological systems and it has been used to improve anaerobic digestion process performance. Bioaugmentation has been investigated for hydrolysis enhancement of lignocellulosic materials such as wheat straw (Peng et al., 2014), corn straw (Zhang et al., 2015) and agricultural residues (Weiß et al., 2016) or for improving methane production of different types of wastewater (Kumar et al., 2015; Tuesorn et al., 2013). Nevertheless, another approach of bioaugmentation would not be only to improve the digestion process but it could be also used to overcome the ammonium/ammonia inhibition of the anaerobic process.

Among the different microorganisms involved in anaerobic digestion, methanogens are the most sensitive to ammonia inhibition (Kayhanian, 1994). More specifically, syntrophic acetate oxidation followed by the hydrogentrophic methanogenesis methabolic pathway seems to be more robust to ammonia toxicity than acetoclastic methanogenic pathway (Yenigün and Demirel, 2013; Chen et al., 2008). This fact was further supported by the work of Fotidis et al., (2014a) who used acetate, labelled in the methyl group, in order to follow the production of $^{14}CH_4$ and $^{14}CO_2$. Those authors found that the acetoclastic methanogenic pathway dominated at low total ammonia level (less than 1.2 g $NH_4^+$-N $L^{-1}$) while syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-CH pathway) became dominant when the total ammonia concentration ranged 2.8–4.57 g $NH_4^+$-N $L^{-1}$.

Given the fact of methanogens sensitivity to ammonium, bioaugmentation with *Methanoculleus bourgensis* (SAO-CH pathway) was applied during the digestion of cattle manure. The results showed an increase of 31% methane yield when the CSTR was working under inhibited steady-state at high ammonia levels (5 g $NH_4^{+-}$N $L^{-1}$) (Fotidis et al., 2014b). Those authors used mesophilic sludge as inoculum and the bioaugmentation process was performed by replacing 11% in two steps (5.5% each) of working volume of

the digester by *Methanoculleus bourgensis* culture ($OD_{600}$ = 0.17-0.19, $\mu_{max}$ = 0.022, under exponential growth phase). The authors concluded that the supplementation of ammonia tolerant methanogenic consortia, especially of fast growing hydrogenotrophic methanogens (e.g. *Methanoculleus bourgensis*), could provide a new solution to alleviate the ammonia inhibitory effect in anaerobic digestion processes. Opposite to that, Westerholm et al., (2012) demonstrated that the bioaugmentation approach did not affect process performance against ammonium inhibition. The later authors operated a CSTR fed with stillage and cattle manure under mesophilic conditions at increasing ammonium concentration from 1.5 to 11 g $NH_4^{+-}N$ $L^{-1}$. In this case, a fixed volume of syntrophic acetate-oxidizing culture (10 mL with concentration of 2.8 $\times 10^{10}$ per mL) was added daily to bioaugment the anaerobic culture. Molecular analysis of the experiment showed absence or low abundance of bioaugmented microorganisms compared to the amount of added microorganisms. The authors attributed this fact to differences between optimal temperature of bioaugmented microorganisms (40-65ºC) and the process temperature (35ºC) and/or the microorganism disability to grow in the process. Therefore, it could be concluded that the success of bioaugmentation approach to alleviate ammonium effects on anaerobic digestion depends on the selection of appropriate microorganisms. Furthermore, not only the microorganisms selection affects the bioaugmentation process but also the addition mode. More specifically, one-time addition of microorganism to bioaugment the seeding inoculum might lead to an unsustainable methane production improvement along the digestion process. The main reason for that non constant improvement is the wash-out of the bioaugmented microorganisms (Angelidaki and Ahring, 2000; Nielsen et al., 2007). Therefore, the periodical microorganisms addition was investigated as an alternative to one-time bioaugmentation for achieving a stable microbial community in digesters (Martin-Ryals et al., 2015). In this context, periodical addition of microorganisms achieved sustained improvements in anaerobic digestion efficiency with methane production 15% higher compared to one-time bioaugmentation.

Up to now, research on the influence of bioaugmentation in anaerobic digestion processes was conducted with substrates rich in nitrogen such as cattle manure (Fortidis et al, 2014b). This option has not been tested yet in new feedstock such is the case of microalgae. Therefore, research on the effect of this approach to alleviate the ammonia/ammonium inhibition on the digestion of reactors fed with protein-rich microalgae should be further investigated as a potential biological tool to improve methane yields.

# CHAPTER 3

# MATERIAL AND METHODS

## 3. Material and Methods

### 3.1 Inocula and substrates

#### 3.1.1. Microalgae strains

*Chlorella vulgaris* (used throughout the thesis), *Scenedesmus* sp. (used in Section 4.1.1) and *Chlamydomonas reinhardtii* (used in Section 4.2.) were studied in this thesis. *C. vulgaris* and *Scenedesmus* sp. were obtained from the wastewater treatment plant of Valladolid (Spain) while *C. reinhardtii* was obtained from the bank SAG Culture Collection at the University of Göttingen (Germany). Finally, in Section 4.1.2, *Scenedesmus* sp. biomass paste was obtained from the University of Almería (Spain).

#### 3.1.2. Collection and storage of sludge and adapted inoculum

The inoculum employed in all experiments was anaerobic sludge collected at the municipal wastewater treatment plant of Valladolid (Spain). Anaerobic sludge presented TS concentration of approximately 14.6±2.3 g $L^{-1}$ and VS/TS ranged 60-67%. The inoculum was kept at room temperature until use.

For the batch assays related to the activity of anaerobes (Section 4.3.4), the anaerobic sludge from the CSTR fed with the enzymatically pretreated protein rich-biomass was employed as inoculum.

### 3.2. Microalgae culture condition

#### 3.2.1. Media used

##### 3.2.1.1 Synthetic media

The microalgae were fed with a modified synthetic Bold's basal medium. More specifically, the modification of this medium consisted on the replacement of sodium nitrate by ammonium chloride as a nitrogen source and tris-acetate as a carbon source. Different solutions were prepared and kept sterilized (120ºC for 20 minutes). The chemical composition of those solutions and quantity used to prepare 1 liter is shown in Table 3.1.

**Table 3.1.** Solutions employed for the preparation of Modified Bolds Basal Medium.

| Stocks | Name | Formula | Stock Concentration (g $L^{-1}$) | Quantity used (mL) |
|---|---|---|---|---|
| **1** | Ammonium chloride | $NH_4Cl$ | 8 | 10 |
| **2** | Calcium chloride dihydrate | $CaCl_2.2H_2O$ | 2.5 | 10 |
| **3** | Magnesium sulfate heptahydrate | $MgSO_4.7H_2O$ | 7.5 | 10 |
| **4** | Potassium phosphate dibasic | $K_2HPO_4$ | 7.5 | 10 |
| **5** | Potassium dihydrogen phosphate | $KH_2PO_4$ | 17.5 | 10 |
| **6** | Sodium chloride | NaCl | 2.5 | 10 |
| **7** | Alkaline EDTA Solution | | | 1 |
| | Disodium EDTA | $Na_2EDTA$ | 50 | |
| | Potassium hydroxide | KOH | 31 | |
| **8** | Acidified Iron Solution | | | 1 |
| | Iron(II) sulfate heptahydrate | $FeSO_4.7H_2O$ | 5.0 | |
| | Sulphuric acid (concentrated) | $H_2SO_4$ | 1.0* | |
| **9** | Boric acid | $H_3BO3$ | 11.4 | 1 |
| **10** | Trace Metal Solution | | | 2 |
| | Zinc sulfate heptahydrate | $ZnSO_4.7H_2O$ | 8.8 | |
| | Manganese(II) chloride tetrahydrate | $MnCl_2.4H_2O$ | 1.4 | |
| | Molybdenum trioxide | $MoO_3$ | 0.7 | |
| | Copper(II) sulfate pentahydrate | $CuSO_4.5H_2O$ | 1.6 | |
| | Cobalt(II) nitrate hexahydrate | $CoNO_3.6H_2O$ | 0.5 | |
| **11** | Trisacetate solution | | | 10 |
| | Tris-(hydroxymethyl) aminomethane | $C_4H_{11}NO_3$ | 242 | |
| | Acetic acid | $CH_3COOH$ | 100* | |

* mL $L^{-1}$

#### 3.2.1.2. Wastewater

Wastewater used in this study was collected from the wastewater treatment plant of Rey Juan Carlos University (Madrid, Spain). The sampling point for wastewater was located just after the primary pretreatment step. This treatment aims at removing settled and floating materials from wastewater by temporarily holding the wastewater in a tank equipped with mechanically driven scrapers. To allow all suspended solids to sediment, fresh wastewater was allowed to settle at room temperature for 24 hours. The supernatants were directly used as cultivation media for microalgae without any treatment or extra-nutrient supplementation. The chemical composition of this wastewater is shown in Table 3.2.

**Table 3.2.** Chemical composition of the wastewater used for *Chlorella vulgaris* cultivation**.**

| | Average | Standard Deviation |
|---|---|---|
| tCOD (mg $L^{-1}$) | 317 | 1.4 |
| sCOD (mg $L^{-1}$) | 218 | 11.3 |
| TS (mg $L^{-1}$) | 580 | 28.3 |
| VS (mg $L^{-1}$) | 280 | 56.6 |
| TSS (mg $L^{-1}$) | 180 | 56.6 |
| VSS (mg $L^{-1}$) | 150 | 42.4 |
| Ammonium (mg N-$NH_4^+$ $L^{-1}$) | 36.3 | 0.5 |
| Nitrate (mg N-$NO_3^-$ $L^{-1}$) | 0.0 | 0.0 |
| Nitrite (mg N-$NO_2^-$ $L^{-1}$) | 0.2 | 0.0 |
| Phosphate (mg P-$PO_4^{-3}$ $L^{-1}$) | 4.2 | 0.0 |
| pH | 7.4 | 0.1 |

### 3.2.2. Cultivation systems

#### 3.2.2.1. Photobioreactors (PBRs)

Different lab-scale and pilot-scale photo-bioreactors (PBRs) were used to cultivate the microalgae biomass used during the development of this thesis. Two lab-scale reactors were used to produce the biomass (Figure 3.1.), namely erlenmeyer flasks with a working volume of 0.5-1 L for the start-up of inocula and photobioreactors with a working volume of 1-3 L for *C. vulgaris and C. reinhardtii* cultivation (used in Sections 4.1.2 and 4.2.1).

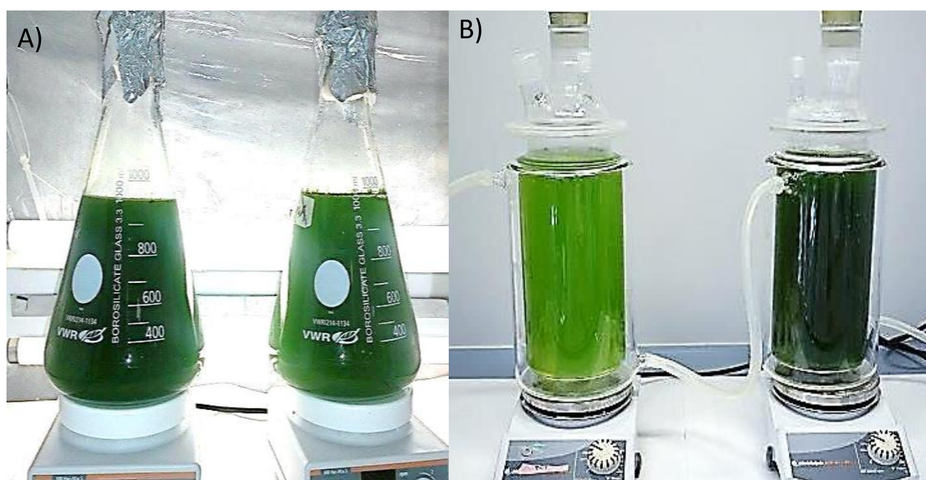



**Figure 3.1.** Cultivation of microalgae biomass (*C. vulgaris*, *Scenedesmus* sp. and *C. reinhardtii*) in lab scale photobioreactors (A) erlenmeyer flasks and (B) photobioreactors.

Pilot scale photobioreactors consisting in tubular reactors with a working volume of 35 L were used for the cultivation of *C. vulgaris* (used in Sections 4.2.6 and 4.3) and 50 L for the cultivation of *C. vulgaris* and *Scenedesmus* sp. (used in Section 4.1.1). *C. vulgaris* used for Section 4.4 was grown in a raceway with a working volume of 200 L. Both reactor configurations can be seen in Figure 3.2. Microalgae were cultivated for 7-10 days at room temperature (22±1ºC).

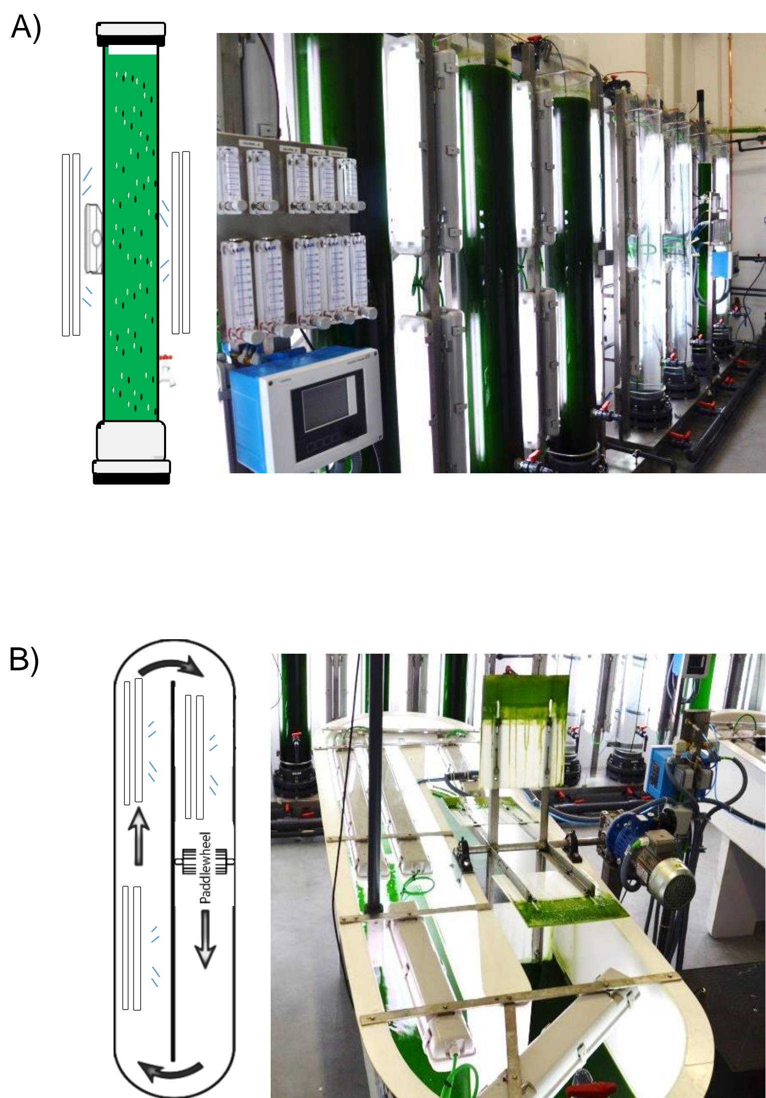



**Figure 3.2.** Cultivation of *Chlorella vulgaris* in pilot scale photobioreactors (A) tubular and (B) raceway.

#### 3.2.2.2. Mixing

To ensure light supply to all cells, avoid nutrients gradient, enhance gaseous transfer and prevent cell settlement, culture mixing was supplied by magnetic stirrers or air bubbling in lab-scale reactors. In the photobioreactors operated at pilot-scale, air blowers and a paddle wheel were used in tubular photobioreactors and raceway, respectively.

#### 3.2.2.3. Illumination

All reactors were in-doors and therefore, light supply was accomplished by using artificial illumination. Fluorescent lamps were placed on both sides of the photo-bioreactors or above the culture media in the case of the raceway (Figure 3.2.). The lamps provided continuous illumination (range 5000-6000 lux).

## 3.3. Harvesting

To harvest microalgae grown on synthetic media, the culture was centrifuged at 5000 rpm for 10 min at 4ºC (Heraeus Multifuge, Germany) after 7-10 days of cultivation. When using wastewater as cultivation media (Section 4.4), 50% of the liquid medium (included *C. vulgaris* biomass) in the raceway was collected and replaced with wastewater once a week. The collected effluents were allowed to settle for 1–2 h and then, the concentrated biomass was centrifuged as described above.

## 3.4. Microalgae biomass pretreatment

In order to achieve an efficient biodegradability, microalgae biomass were subjected to different pretreatments prior to anaerobic digestion. Thermal and enzymatic pretreatments were applied and their influence on microalgae biomass in terms of cell wall disruption and organic matter solubilisation were investigated in this thesis. The pretreatments procedures are summarized in the block diagram shown in the Figure 3.3.

### 3.4.1. Thermal pretreatment

Thermal application was applied for three main purposes:

**1- Biomass pretreatment (without biocatalysts)**

Microalgae biomass (*C. vulgaris* and *Scenedesmus* sp., Section 4.1) at biomass load of 50 g $L^{-1}$ were subjected to low temperature thermal pretreatment in the attempt to release hydrolytic enzymes from microalgae itself to the medium (autohydrolysis). With this approach, no further catalysts would be required. As enzymes can work only within a certain temperature range and most of them work best at temperature around 50ºC, autohydrolysis pretreatment was conducted at that temperature (Carvajal et al., 2013). Autohydrolysis was conducted by incubating microalgae biomass in a water bath under continuous stirring at 100 rpm for 24 and 48 h.

**2- Thermal application prior to enzymatic hydrolysis**

This strategy was followed as an attempt to increase accessibility and performance of enzymes for microalgae biomass hydrolysis (Section 4.2.2). In this context, a first thermal application at mild temperatures (75ºC for 30 min) was applied to *C. vulgaris* and C. *reinhardtii* at biomass loads of 16 g $L^{-1}$. Afterwards, the thermal hydrolysates were cooled down to room temperature and the enzymatic hydrolysis trials were carried out as described in Section 3.4.2.

**3- Thermal application for enzymes deactivation**

In order to deactivate the enzyme after hydrolysis, the produced hydrolysates after all enzymatic pretreatments performed during the development of this thesis were subjected to 75ºC for 30 minutes (Romero-Garcia et al., 2012).

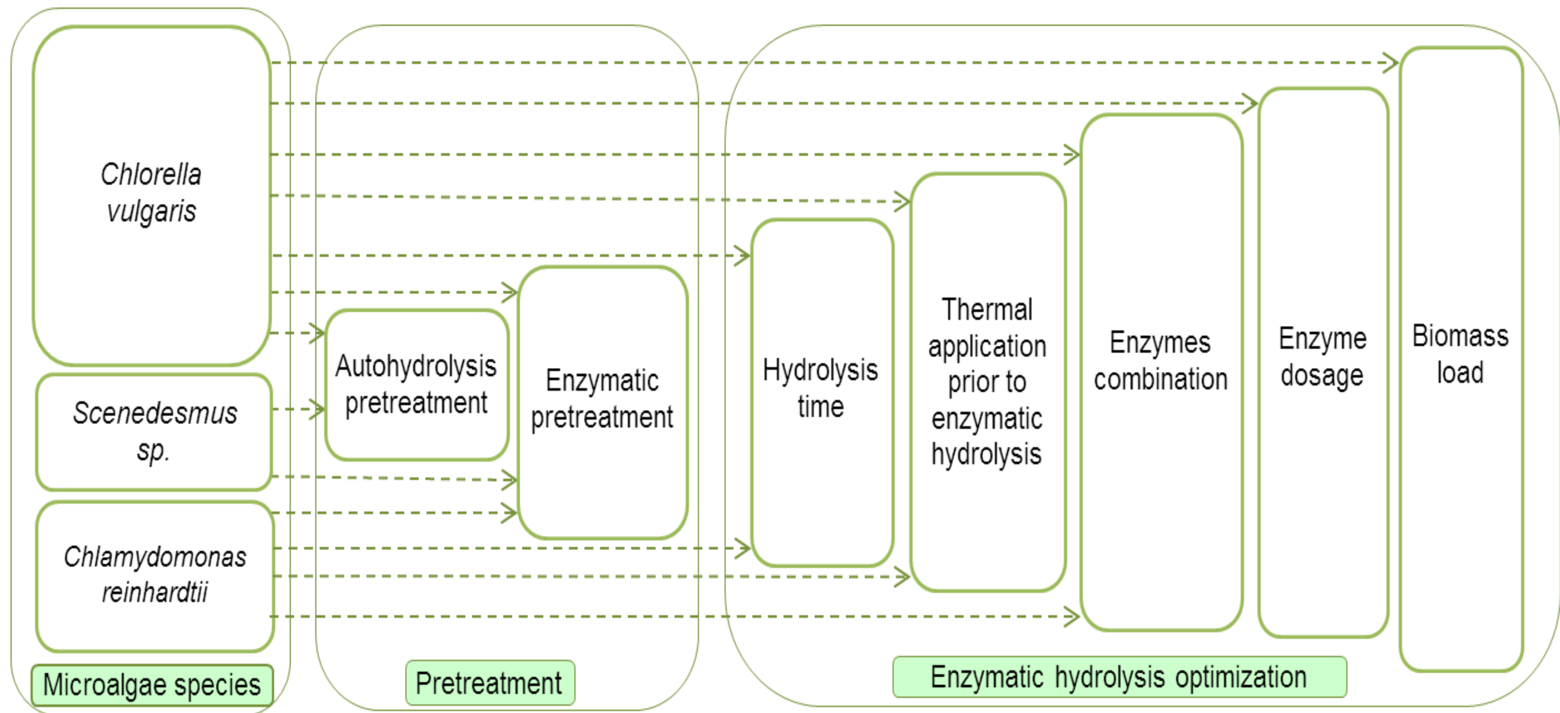



**Figure 3.3.** Block-diagram of microalgae biomass pretreatment employed during the development of this thesis prior to anaerobic digestion.

### 3.4.2. Enzymatic pretreatment

#### 3.4.2.1. Used enzymes

Carbohydrase enzymes (namely, Celluclast 1.5 L, Viscozyme L and Pectinex-Ultra SP-L) and protease (namely, Alcalase 2.5 L) were used for the hydrolysis of microalgae biomass. All these enzymes were commercially available and their characteristics and activities, according to the information provided by the supplier Novozyme (Denmark), are summarized in Table 3.3.

Enzyme loadings and hydrolysis conditions (temperature and pH) were applied in accordance with the supplier recommendation. In this context, enzymatic loadings were 0.25, 0.3, 0.25 and 0.2 mL enzymatic cocktail per g of substrates (DW) for Celluclast, Viscozyme, Pectinase and Alcalase, respectively. The pHs at which enzymatic hydrolysis was performed were 5, 5.5, 4.5 and 8 for Celluclast, Viscozyme, Pectinase and Alcalase, respectively. Throughout the enzymatic hydrolysis, pH was adjusted with chemical reagents (HCl and NaOH) when required and temperature was kept at 50ºC.

#### 3.4.2.2. Enzymatic pretreatment optimization

##### 3.4.2.2.1. Thermal application prior to enzymatic hydrolysis

Thermal application prior to enzymatic hydrolysis was conducted as reported in Section 3.4.1. In this context, raw, enzymatic hydrolysis and thermally pretreated enzymatic hydrolysis of *C. vulgaris* and *C. reinhardtii* biomass were compared in terms of organic matter released in the soluble phase in order to study the benefits that the additional thermal pretreatment can provide on opening up the microalgae cell structure for the enzyme activity (Section 4.2.1.).

**Table 3.3.** List of enzymes and characteristics according to the information provided by the supplier (Novozyme-Denmark).

| Commercial name | Type | Source | Composition | Activity |
|---|---|---|---|---|
| **Celluclast 1.5 L** | Carbohydrase | *Trichoderma reesei* | a broad spectrum of cellulolytic enzyme activities, most notably cellobiohydrolases (CBHs) and endo-1,4-β-glucanases (EGs). | 700 EGU* $g^{-1}$ |
| **Viscozyme L** | Carbohydrase | *Aspergillus aculeatus* | a range of carbohydrases including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase. It also breaks down branched pectin-like substances found in plant cell walls. | 100 FBG** $g^{-1}$ |
| **Pectinex-Ultra SP-L** | Carbohydrase | *Aspergillus niger* | a mixture of enzymes. The main enzymes it contains are pectintranseliminase, polygalacturonase and pectinesterase. As a side activity, *Pectinex* also contains small amounts of hemicellulases and cellulases. | 9500 PGU*** $mL^{-1}$ |
| **Alcalase 2.5 L** | Protease | *Bacillus licheniformis* | An endo-protease of the serine type which can hydrolyse most peptide bonds within a protein molecule. | 2.5 AU-A**** $g^{-1}$ |

Abbreviations: *EGU, EndoGlucanase Unit; **FBG, Fungal Beta-Glucanase Units;***PGU, PolyGalacturonase Unit;****AU-A, AU, Anson Unit with Subtilisin A as major enzyme component

#### 3.4.2.2.2. Carbohydrase and protease combination

The highest carbohydrate hydrolysis was obtained with Viscozyme and the highest organic matter hydrolysis was achieved with Alcalse. In accordance to the good results achieved after the hydrolysis conducted with both enzymatic cocktails (Section 4.1.2), the potential synergistic effect of both enzymes was assessed by combining Viscozyme L and Alcalase 2.5 L. Due to the different pHs at which those enzymatic cocktails present maximum activity, the hydrolysis of this combination was conducted in two stages. Based on the organic matter released in the soluble phase (sCOD) after applying Viscozyme L, the follow up of the soluble COD revealed increasing values during the first 3 hours. After this period of time, the soluble COD was constant. In this manner, the first hydrolytic stage adding Viscozyme lasted for 3 hours and it was conducted under the recommended operating conditions at 50ºC and pH 5.5. In the second stage, the pH was risen to 8.0 with NaOH (4 M) before adding the enzyme Alcalase. Similarly to Viscozyme, the follow up of the organic matter release over time revealed that the enzyme activity was negligible after 2 hours. Thus, the overall enzymatic pretreatment lasted 5 hours.

#### 3.4.2.2.3. Optimization of protease dosage for *C. vulgaris* biomass pretreatment

According to the promising results obtained in organic matter release and methane yields after the addition of protease to *C. vulgaris* (Sections 4.1.2 and 4.2.4), this pretreatment was optimized to achieve the highest hydrolysis efficiency at the lowest cost possible. To achieve that goal, microalgae biomass (16 g $L^{-1}$) were hydrolyzed at different Alcalase dosages. The enzyme dosage was calculated according to the protease activity declared by the supplier. The highest protease dosage (0.585 AU g $DW^{-1}$) corresponded to the best result pointed out in Section 4.1.2. This was considered the highest dosage level and two additional lower dosages were tested on *C. vulgaris* biomass hydrolysis (0.146, 0.293 and 0.585 AU g $DW^{-1}$). Hydrolysis was conducted in an orbital shaker at 130 rpm and incubated at 50ºC. Enzymatic pretreatment time of 3 hours was selected based on optimal

hydrolysis time for this biocatalyst on *C. vulgaris* biomass. In accordance to the supplier recommendations, the pH was adjusted to 8 by adding NaOH (4 M). Along the hydrolysis time, pH was adjusted on demand.

#### 3.4.2.2.4. Optimization of *C. vulgaris biomass* loads

After determining the optimum protease (Alcalase) dosage (0.585 AU g $DW^{-1}$), different *C. vulgaris* biomass loads were tested for further optimization of the protease hydrolysis pretreatment. In this manner, biomass loads were increased 2 and 4-fold (namely, 32 g $L^{-1}$ and 65 g $L^{-1}$) and hydrolyzed at the optimum enzyme dosage (Section 4.2.6). The purpose of this experiment was to determine the biomass load that can be treated without diminishing the hydrolysis efficiency which is a key parameter that can greatly affect the economic feasibility of the process. Since the high biomass load may cause an increase in viscosity that limits efficient mixing of the enzymes with the biomass and consequently the hydrolysis efficiency will be decreased.

### 3.5. Hydrolysis efficiency

The microalgae biomass for each hydrolysis conducted during this thesis was analyzed for total and soluble COD (all experiments), protein (Section 4.2.4) and carbohydrate (Sections 4.1.1.2; 4.1.2.2.2 and 4.2.4) before and after the pretreatments. Samples were taken out periodically (each hour) during enzymatic pretreatments in order to study the effect of enzymes on organic matter, protein and carbohydrate solubilisation as a function of time. These parameters were chosen since COD solubilized is directly linked to total organic matter released while both carbohydrates and proteins are the main components (>85%) of the total microalgae dry weight.

All samples were measured in duplicate. The hydrolysis efficiency was defined according to the following equation:

$$\% \, Hydrolysis \, efficiency = \frac{sCOD \, concentration \, after \, treatment - sCOD \, concentration \, in \, raw \, biomass}{tCOD \, concentration - sCOD \, concentration \, in \, raw \, biomass} * 100$$

where sCOD is soluble chemical oxygen demand and tCOD is total chemical oxygen demand. The efficiency of protein and carbohydrate released during the biological pretreatment was also measured by using the same formula, but considering the soluble and total fraction of protein and carbohydrate instead of COD.

## 3.6. Anaerobic digestion

### 3.6.1. Biochemical methane potential (BMP)

Biomethane potential tests were used to compare the anaerobic biodegradability of raw biomass with regard to the pretreated microalgae biomass. According to González-Fernández and Garcia-Encina (2009), microalgae biomass and anaerobic sludge were mixed to attain a ratio of 0.5 g chemical oxygen demand (COD) microalgae biomass (substrate)/volatile solids (VS) anaerobic sludge (as inoculum). This ratio ensures an optimal organic matter conversion into methane, avoiding organic matter overloading or underestimation of anaerobes activity. The amounts of sludge and substrate (microalgae) were calculated to obtain a final liquid volume of 70 mL on glass bottles of 120 mL (Figure 3.4.A). To provide enough buffering capacity during digestion, calcium bicarbonate was added. The pH was adjusted at 7-7.5 in all the reactors before starting anaerobic assays. Oxygen was removed by flushing the headspaces of the bottles with helium. After the set-up of each reactor, reactors were incubated in an orbital shaker at 130 rpm and 35ºC. To calculate endogenous methane production, blanks containing only anaerobic sludge were run. The mesophilic incubation of the reactors lasted until the biogas production reached steady state (period characterized by a stable methane production, approximately 3-5 weeks). The volume of biogas produced by the substrates was calculated by measuring the pressure of the bottle's headspace. In this context, the gas productions were expressed in standard temperature (0ºC) and pressure (1 atm) conditions (STP conditions) according to the following equations:

$$P.V = n.R.T \quad (1)$$

where:

P is pressure variation during the process (bar)

V is reactor volume (L)

n is amount of substance of generated gas (mole),

R is gas constant ($bar*L*K^{-1}*mole^{-1}$),

T is process temperature (= 35 + 273 Kelvin).

The produced biogas was recalculated to normal pressure and temperature (0 $^{o}$C and 1 atm).

**$P^{o}.V^{o}=n.R.T^{o}$** (2)

where:

$P^{o}$ is reference pressure (1atm = 1.013 bar),

$V^{o}$ is biogas production at 0$^{o}$C and 1 atm, during the test

$T^{o}$ is standard temperature (0$^{o}$C, 273 Kelvin)

By Substituting n (as it is fixed) in two former equations:-

**$PV/T = P^{o}V^{o}/T^{o}$** (3)

Therefore, **$V^{o} = P.V.T^{o}/P^{o}.T$** (4)

**3.6.2. Semi-continuous anaerobic digestion**

CSTRs with a working volume of 1 L were used as digesters (Figure 3.4.B). In order to prevent any photosynthetic activity, the digesters were covered with aluminum foil. The digesters were maintained at mesophilic temperature (35$^{o}$C) by circulating water through a water jacket. Constant mixing was provided by a magnetic stirrer. The same volume was daily withdrawn (effluent) and added (substrate) to the digesters using plastic syringes. pH was monitored every day, immediately after withdrawing effluent, but not controlled. The organic loading rates (OLR) for the whole experimental period was 1.5 g COD $L^{-1}$ $d^{-1}$. The hydraulic retention time (HRT) was 15 d in the case of the CSTR fed with raw biomass and 20 d for the CSTR fed with enzymatically pretreated *C. vulgaris* biomass (Alcalase or Viscozyme). Biogas volume was measured by water displacement every day. The methane

content was analyzed once a week. Steady state was characterized by a stable gas production and COD concentration of the effluents digesters.

After determining the optimum pretreatment to enhance biogas production, an additional experiment with a CSTR fed with protease pretreated *C. vulgaris* biomass was conducted in order to examine the possibility of doubling the OLR applied to the reactor (3 g COD $L^{-1}$ $d^{-1}$) and diminish the HRT to 15 d.

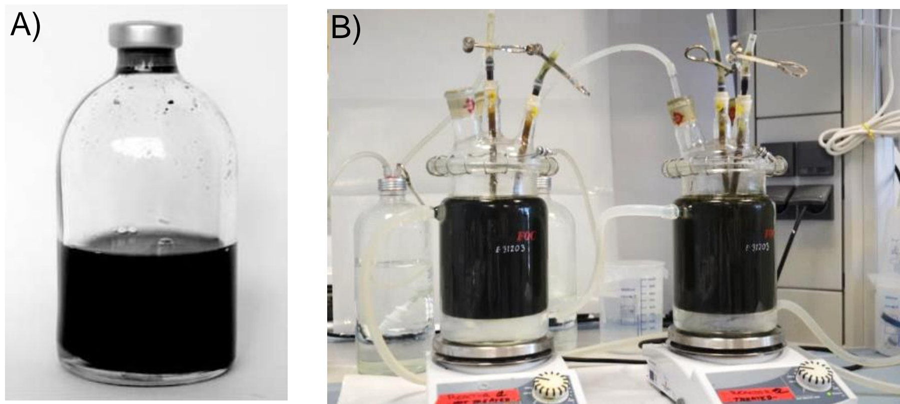



**Figure 3.4.** Anaerobic digestion reactors used for microalgae degradation (A) BMP reactor and (B) semi-continuous fed CSTR**.**

### 3.7. Cell monitoring

#### 3.7.1. Optical microscope

Microalgae identification (*C. vulgaris* used throughout the thesis and *Scenedesmus* sp. used in Section 4.1.1) was carried out by microscopic examination (OLYMPUS IX70, USA) of culture broth samples according to Phytoplankton Manual (Sournia, 1978).

Raw and pretreated microalgae biomass (*C. vulgaris*, *Scenedesmus* sp. and *C. reinhardtii*) were monitored using a magnification of 100x in a microscope Olympus CX41RF (Japan).

#### 3.7.2. Transmission electron microscopy (TEM)

To examine raw and protease pretreated *C. vulgaris* biomass at higher resolution, transmission electron microscopy (TEM) was used. Microalgae biomass was immersed in 3% glutaraldehyde buffered to pH 7.4 with 1X phosphate buffer saline (PBS) and kept at ambient temperature for 2 h. After that, several washing were performed in PBS 1X. The samples were post-fixed in osmium 1% and potassium ferricyanide 0.8% and kept at 4ºC for 1 h. Fixed samples were dehydrated in a series of graded ethanol and embedded in LX 112 resin (Ladd Research Industries). Ultrathin Sections were stained with uranyl acetate and lead citrate and examined by TEM (JEOL 1230) at 80 kV.

### 3.8. Analytical methods

#### 3.8.1. Total solids (TS), total suspended solid (TSS), volatile solids (VS) and volatile suspended solids (VSS)

TS, TSS, VS and VSS content were measured according to Analytical Standard Methods (Eaton et al 2005). Briefly, TS was determined as the residue after drying fixed volume of microalgae culture at 105ºC for 24 h and VS was determined as the loss after ignition at 550ºC for 3 h. TSS and VSS were measured by filtering a fixed volume of microalgae culture through a

glass fibre prefilters (Merck) and determining the residue after drying at 105ºC for 3 h and the loss after ignition at 550ºC for 30 min, respectively.

### 3.8.2. Total (tCOD) and soluble (sCOD) chemical Oxygen Demand

The chemical oxygen demand (COD) content is used to measure organic matter content of the samples. This parameter describes the amount of oxygen that is needed to completely oxidize the organic matter content of a sample under aerobic conditions. Test kit (Merck, ISO 15705) and a Spectrophotometer (Spectroquant® pharo 100, EU) were used for determining COD total (tCOD) and soluble (sCOD). Soluble COD was determined after samples centrifugation at 14,600 rpm for 5 min (Mini-spin Eppendorf 5424).

### 3.8.3 Total Kjeldahl Nitrogen (TKN) and ammonium nitrogen ($N\text{-}NH_4^+$) determination

TKN was determined according to Kjeldahl method (Kjeldahl, 1883). This method involves digestion, distillation and titration. A fixed volume of microalgae sample was digested with 12 mL sulphuric acid (95%) and catalyst mixture ($K_2SO_4$ and $CuSO_4$) at 420ºC for 1 h. The digestion step was conducted in FOSS Tecator TM scrubber. After digestion, the sample was distilled using Kjeltce TM 8200 autodistillation unit. The digestion fraction was made alkaline with 50 mL of sodium hydroxide solution (40%), and the released ammonia was steam distilled into a receiver filled with 25 mL of 4% boric acid with Kjeldahl indicator. Lastly, the contents were titrated with hydrochloric acid. Nitrogen content was calculated according to the equation below:

$$nitrogen\,\% = \frac{(\mathrm{T} - \mathrm{B}) * \mathrm{N} * 14.007 * 100}{\mathrm{W}}$$

where:

T: titrated volume of hydrochloric acid for sample, mL

B : titrated volume of hydrochloric acid for blank, mL

N : normality of hydrochloric acid14.007 - molar mass of N, mg $mmol^{-1}$

W : sample weight, mg

Regarding ammonium measurement, a test kit (Merck, ISO 15705) and a Spectrophotometer (Spectroquant® pharo 100, EU) were used for determining ammonium-nitrogen in soluble phase after samples centrifugation at 14,600 rpm for 5 min (Mini-spin Eppendorf 5424).

### 3.8.4. Proteins content determination

Proteins content was estimated by multiplying the total Kjeldahl nitrogen by a correction factor of 5.95 (González López et al., 2010).

### 3.8.5. Carbohydrates content determination

Carbohydrate content in liquid samples was determined by the phenol sulphate method (Dubois et al., 1956). Briefly, 200 µl of liquid sample was diluted up to sugars concentration range between 0.1 and 0.4 mg $mL^{-1}$. Deionized water, as blank sample, and glucose standards solution to prepare a calibration curve were also employed. Then, 50 µl of phenol solution (at concentration of 5% v/v) and 5 mL of sulphuric acid were added to each sample and mixed. After 30 minutes, the amount of total soluble sugars was determined by using a Spectrophotometer (wavelength of 485 nm) (Spectro-star Omega S/N 415-1414, Germany).

### 3.8.6. Ash content determination

The ash content (inorganic matter) was calculated as follows:

%Ash = 100 - %VS

### 3.8.7. Lipids content determination

Lipids were estimated as the remaining fraction of TS after the determination of proteins, carbohydrates and ash.

### 3.8.8. pH measurement

The pH was measured using a pH meter (Crison Basic 20+, EU). The pH meter was calibrated regularly with pH 4.01, 7.0 and 9.21 buffers (HANNA, HI).

### 3.8.9. Volatile Fatty Acids (VFAs) determination

The samples were centrifuged at 14,600 rpm for 5 min (Mini-spin Eppendorf 5424), followed by filtration at 0.2 μm (Nylon membrane). 2 mL of supernatant were transferred to a vial prior to the analysis by high-performance liquid chromatography. VFAs were analyzed by high-performance liquid chromatography in an Agilent 1260 chromatograph equipped with UV–Vis detector (RID and DAD) and Bio-Rad column (Aminex HPX-87H). The mobile phase was sulphuric acid (0.005 M) with a flow rate of 0.35 ml $min^{-1}$.

### 3.8.10. Nitrate, nitrite and phosphate determination

The samples were centrifuged at 14,600 rpm for 5 min (Mini-spin Eppendorf 5424), followed by filtration at 0.2 μm (Nylon membrane). 2 mL of supernatant were transferred to a vial prior to the analysis by ionic chromatography. Nitrate, nitrite and phosphate were measured by ionic chromatography 930 Compact IC Flex (Metrohm) equipped with Metro Sep A sup 5-250/4.0 column maintained at 25ºC, with a conductivity detector (suppressed CE J006). $Na_2CO_3$ (3.2 mM) and $NaHCO_3$ (1.0 mM) were used as mobile phase at flow rate of 0.7 mL $min^{-1}$ and pressure of 10.8 MPa.

### 3.8.11. Gas composition in BMP and CSTRs assays

Gas composition was determined by gas chromatography (Agilent 7820A) equipped with HP-PLOT Q column. Helium was used as the carrier gas. The injection port temperature was set at 250ºC, the oven at 40ºC and the detector temperature at 275ºC.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4. Results and discussion

Three different microalgae were used in this thesis, namely *Chlorella vulgaris*, *Scenedesmus* sp. and *Chlamydomonas reinhardtii*. From an economic point of view, microalgae biogas production should be ideally coupled to wastewater bioremediation. In this way, microalgae exert the dual purpose of contaminants removal from wastewater while producing biomass that could be valorized for energy production (biogas). *Chlorella vulgaris* and *Scenedesmus* sp. are two common and robust microalgae easy to grow in wastewater. Those microalgae were selected in accordance to their easiness to be cultivated outdoors and their different cell wall composition (Posadas et al., 2015b; Lam and Lee, 2014). For comparison purposes, an additional microalgae strain was tested, namely *Chlamydomonas reinhardtii. C. vulgaris* and *Scenedesmus* sp. have a rigid cell wall while the cell wall of *C. reinhardtii* is weaker (Mussgnug et al., 2010). As a result, the first two strains are difficult to degrade anaerobically while *C. reinhardtii* should be easier.

As microalgae composition (macromolecular profile) varies drastically based on many parameters such as microalgae strain and cultivation conditions, information regarding those parameters is given for each experiment developed throughout the thesis. Moreover, when focusing on the use of biocatalyst as pretreatment to hydrolyze microalgae biomass, the knowledge of the macromolecular profile seems crucial for an appropriate hydrolysis of the organic matter. For all evaluated pretreatments, data related to organic matter (COD, carbohydrates or proteins) solubilisation is firstly discussed, and then, potential improvements of methane yields are investigated for each case.

### 4.1. Microalgae pretreatment

The effectiveness of pretreatment methods on biogas production depends on the characteristics of microalgae, i.e., the toughness and structure of the cell wall, and their macromolecular composition. Catalyst-free pretreatments (autohydrolysis) at low temperature have gained interest to improve the

organic matter bioavailability. This pretreatment is envisaged as a low energy and low cost process to improve the anaerobic digestion of these substrates. Autohydrolysis is a biological response of the microorganisms induced by heat. For instance, mild temperatures (<55ºC) and limited dissolved oxygen may favor the release of hydrolytic enzymes to the medium and hence no further catalysts are required (Carvajal et al., 2013). To this end, microalgae feedstocks were subjected to autohydrolysis as an attempt to disrupt the cell biomass structure and increase its bioavailability for anaerobic microorganisms.

#### 4.1.1. Autohydrolysis pretreatment

The aim of this study was to evaluate the effect of low temperature (50ºC) autohydrolysis on *Chlorella vulgaris* and *Scenedesmus* sp. biomass disruption at different incubation times (24 and 48 h). Special attention was given to the solubilisation of both organic matter (COD) and carbohydrates. In order to assess the effect on methane production after autohydrolysis, raw and pretreated biomass were subjected to anaerobic digestion.

##### 4.1.1.1. Microalgae biomass and macromolecular composition

The chemical characterization of *Chlorella vulgaris* and *Scenedesmus* sp. biomass was quite similar. Organic matter content was higher than 90% and thus ash content (inorganic matter) ranged 5-10%. Carbohydrate fraction accounted approximately 23% and 20% of the dry weight biomass of *C. vulgaris* and *Scenedesmus* sp., respectively. Both biomass displayed low sCOD/tCOD ratio (0.8-1%), which confirmed that that the cell walls were mostly intact (Table 4.1).

**Table 4.1.** Chemical characterization of *Chlorella vulgaris* and *Scenedesmus* sp.

| Chemical parameters | Average (± standard deviation) | |
|---|---|---|
| | *C. vulgaris* | *Scenedesmus* sp. |
| TS (g $L^{-1}$) | 56.0±1.1 | 44.5±1.4 |
| VS (g $L^{-1}$) | 50.2±1.0 | 42.2±1.0 |
| tCOD (g $L^{-1}$) | 78.8±0.1 | 73.0±0.1 |
| sCOD (g $L^{-1}$) | 6.5±0.2 | 7.01±0.1 |
| Carbohydrate (% mg g $DW^{-1}$) | 23.0 | 20.0 |
| Ash (% mg g $DW^{-1}$) | 10.3 | 5.2 |

#### 4.1.1.2. Organic matter solubilisation

In order to determine the effectiveness of autohydrolysis for breaking down the cell wall, solubilisation of organic matter (COD) was examined. This parameter is widely used to estimate the extent of biomass disruption of different substrates (Carrère et al., 2008; González-Fernández et al., 2008). As a matter of fact, the amount of solubilised COD seems to be directly linked to methane production enhancement (Passos et al., 2013; Carrère et al., 2008). The results showed that biomass autohydrolysis was strain specific. 16% and 6% COD solubilisation was observed for *Chlorella* and *Scenedesmus*, respectively (Table 4.2). These results are in agreement with Passos et al., (2013). Those authors reported an increase of 5-fold soluble COD when treating microalgae biomass at 55°C for 15 h. In the present study, the autohydrolysis pretreatment increased soluble COD by 2–3-fold. Compared to *Chlorella*, the cell wall rigidity of *Scenedesmus* could be responsible for the lower COD solubilisation achieved during autohydrolysis. This fact could be explained by the different cell wall structure. Species of genus *Scenedesmus* have a complex cell wall composed of individual chemicals such as sporopollenine distributed in several layers with a particular arrangement, which gives it an extreme resistance (Burczyk and

Dworzanski 1988). These ultrastructure characteristics confer greater resistance to *Scenedesmus* compared to *Chlorella*.

None of the microalgae biomass showed any COD solubilisation enhancement at increasing incubation time. According to González-Fernández et al., (2013), prolonged thermal treatment did not improve COD hydrolysis yields. Note worth to mention that those results were attained at higher temperatures (70–90°C). Nevertheless, it seems likely that the same conclusion stands for lower temperatures (50°C).

#### 4.1.1.3. Carbohydrate solubilisation

Since *Chlorella* sp. and *Scenedesmus* sp. are traditionally characterized by carbohydrate-based cell walls (Cheng et al., 2013; Takeda, 1996), the determination of carbohydrates solubilisation was used as a potential tool to evaluate the pretreatment efficiency.

*C. vulgaris* and *Scenedesmus* sp. raw biomass contained total carbohydrate of 23 (2% soluble) and 20% (0.8% soluble) dry weight, respectively. Opposite to what it was observed for COD solubilisation, incubation time affected carbohydrates release. Autohydrolysis pretreatment led to different profiles of carbohydrates solubilisation. *C. vulgaris* exhibited 10.8 and 15% carbohydrates solubilisation for incubation times of 24 and 48 h (Table 4.2.). With regard to *Scenedesmus* sp. biomass, the carbohydrate solubilised was higher than the attained for *C. vulgaris.* In this case, *Scenedesmus* sp. achieved 30% carbohydrates solubilisation regardless the incubation time. As mentioned previously for COD solubilisation, these differences observed in carbohydrates solubilisation could be due to differences in cell wall disruption efficiency or exopolymeric compounds release. This fact still needs to be elucidated.Overall, the release of carbohydrates by autohydrolysis treatment was specie dependent. Carbohydrates solubilisation by autohydrolysis pretreatment was markedly higher for *Scenedesmus* than for *Chlorella* biomass Exposure time did not affect carbohydrate release during *Scenedesmus* sp. autohydrolysis, while *Chlorella vulgaris* biomass increased soluble carbohydrates from 10.8 to 15%.

**Table 4.2.** COD and carbohydrates solubilisations during autohydrolysis of the two microalgae strains (*Chlorella vulgaris* and *Scenedesmus* sp.).

| | Pretreatment | % COD solubilised | % Carbohydrates solubilised |
|---|---|---|---|
| *Chlorella vulgaris*. | 24 h at 50 °C | 16.2 | 10.8±0.1 |
| *Chlorella vulgaris*. | 48 h at 50 °C | 17.0 | 14.9±0.1 |
| *Scenedesmus* sp. | 24 h at 50 °C | 5.7 | 31.3±0.5 |
| *Scenedesmus* sp. | 48 h at 50 °C | 5.7 | 30.8±0.5 |

#### 4.1.1.4. Effect of autohydrolysis pretreatment on methane production

##### 4.1.1.4.1. *C. vulgaris* biomass

The methane production over an incubation period of 38 days is shown in Figure 4.1. Methane produced by *C. vulgaris* biomass exhibited highest yield after 48 h autohydrolysis. This result is probably due to the higher carbohydrate solubilisation registered under this incubation time compared to 24 h (Table 4.2.). This would be in agreement with Mendez et al., (2014a) who elucidated a close relation between carbohydrate solubilisation and methane yield. With regard to raw biomass, 48 h incubation time resulted in 10% methane production increase. The low methane production enhancement achieved with autohydrolysis is in accordance with Passos et al., (2013). Those authors achieved 13% enhancement when pretreating microalgae biomass at 55°C for 15 h while increasing temperature to 75°C concomitantly increased methane yield by 42%.

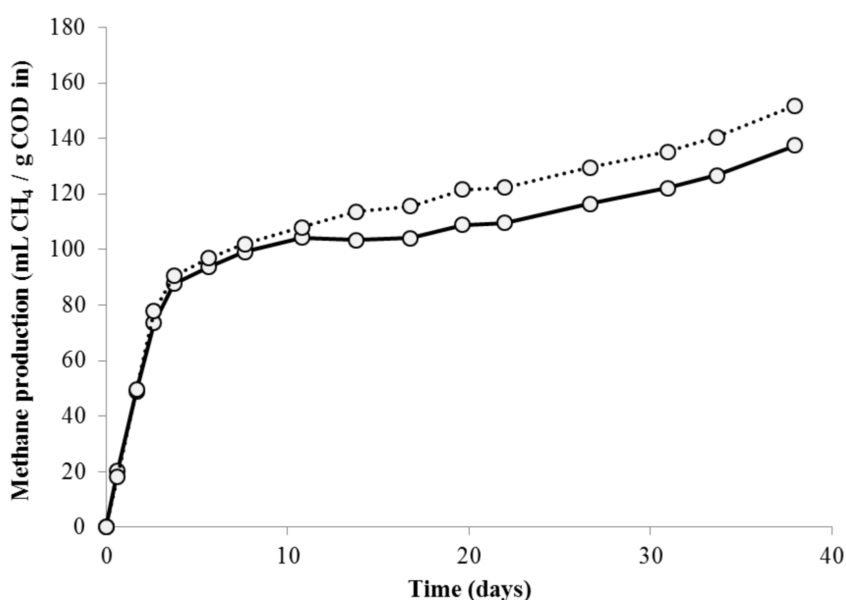



**Figure 4.1.** Methane production achieved by *Chlorella vulgaris* biomass pretreated with autohydrolysis (continuous and dashed lines corresponded to 24 and 48 h pretreatment, respectively).

#### 4.1.1.4.2. *Scenedesmus* sp. biomass

In the case of *Scenedesmus* biomass, methane production was quite similar for 24 and 48 h incubation at 50°C. With regard to raw biomass, no improvement was registered after autohydrolysis (Figure 4.2.).

Overall, methane yield enhancement was quite low compared to other studies (Mendez et al., 2013; 2014a). Out of the two microalgae biomass, only the anaerobic digestion of *C. vulgaris* provided an increase in methane production (10%). This methane production corresponds to anaerobic biodegradabilities of 40–45%. The low methane production enhancement was ascribed to the fact that the organic matter solubilisation was probably mediated by exopolymers released during the pretreatments rather than by cell wall breakage. As it can be seen in Figure 4.3., cells were aggregated in flocs while some other cells kept their original shape.

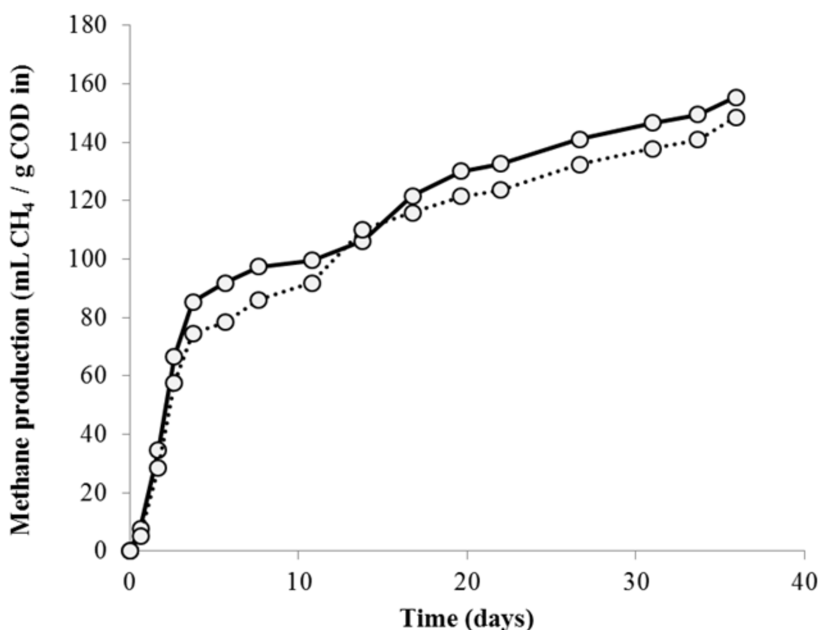



**Figure 4.2.** Methane production achieved by *Scenedesmus* sp. biomass pretreated with autohydrolysis (continuous and dashed lines corresponded to 24 and 48 h pretreatment, respectively).

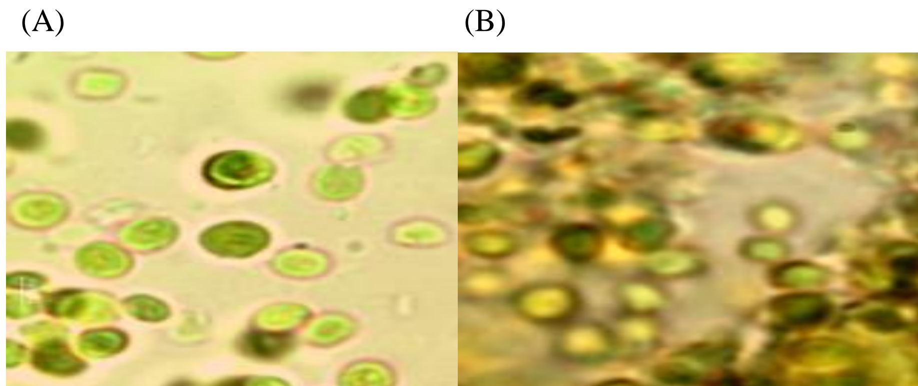



**Figure 4.3.** *Scenedesmus* sp. microscopic picture before (A) and after (B) pretreatment.

As it can be concluded from the above results, the autohydrolysis pretreatment was unable to break down efficiently the complex cell wall of the microalgae studied. Moreover, the low methane yield enhancement reached by autohydrolysis pretreatment revealed that microalgae biomass required to be pretreated by alternative approaches to maximize hydrolysis effectiveness and justify the pretreatments costs.

Enzymatic pretreatment could be a promising method to improve microalgae hydrolysis due to its low energy consumption. In this context, enzymatic pretreatment, usually conducted at mild temperature, could be an interesting alternative to energy-consuming pretreatments for enhancing the disruption/hydrolysis of microalgae biomass (cell walls and other biopolymers). Additionally, enzymatic pretreatment does not involve the use of chemical compounds, thus preventing the formation of inhibitory by-products. The overall process cost of enzymatic pretreatment may be lower than other pretreatments such is the case of thermochemical application. With regard to those later ones, enzymatic hydrolysis avoids equipment corrosion and occurs under mild temperatures. Likewise, enzymes can be produced by a wide range of bacteria and fungi (Miao et al., 2013; Ahamed and Vermette, 2008). So far, very little work has been done on the effect of enzymatic hydrolysis on microalgae cell disruption/hydrolysis for methane production purposes.

### 4.1.2. Enzymatic pretreatment

In order to evaluate the hydrolytic efficiencies of different types of enzymes on microalgae biomass, this study aimed at assessing commercial enzymatic cocktails (carbohydrases and proteases) in terms of organic matter solubilisation and biogas production. After this enzymatic cocktails screening, the best enzyme activity was selected to optimize biogas production using microalgae biomass as substrate.

#### 4.1.2.1. Microalgae biomass: macromolecular composition

The effectiveness of different biological catalysts on microalgae cell hydrolysis and methane production potential was evaluated using two microalgae strains displaying different macromolecular profiles. *Chlorella vulgaris* and *Scenedesmus* sp. were grown in mineral media (modified BBM and fertilizers, respectively). Both microalgae exhibited totally different macromolecular distribution (Table 4.3.).

**Table 4.3.** Chemical characterization of *Chlorella vulgaris* and *Scenedesmus* sp.

| Chemical parameters | Average (± standard deviation) | |
|---|---|---|
| | *C. vulgaris* | *Scenedesmus* sp. |
| TS ($g\ L^{-1}$) | 16.3±0.1 | 16.2±0.2 |
| VS ($g\ L^{-1}$) | 15.3±0.1 | 14.5±0.1 |
| tCOD ($g\ L^{-1}$) | 24.5±0.1 | 22.7±0.1 |
| sCOD ($g\ L^{-1}$) | 1.242±0.2 | 0.64±0.1 |
| Protein (% mg g $DW^{-1}$) | 63.4±0.5 | 33.1±0.1 |
| Carbohydrate (% mg g $DW^{-1}$) | 25.7±2.9 | 34.6±3.0 |
| Lipids and others (% mg g $DW^{-1}$) | 4.8±1.1 | 21.8±3.7 |
| Ash (% mg g $DW^{-1}$) | 6.2±0.1 | 10.5±0.7 |

Proteins and carbohydrates accounted for 63% and 26% of the dry weight (DW) biomass of *C. vulgaris*, respectively (Table 4.3.). In the case of *Scenedesmus* sp., carbohydrate and protein fraction was 33 and 34% of the DW. Therefore, only 5% of the DW was assumed to be lipid fraction for *C. vulgaris* while this value rose to 22% for *Scenedesmus* sp. The different macromolecular distribution of both microalgae and their different growing media was useful to determine whether these differences were key parameters to determine biocatalysts efficiency.

#### 4.1.2.2. Organic matter solubilisation during enzymatic pretreatment

Since the predominant macromolecules in both microalgae were proteins and carbohydrates (Table 4.3.), the effect of proteases (Alcalase 2.5L) and carbohydrases (Celluclast 1.5, Viscozyme and Pectinase) on biomass hydrolysis and methane production was evaluated.

##### 4.1.2.2.1. Soluble COD (sCOD)

Organic matter solubilisation was followed by measuring the COD released to the soluble phase over time (Figure 4.4.). The initial sCOD accounted for 1% of the total organic matter in the case of *C. vulgaris* (Table 4.3.). The temperature control assay provided the lowest hydrolysis (18%). This value is in agreement with those obtained in *C. vulgaris* thermal autohydrolysis at 55ºC for 24 h (Section 4.1.1.1). Organic matter hydrolysis registered after carbohydrases treatment (25-29%) was lower than that observed for proteases (Figure 4.4.A). The highest COD solubilisation was observed after enzymatic hydrolysis conducted by means of Alcalase 2.5L (a serine endopeptidase consisting primarily of subtilisin A). The COD hydrolysis efficiency with proteases was 47%. This value was similar to that attained by Romero-Garcia et al., (2012). These authors achieved hydrolysis efficiency of 42% when subjecting *Scenedesmus almeriensis* to commercial enzymes (Alcalase and Flavourzyme for 4 h). Maximum COD solubilisation was observed after 1 h of enzymatic hydrolysis and it, then, remained almost constant for most of the biocatalysts tested (except for the pectinase pretreated biomass, which showed increasing hydrolysis throughout the experiment time).

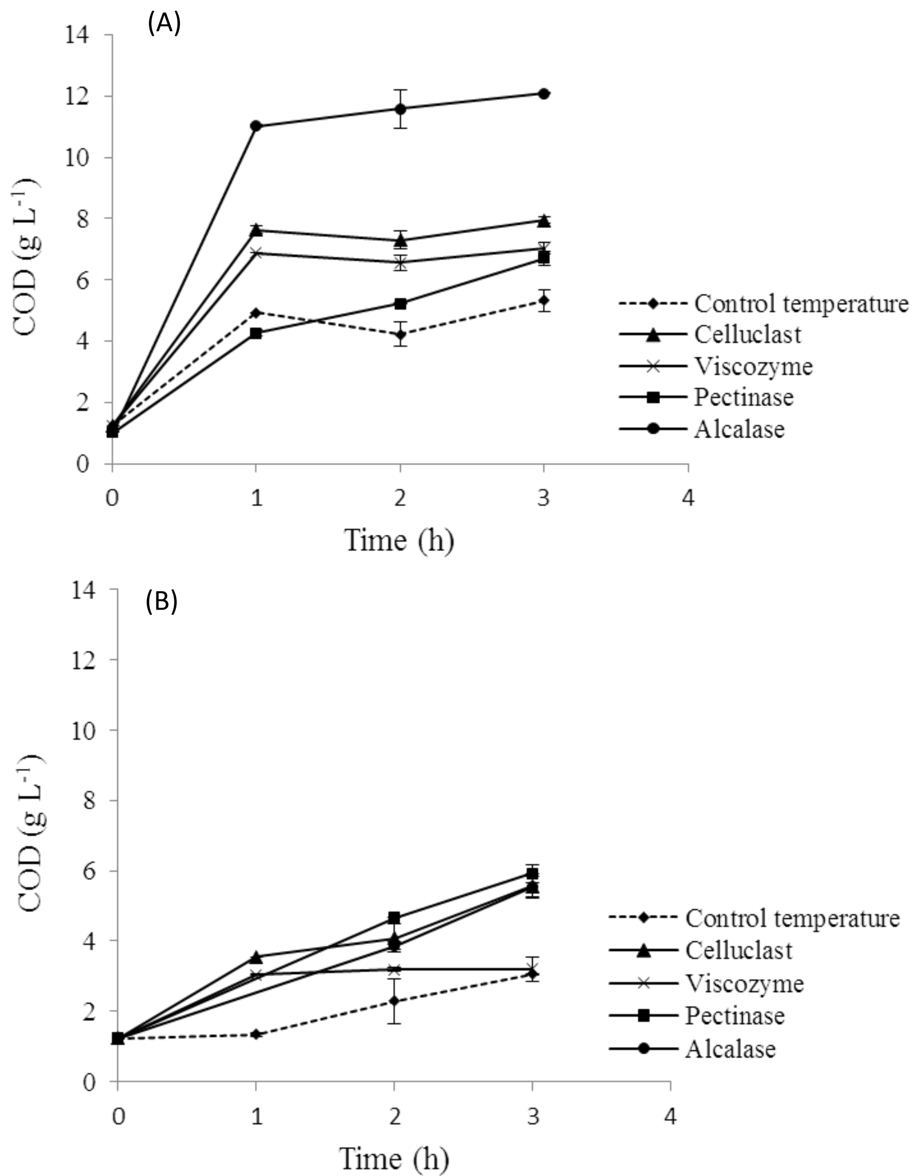



**Figure 4.4.** Organic matter solubilisation over time with different enzymatic pretreatments applied to *Chlorella vulgaris* (A) and *Scenedesmus* sp. (B).

As it can be seen in Figure 4.4.B., the organic matter solubilisation in the case of *Scenedesmus* sp. exhibited a different trend. The initial sCOD for *Scenedesmus* sp. was 5% of the total COD in the soluble phase (Table 4.3.). In contrast to *C. vulgaris*, the COD hydrolysis of *Scenedesmus* sp. was continuous over time and thus, the protease trial was allowed to continue longer (as described later). In this case, the exception was the trial with Viscozyme where sCOD remained constant after 1 h of hydrolysis. This biocatalysts cocktail, together with the temperature control trial, mediated the lowest COD solubilisation (8%). The hydrolysis efficiencies recorded for cellulase and for pectinase were around 20% after 3 h of hydrolysis.

In contrast to *C. vulgaris*, the hydrolysis conducted with protease on *Scenedesmus* sp. biomass did not exhibit high hydrolysis efficiency. In this case, 15% organic matter hydrolysis was calculated for the protease after 3 h while this value was increased to 30% after 8 h hydrolysis. In this manner, due to the lower organic matter hydrolysis observed for *Scenedesmus* sp. compared with *C. vulgaris*, it can be inferred that higher organic matter hydrolysis could be obtained at longer hydrolysis time. The hydrolysis efficiencies obtained for *Scenedesmus* sp. were 1.5–2.8-fold lower than those obtained for *C. vulgaris* (Figure 4.4.). Hydrolysis efficiencies are genus specific due to the differences in cell wall and biomass composition. Even though both microalgae were traditionally characterized by a carbohydrate-based cell wall containing hemicellulose (Takeda, 1991; 1996), the cell wall of *Scenedesmus* species seems to be more resistant due to the trilayer structure formed by components such as sporopollenin (Burczyk and Dworzanski, 1988). This very hard cell wall could be one of the reasons for the reduced COD solubilisation attained during hydrolysis of *Scenedesmus* sp. when compared with *Chlorella*. As a matter of fact, the harder cell wall of *Scenedesmus* in comparison with that of *Chlorella* has been reported in several studies (González-Fernández et al., 2012; Mussgnug et al., 2010). In addition, *Scenedesmus* sp. used in this study exhibited an average lipids content of 22% while the biocatalysts employed were carbohydrases and protease. It remains to be seen what the effect of lipases is on this type of substrate (out of the scope of this investigation).

#### 4.1.2.2.2. Soluble carbohydrates

The solubilisation of carbohydrates was investigated since several authors claimed that the carbohydrates fraction is responsible for the hard cell wall exhibited by some microalgae (Takeda 1991; 1996; Pieper et al., 2012; Cheng et al., 2013). Thus, it seems likely that carbohydrates follow-up during hydrolysis could help gain insights on the efficiency of cell hydrolysis.

Soluble carbohydrate (sCH) determined in raw *C. vulgaris* and *Scenedesmus* sp. accounted for 2.8% and 0.4% of the total carbohydrate, respectively (Table 4.3., Figure 4.5). Carbohydrates solubilisation ranged from 15–20% for all tested biocatalysts (exception made for Viscozyme). Regardless of the microalgae genera, the highest carbohydrate hydrolysis was obtained with Viscozyme (Figure 4.5.). 84% and 36% of the total carbohydrate of *C. vulgaris* and *Scenedesmus* sp. was available in the soluble phase after 3 h of hydrolysis. The high carbohydrate solubilisation recorded for *C. vulgaris* indicated that this enzymatic cocktail was more suitable for the cell carbohydrates of this particular microalga, while the carbohydrate composition of *Scenedesmus* cells seemed to be more recalcitrant.

The positive effect of Viscozyme may be attributed to its multiple activities including β-glucanase, xylanase, cellulase and hemicellulase. On the other hand, celluclast [a 1,4-(1,3:1,4)-β-D-Glucan 4-glucano-hydrolase] provided 48% solubilisation of the total carbohydrates of *C. vulgaris* (Figure 4.5.A), while the other carbohydrases (Pectinase) and the protease (Alcalase 2.5L) mediated 30–40% carbohydrates solubilisation. Once again, a different trend could be observed for *Scenedesmus* sp. In this latter case, cellulases and the rest of the biocatalyst resulted in low carbohydrates solubilisation. As a matter of fact, the addition of enzymes did not increase the carbohydrates solubilised with regard to the thermal treatment used for temperature incubation control (Figure 4.5.B). In the case of the protease, the carbohydrates solubilisation could be attributed to the glycoproteins present in microalgae cell walls (Popper and Tuohy, 2010) and the release of internal carbohydrates.

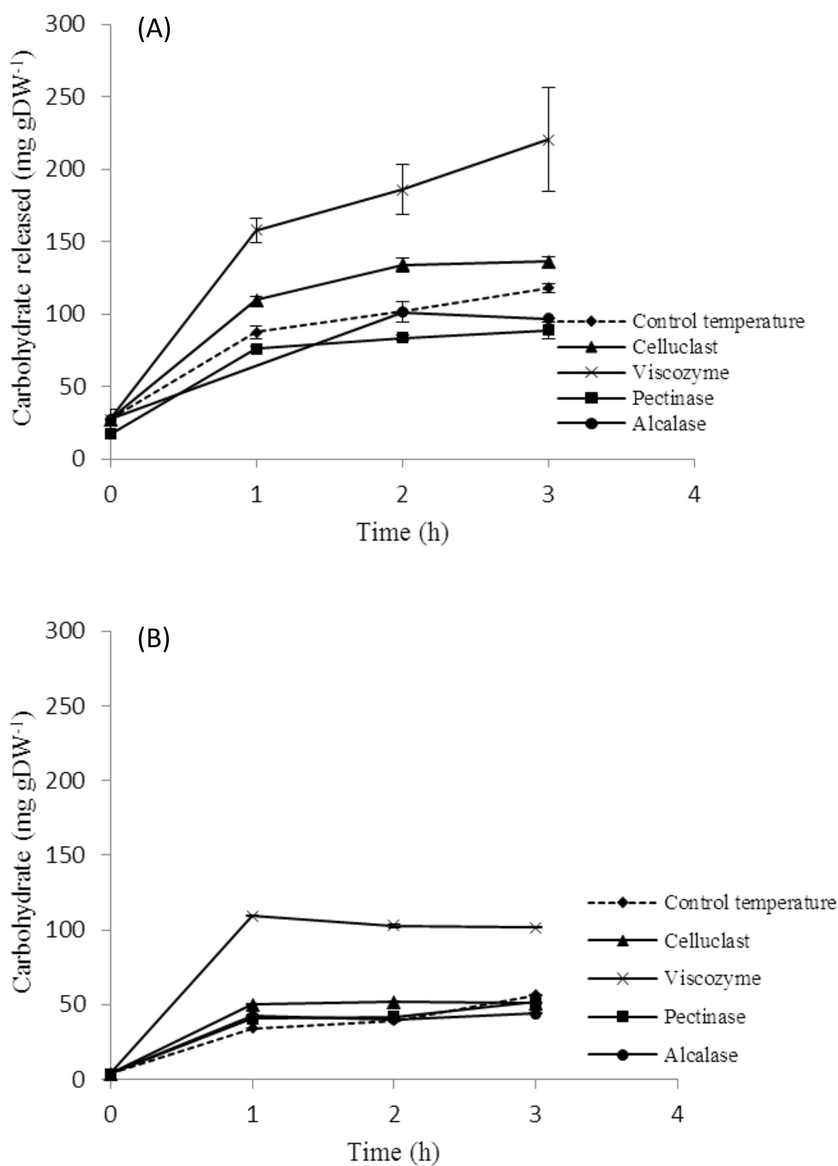



**Figure 4.5.** Carbohydrate solubilisation over time with different enzymatic pretreatments applied to *Chlorella vulgaris* (A) and *Scenedesmus* sp. (B).

#### 4.1.2.3. Methane production potential of raw and enzymatically pretreated microalgae biomass

The effectiveness of enzymatic hydrolysis on both microalgae was investigated with regard to the anaerobic digestion of those pretreated substrates and compared with the raw biomass (Figure 4.6.). Methane content in the biogas was stable throughout digestion time (69.5±1.6%). In the case of *C. vulgaris*, the raw biomass achieved methane yield of 142 mL $CH_4$ g COD $in^{-1}$. Assuming complete biodegradation of 350 mL $CH_4$ g COD $in^{-1}$, the anaerobic biodegradability of this microalga corresponded to 42%. This value was in the same range reported in previous studies (Mendez et al., 2013; Kumar et al., 2014).

Methane production by the enzymatically hydrolyzed biomass was substantially higher than that obtained by the non-treated biomass. Of the screened carbohydrases, the enzymatic cocktails Celluclast and Viscozyme increased methane production to 170 mL $CH_4$ g COD $in^{-1}$ (14% enhancement). This result was in agreement with methane yield reported by Passos et al., (2016). Those authors achieved 8-15% methane yield increase in microalgae biomass experiments treated with carbohydrase (namely, cellulase and an enzyme mixture composed of cellulase, glucohydrolase and xylanase).

In contrast to the low methane yield enhancement (1.2-fold) obtained with carbohydrases, the effect of pectinase on *C. vulgaris* resulted in greater increase (1.54-fold). This result confirmed that the carbohydrates hampering an efficient anaerobic digestion of *C. vulgaris* were the uronic acids contained in the cell wall. This finding was supported by other studies that also demonstrated that cellulase, lysozyme, xylanase and amylase biocatalysts did not have any significant effect on microalgae cell wall hydrolysis (Kim et al., 2014; Gerken et al., 2013). While the three tested carbohydrases mediated similar COD solubilisation (Figure 4.4.A), the cellulases exhibited the highest carbohydrate solubilisation (Figure 4.5.A). Despite the 85% carbohydrates solubilised after 3 h of hydrolysis, the methane yield enhancement was low. It can thus be inferred that the carbohydrate fraction was not directly responsible for the low *C. vulgaris* digestibility.

The improvement registered for biomass hydrolyzed by carbohydrases was low when compared with the values attained for *C. vulgaris* pretreated with proteases. Methane yield achieved by *C. vulgaris* pretreated with protease was 248 mL $CH_4$ g COD $in^{-1}$. With regard to the raw biomass, the use of this biocatalyst provided a 1.72-fold enhancement of methane yield. At this point, it should be stressed that the biomass hydrolyzed with protease gave the highest methane yield concomitantly with the highest organic matter solubilisation (Figure 4.6.A and Figure 4.4.A).

Methane yield is dependent on biomass chemical composition. In this context, lipids, proteins and carbohydrates may attain methane yields of 1.014, 0.496 and 0.415 L $CH_4$ g VS $in^{-1}$, respectively (Angelidaki and Sanders, 2004). Even though lipid fraction exhibits the highest methane yields, methane productivity is lower than for the other two macromolecules due to the lower hydrolysis constant. In this sense, Pavlostathis and Giraldo-Gomez (1991) reported hydrolysis times of 0.18, 0.43 and 3.2 days for carbohydrates, proteins and lipids, respectively. Therefore, macromolecular profile affects methane yield and productivity. This is the case for instance of *C. vulgaris*. Despite of the different macromolecular distribution determined in different studies, anaerobic biodegradability was kept in the narrow range of 42–45%. More specifically, protein content of *C. vulgaris* in this study was 63%, while the same biodegradabilities were attained for *C. vulgaris* that exhibited 35–40% protein content (Mendez et al., 2013). Therefore, the biodegradability of raw *C. vulgaris* was approximately 42–48%, regardless the protein biomass content. In addition, it can be inferred that methane yield achievable using microalgae could be more dependant on cell wall characteristics rather than on macromolecular composition.

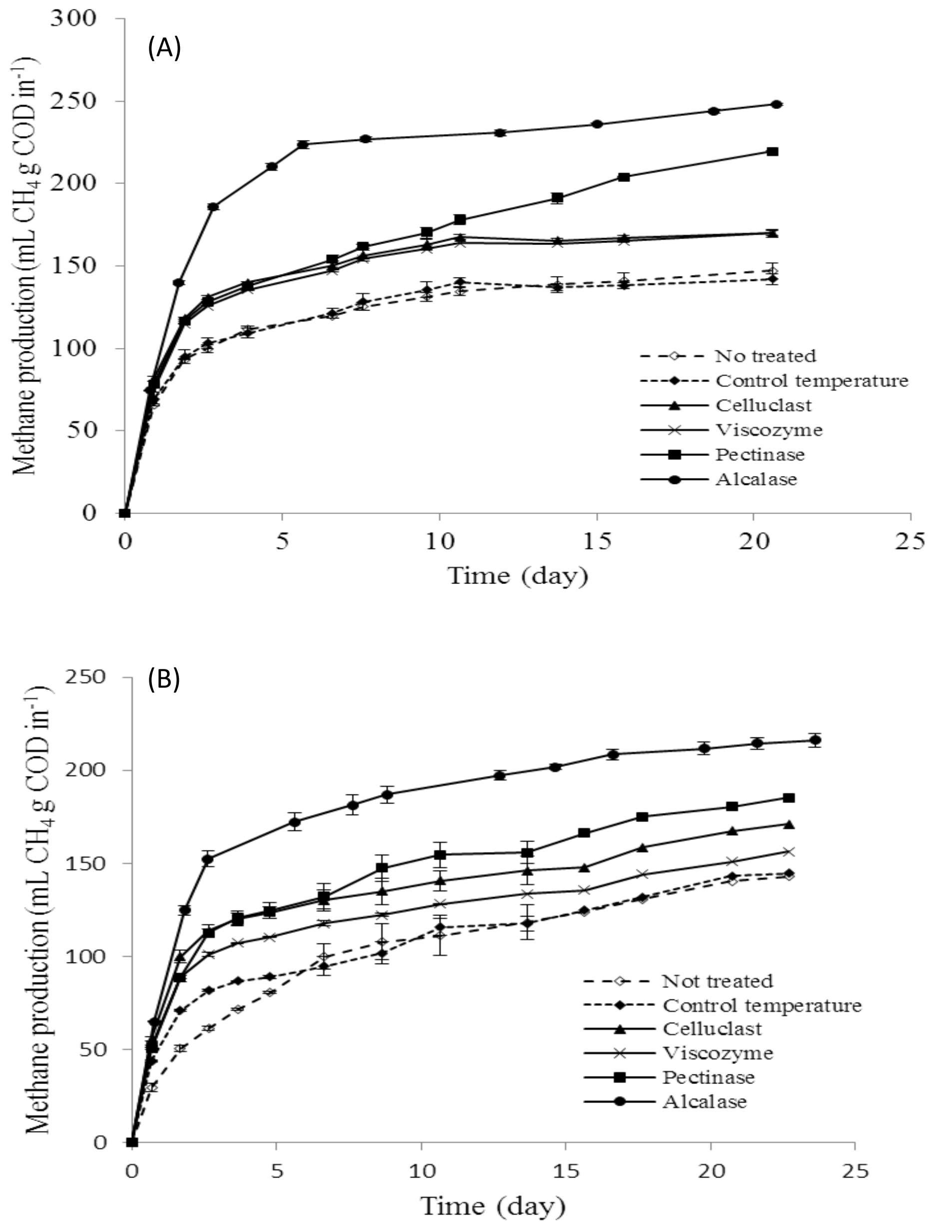



**Figure 4.6.** Cumulative methane production achieved by raw biomass (not treated), control temperature (autohydrolysis), and different biomass pretreated enzymatically of *Chlorella vulgaris* (A) and *Scenedesmus* sp. (B)

Despite the different macromolecular composition, raw *Scenedesmus* sp. presented similar methane yield (141.6 mL $CH_4$ g COD $in^{-1}$, Figure 4.6.B) to *C. vulgaris*. However, the productivities were different in the two microalgae. In the case of *C. vulgaris*, 90% of the total methane yield was achieved after 10 days of digestion, while *Scenedesmus* sp. required 17 days. Moreover, raw *Scenedesmus* sp. steadily produced methane throughout the digestion time. *Scenedesmus* sp. required longer retention times for an efficient conversion. This microalga has been studied before as a potential substrate for anaerobic digestion and the results of these investigations raised the need of an efficient pretreatment to increase its biodegradability (González-Fernández et al., 2012a; b). In the particular case of the *Scenedesmus* sp. employed herein, the lipids content (22%, Table 4.3.) could also contribute to the delayed methane production since, of the three macromolecules, lipids are the fraction that requires the longest retention time for bioconversion to methane (Pavlostathis and Giraldo-Gomez, 1991). As observed for *C. vulgaris*, the use of biocatalysts before anaerobic digestion provided similar methane production profiles.

*Scenedesmus* sp. hydrolyzed with pectinase enhanced methane production by 1.3-fold compared with the raw material. In this manner, this carbohydrase mediated the highest methane yield improvement, followed by the biomass pretreated with Celluclast and Viscozyme. These enzymatic cocktails gave an enhancement of 1.2–1.3-fold. Even though the carbohydrates solubilised during enzymatic hydrolysis were considerably lower than the values recorded for *C. vulgaris* (Figure 4.5.), the increase in methane yield was similar for both microalgae biomass. Once again, this fact suggested that carbohydrates were not directly responsible for the optimized anaerobic digestion of this substrate. In the case of the biomass hydrolyzed with protease, the methane yield reached 216 mL $CH_4$ g COD $in^{-1}$, which corresponded to 62% anaerobic biodegradability. At this point, it should be stressed that the biomass subjected to the biomethane production test was hydrolyzed with protease for 8 h (organic matter released was 31% compared with 15% registered after 3 h hydrolysis). The methane yield enhancement registered for *Scenedesmus* sp. treated with protease was 20 percentual points lower than that observed for *C. vulgaris*. Likewise, the organic matter solubilised by this biocatalyst was

also lower for *Scenedesmus* sp. (Figure 4.6.). However, in contrast to *C. vulgaris* methane productivities, it should be noted that methane was produced throughout the anaerobic assay. Therefore, it may be hypothesized that *Scenedesmus* sp. could provide similar methane yields to *C. vulgaris* when longer digestion times are allowed.

Biomass subjected to temperature incubation (50ºC), as a control of the temperature used during enzymatic hydrolysis, did not improve the biogas production compared to the raw biomass (Figure 4.6.). This result suggested that, even if low temperature solubilised a part of the organic matter and carbohydrates (Figures 4.4. and 4.5.), the degree of solubilisation was not sufficient to enhance methane production. Even though the solubilisation achieved in COD and carbohydrates terms was similar to that for biomass enzymatically pretreated, the chemical composition of the organic matter released could be different. The organic matter released during heat application did not contribute to methane yield enhancements (Figure 4.6.). Previous results in Section 4.1.1.3 have also shown a similar trend. With regard to other pretreatments evaluated to optimize microalgae anaerobic digestion, thermal hydrolysis evidenced a direct link between carbohydrates solubilisation and methane yield improvement (Mendez et al., 2014a). Nevertheless, this relation did not hold for other microalgae or other pretreatments (Mendez et al., 2014b). Taking into consideration data presented here, carbohydrates solubilisation did not rule methane production. Microalgae biomass hydrolysis conducted with different biocatalysts showed that the highest hydrolysis efficiency in COD terms resulted in highest biodegradability.

Overall, both microalgae showed highest methane production after protease hydrolysis. At this point, it should be stressed that this conclusion holds for the strains studied herein, while microalgae interspecies specificities towards enzymes may provide different results (Gerken et al., 2013). Likewise, it is also important to note that the observed behavior did not result exclusively from cell wall breaking down but also from hydrolysis of the internal cell components. Microalgae cell wall proteins have not been studied extensively, as is the case with carbohydrates. Cell walls of the studied genera contain 2–

16% protein (Blumreisinger et al., 1983). In fact, both studied microalgae belong to the same phylum, but carbohydrate cell wall composition is somewhat different (Takeda 1991; 1996).

The present study confirmed that, of the carbohydrate polymers, cellulose was probably not the polymer conferring cell wall rigidity, but uronic acids may play a significant role in carbohydrates cell hardness. Nevertheless, carbohydrases did not provide the best results. In the biogas production context, microalgae biomass disruption seems to be more effective with proteases. Even though methane yield of carbohydrates and proteins calculated theoretically is quiet close (Angelidaki and Sanders 2004), the hydrolysis rate determined experimentally was 0.78 and 0.65 $d^{-1}$ for starch (carbohydrate) and bovine serum albumin (protein), respectively (Elbeshbishy and Nakhlaa, 2012). The lower hydrolysis rate of proteins, together with the fact that the protease action provided the highest methane yield for both microalgae biomass led to the conclusion that cell proteins are directly linked to the low anaerobic biodegradability of this substrate. This conclusion was in line with the one reported by Miron et al., (2000) and Mottet et al., (2010) who also pointed out that carbohydrates are more easily hydrolyzed or decomposed than proteins in the case of activated and primary sludge.

### 4.2. Pretreatment optimization

Given the promising results attained with Viscozyme and Alcalase, this study was designed to optimize the enzymatic pretreatment by testing the effect of a hydrothermal pretreatment prior to the enzymatic hydrolysis and the combination of carbohydrases and proteases on biomass hydrolysis. Thermal application before enzymatic hydrolysis was studied to circumvent the potential impediments that the enzyme may find for an appropriate performance derived from the low accessibility of microalgae components at enzymatic attack. On the other hand, the combination of both cocktails was investigated since some studies have shown that proteases combined with carbohydrase can provide a synergistic effect, and thus enhancing considerably hydrolysis extent (Reichardt, 1993; Alvira et al., 2011). In order to check if these two enzymes provided additional benefits on hydrolysis when

compared to the hydrolysis conducted with the enzymes independently, their combination was studied as well. Since microalgae cell walls are typically composed of microfibrillar polysaccharides embedded in a matrix of proteoglycans, the sequential use of catalysts employed was firstly the application of Viscozyme, releasing glucans components and in a second stage, Alcalase was added.

A set of experiments was carried out on two different microalgae biomass: *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. These microalgae were chosen since *C. vulgaris* has a rich-carbohydrate cell wall (Pieper et al., 2012) while *C. reinhardtii* has a cell wall with high glycoprotein content (Adair and Snell, 1990) and poor in cellulose (Reichardt, 1993). Therefore, those two microalgae strains represent two different biomass models.

### 4.2.1. Microalgae biomass composition

The chemical characterization of both biomass is summarized in Table 4.4. Both microalgae biomass exhibited quite a similar macromolecular distribution. Protein fraction constitutes the major macromolecule of the dry biomass weight, accounting approximately for 64% in both biomass. The second predominant macromolecule was carbohydrates and accounted approximately 22% of the DW. The initial sCOD of *C. vulgaris* accounted for 14% of the total organic matter while *C. reinhardtii* exhibited 30% of the total COD in the soluble phase. *C. reinhardtii* also contained higher amounts of total and soluble carbohydrates and proteins (Table 4.4).

**Table 4.4.** *Chlorella vulgaris* and *Chlamydomonas reinhardtii* chemical characterization.

| Chemical parameters | Average (± standard deviation) | |
|---|---|---|
| | *C. vulgaris* | *C. reinhardtii* |
| TS (g $L^{-1}$) | 15.8±0.3 | 15.2±0.3 |
| VS (g $L^{-1}$) | 14.5±0.6 | 13.3±0.1 |
| Total COD (g $L^{-1}$) | 22.3±1.9 | 20.5±0.4 |
| Soluble COD (g $L^{-1}$) | 3.1±0.2 | 6.2±0.3 |
| Total carbohydrates (% mg g $DW^{-1}$) | 18.6±0.0 | 22.6±2.2 |
| Soluble carbohydrates (% mg g $DW^{-1}$) | 6.0±0.6 | 9.7±0.1 |
| Total proteins (% mg g $DW^{-1}$) | 63.4±2.3 | 64.8±0.8 |
| Soluble proteins (% mg g $DW^{-1}$) | 19.5±4.6 | 30.4±4.8 |
| Lipids and others (% mg g $DW^{-1}$) | 18.0 | 12.6 |

**4.2.2. Effect of hydrothermal pretreatment before enzymes addition**

Figure 4.7 shows time course of organic matter solubilisation in experiments with hydrothermal incubation (75°C for 30 min) previous to enzymatic attack of *C. vulgaris* and *C. reinhardtii* biomass. After thermal pretreatment, enzymatic hydrolysis was performed with Viscozyme and Alcalase. As it can be seen in Figure 4.7, negligible changes were recorded in all tests and no synergistic effects of hydrothermal pretreatment before enzymatic hydrolysis were observed. Therefore, it may be concluded that enzymes did not have limitations to access cell wall components to be hydrolyzed.

### 4.2.3. Combined effect of carbohydrases and proteases on microalgae biomass hydrolysis

The effect of carbohydrases (Viscozyme), proteases (Alcalase) and combinations of both enzymes on sCOD is shown in Figure 4.7. In all experiments maximum hydrolysis was reached after 2 h and remained constant onwards (Figure 4.7). These results were in agreement with literature dealing with this topic. These studies showed a constant degree of hydrolysis after 2 h when using Alcalase on *Scenedesmus almeriensis* (Romero-Garcia et al., 2012) and Viscozyme on *Dunaliella tertiolecta* (Lee et al., 2013). For both biomass, the highest impact on COD solubilisation was observed for the hydrolysis carried out with protease (Alcalase). Hydrolysis carried out with Alcalase increased 4-fold sCOD in experiments with *C. vulgaris* and 2.4-fold in *C. reinhardtii*. The same trend was observed with carbohydrase (Viscozyme), although its effect on sCOD was lower than Alcalase. The sCOD doubled its value after 2 h of hydrolysis in experiments with *C. vulgaris* while the addition of Viscozyme resulted in an increase of 1.6-fold in *C. reinhardtii*. This fact might be explained by the higher amount of proteins compared to carbohydratespresent in both microalgae strains. The lower enzymatic efficiencies obtained in experiments with *C. reinhardtii* were ascribed to the fact that this microalgae biomass presents higher sCOD in the raw material. *C. reinhardtii* has been reported to present the ability to secrete large amount of exopolysaccharides (Bafana, 2013) and thus, the high sCOD registered in the raw biomass may be due to the presence of these exopolymers.

When the combination of both enzymes was applied on *C. vulgaris*, after Viscozyme addition, sCOD increased by 2.2-fold after 2 h of hydrolysis. After this time, the degree of solubilisation remained constant until Alcalase was added. After 5 h of hydrolysis, the combination of Viscozyme + Alcalase mediated slightly higher hydrolysis efficiency (67.5%) than Alcalase alone (57.4%). Similar to the sCOD attained for *C. vulgaris*, the combination of both enzymes supported slightly higher hydrolysis efficiencies on *C. reinhardtii*. In this case, maximum COD released accounted for 86% of the total COD.

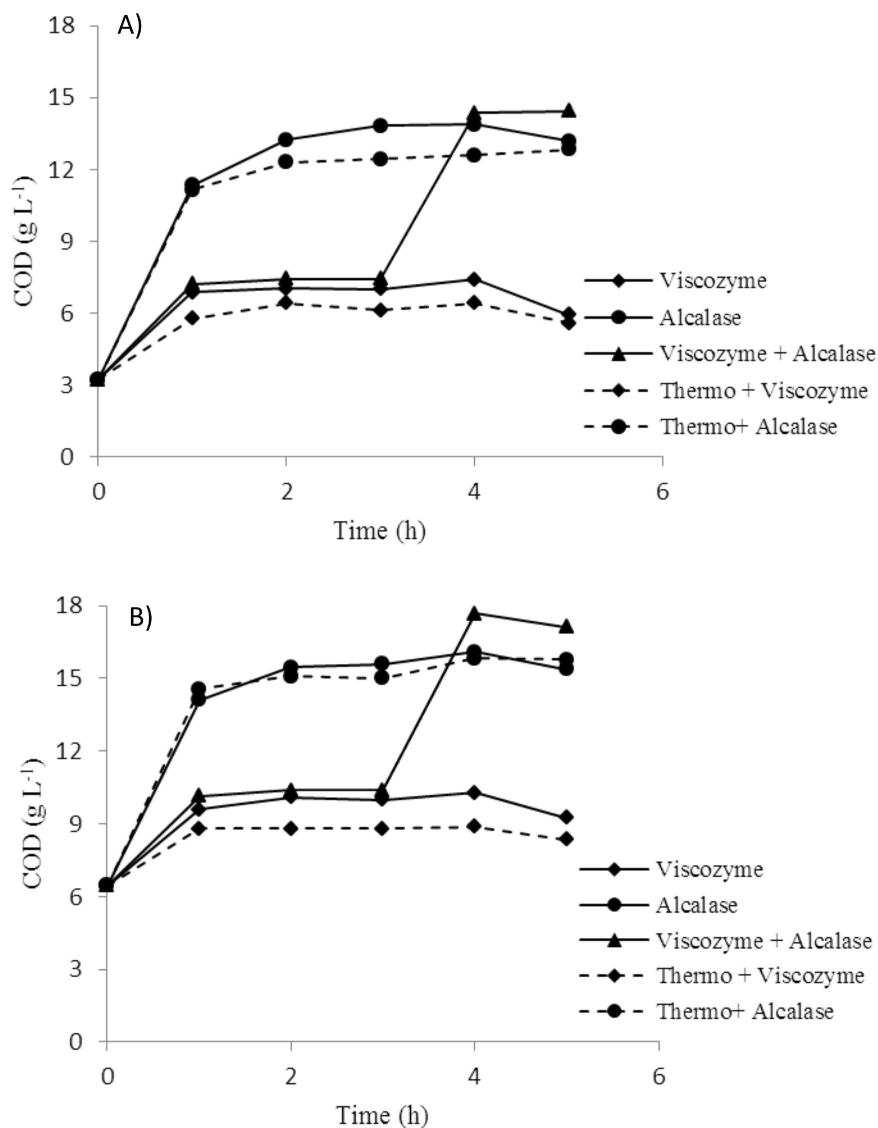



**Figure 4.7.** Time course organic matter solubilisation concentration upon the different enzymatic trials applied to (A) *C. vulgaris* and (B) *C. reinhardtii*. Discontinuous lines refer to the trials in which thermal pretreatment was applied before enzymatic hydrolysis.

### 4.2.4. Carbohydrate and protein solubilisation

As carbohydrates and proteins represent 86% of the total microalgae DW, the increase in soluble carbohydrate and soluble protein were monitored along hydrolysis reaction time (5 h) to evaluate the solubilisation kinetics of the enzymatic hydrolysis.

The carbohydrate content in the soluble phase obtained during enzymatic hydrolysis is shown in Figure 4.8. Soluble carbohydrate of the raw *C. vulgaris* and *C. reinhardtii* accounted for 32% and 42% of the total carbohydrate, respectively (Table 4.4.). The highest soluble carbohydrates hydrolysis (160 and 220 mg g $DW^{-1}$ for *C. vulgaris* and *C. reinhardtii,* respectively) was obtained with the carbohydrase (Viscozyme) after 2 h (Figure 4.8.) and no increments were observed in soluble carbohydrates measured after 2 h reaction. This enzymatic mixture degrades chemical bonds of cell walls and membranes resulting in carbohydrates solubilisation. The addition of Viscozyme produced 86% and 96% solubilisation of the total carbohydrates for *C. vulgaris* and *C. reinhardtii*, respectively. Protease addition increased soluble carbohydrates from 60 to 100 and from 80 to 120 mg g $DW^{-1}$ in the experiments with *C. vulgaris* and *C. reinhardtii* biomass, respectively. This slight increase was attributed to the concomitant release of easily hydrolysable carbohydrates solubilised together with proteins (glycoproteins). Carbohydrate solubilisation registered in the present study was much higher than the reported with other pretreatments employed to enhance methane production of this biomass. While thermal treatment at 120°C for 30 min resulted in 35.2% carbohydrate solubilisation (Mendez et al., 2013), the increase of temperature up to 180°C for 10 min reached 69% (Mendez et al., 2014a).

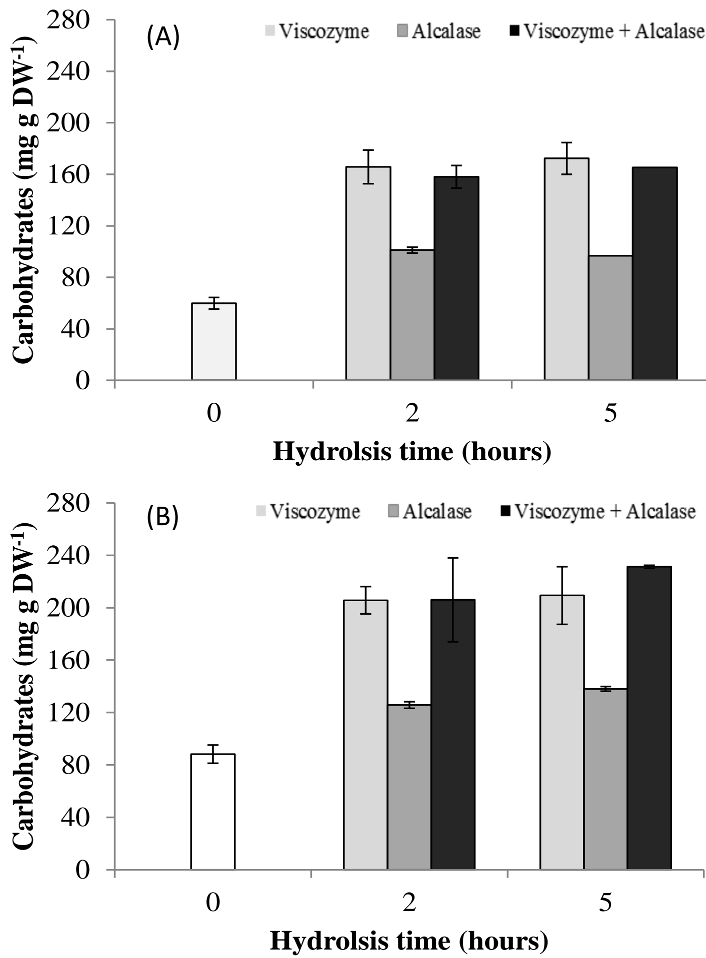



**Figure 4.8.** Time course carbohydrates solubilisation upon the different enzymatic trials applied to (A) *C. vulgaris* and (B) *C. reinhardtii*.

Regarding protein solubilisation, the values attained during enzymatic hydrolysis are shown in Figure 4.9. In all experiments after 2 h reaction time, proteins solubilisation was constant. These results are in accordance with data provided by Romero-Garcia et al., (2012) who pretreated *S. almeriensis* with Alcalase reaching maximum protein content in the soluble fraction after 2 h. Alcalase addition to *C. vulgaris* biomass supported an increase of more than 3-fold proteins solubilisation after 2 h of hydrolysis (Figure 4.9.A). On the other hand, Viscozyme addition provided only 1.9-fold protein solubilisation compared to the raw biomass after 2 and 5 h hydrolysis. In the case of *C. reinhardtii*, soluble protein enhancement was almost doubled with the hydrolysis conducted with the protease and reached similar hydrolysis efficiency as recorded for *C. vulgaris*.

Overall, both enzymes (carbohydrase and protease) showed high hydrolysis efficiencies, rendering almost all the particulate carbohydrates and proteins available in the soluble phase. This fact is important since references in the literature have reported prevailing carbohydrates solubilisation over proteins (Mendez et al., 2013; 2014a). Usually, proteins are converted to other complex molecules during those harsh pretreatments and hence underestimated.

There was no apparent synergistic effect between carbohydrases and proteases since the sequential addition of Viscozyme and Alcalase did not increase solubilisation of both carbohydrates and proteins. In fact, the carbohydrase addition previous to protease treatment delayed protein solubilisation of both microalgae.

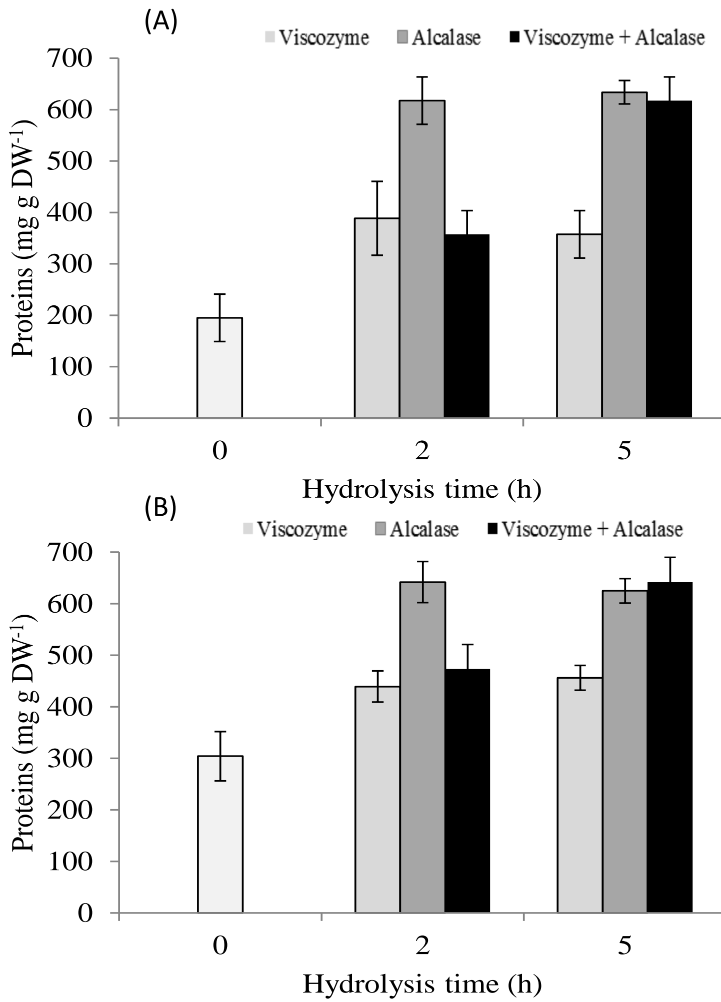



**Figure 4.9.** Time course of proteins solubilisation upon the different enzymatic trials applied to (A) *C. vulgaris* and (B) *C. reinhardtii*.

### 4.2.5. Methane production potential of enzymatically pretreated and raw microalgae biomass

#### 4.2.5.1. Effect of carbohydrases on methane production from *C. vulgaris* and *C. reinhardtii*

Regarding methane content of biogas, no differences were observed among pretreated biomass and raw material. The percentage of methane in the biogas ranged 72±0.7%. These values were in the range of previous studies dealing with anaerobic digestion of microalgae (Mendez et al., 2013; 2014). Methane production for the raw *C. vulgaris* biomass reached 190.6±2.3 mL $CH_4$ g COD $in^{-1}$ (Figure 4.10.A). This value corresponded to 54% anaerobic biodegradability which was relatively high compared with other studies (Mendez et al., 2013; Ras et al., 2011).

All enzymatic hydrolysis enhanced, at different extent, methane production while thermal pretreatment (70°C for 30 min) did not have any effect. In experiments with *C. vulgaris* pretreated with carbohydrasas (Viscozyme), in spite of the high carbohydrates solubilisation observed (86%), the methane production enhancement was low (14%) (Figure 4.8.A). The use of thermal application previous to enzymatic hydrolysis provided similar methane yields. These data, together with the carbohydrates release during carbohydrase treatment suggested that cell wall carbohydrates were hampering the methane production of this biomass to a minor extent. Composition of *C. vulgaris* cell wall is still not clear. It has been traditionally recognized that this microalgae possesses a carbohydrates-based cell wall, mainly composed of cellulose and hemicellulose (Cheng et al., 2013; Takeda 1991; 1996). However, recent studies have found that cellulases and amylases were not effective in hydrolyzing of *C. vulgaris* cell wall (Kim et al., 2014). To verify which enzyme would be more efficient for cell wall degradation, these authors tested pectinase, cellulase, amylase, xylanase, β-glucosidase, chitinase, lysozyme, and sulfatase. Their results confirmed that only pectinase had a

significant effect on the degradation of polysaccharides from the cell wall of *C. vulgaris*. Similarly, another recent publication, reached the same conclusion (Gerken et al., 2013). It seems likely that *C. vulgaris* does not have a cellulose rigid cell wall but rather uronic acids and aminosugars are conferring this microalgae its hardness. Gerken et al., (2013) concluded that *Chlorella* is typically most sensitive to chitinases and lysozymes (enzymes that degrade polymers containing N-acetylglucosamine). In the present study, the commercial enzyme mixture employed (Viscozyme) was composed by arabanase, cellulose, β-glucanase, hemicellulase and xylanase.

With regard to the anaerobic assays of *C. reinhardtii,* methane content of the produced biogas was in the same range as *C. vulgaris*. Raw C*. reinhardtii* yielded 263.1±0.1 mL $CH_4$ g COD $in^{-1}$, which corresponded to 75% anaerobic biodegradability. This value was in agreement with Mussgnug et al., (2010) and higher than the value reported by Passos et al., (2013). This later study employed a mixture of microalgae mainly composed by *Chlamydomonas* and *Nitzchia*. Diatoms, as *Nitzchia*, have a silica cell wall that confers them quiet a resistance and, therefore, methane production achieved might be lower. While in *C. vulgaris*, Viscozyme addition previously to anaerobic digestion provided a slight increase in methane production, the enhancement was negligible in *C. reinhardtii* biomass. The biomass hydrolyzed with Viscozyme and thermal application prior to Viscozyme addition resulted in 255.7±9.4 mL $CH_4$ g COD $in^{-1}$ and 279.4±1.1 mL $CH_4$ g COD $in^{-1}$, respectively (Figure 4.10.B). In this manner, even though Viscozyme resulted in organic matter solubilisation (Figure 4.7.A), and more specifically carbohydrates solubilisation (Figure 4.8.B), it may be concluded that the release of carbohydrates did not enhance methane production because these compounds were already easily bioavailable in the raw biomass. The reason for the negligible methane enhancement when using Viscozyme is the cell wall composition of *C. reinhardtii*. As a matter of fact, this green microalga lacks of cellulose (Adair and Snell, 1990) while its structural support is made of glycoproteins and proteoglycans (Reichardt, 1993).

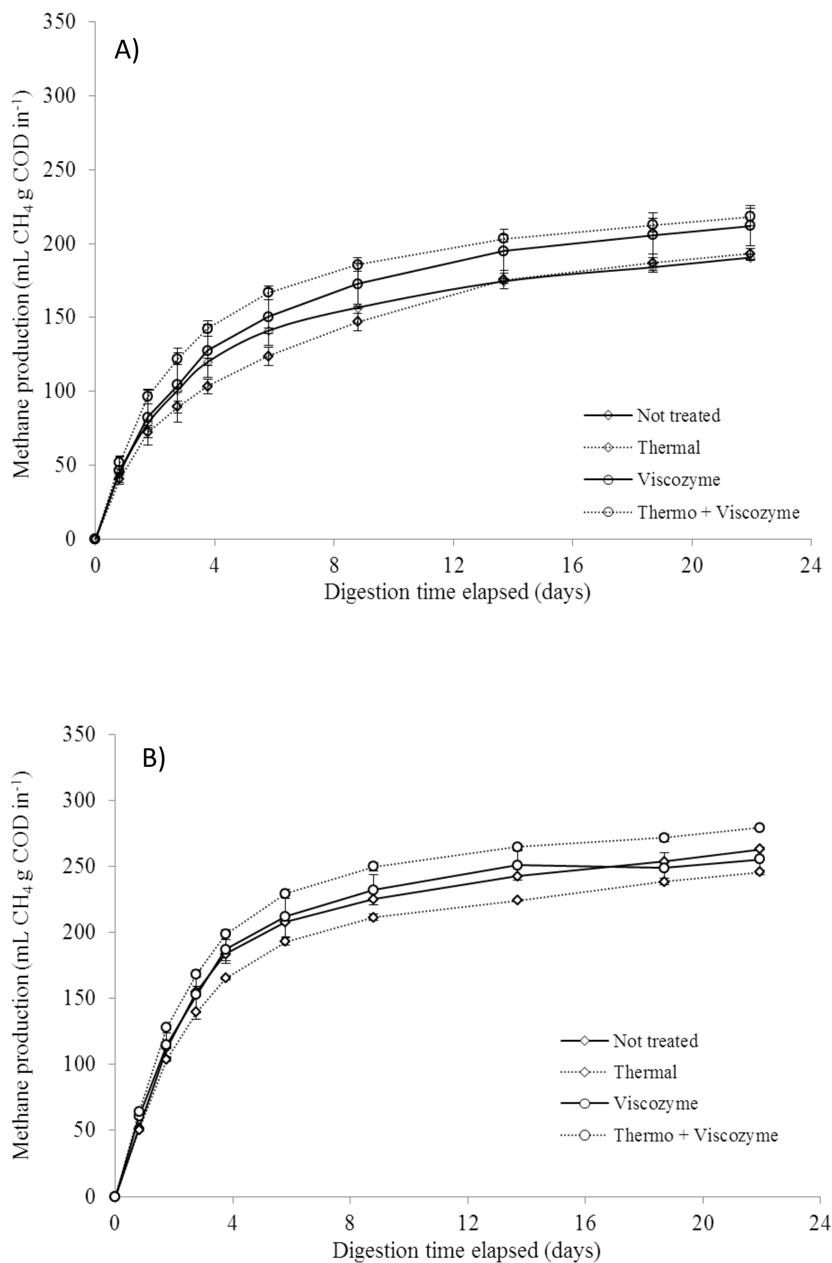



**Figure 4.10.** Cumulative methane yield achieved by carbohydrase pretreatment applied to (A) *C. vulgaris* and (B) *C. reinhardtii*. Discontinuous lines refer to the trials in which thermal pretreatment was applied before enzymatic hydrolysis.

### 4.2.5.2. Effect of proteases on methane production from *C. vulgaris* and *C. reinhardtii*

Protease hydrolysis supported much higher methane production in both microalgae biomass when compared to raw biomass and carbohydrases addition (Figure 4.11.). Compared to raw biomass, the addition of Alcalase to *C. vulgaris* biomass enhanced 51% methane production (287.0±1.3 mL $CH_4$ g COD in$^{-1}$, Figure 4.11.A). The combination of Viscozyme and Alcalase exhibited similar results, while the thermal pretreatment previous the addition of the enzyme decreased methane production to 270.8±5.7 mL $CH_4$ g COD in$^{-1}$. This value was higher to those obtained in other studies pretreating this particular biomass for methane production. In this context, thermo-acid pretreatment (120°C with 2 N $H_2SO_4$) yielded 65% anaerobic biodegradability, while thermal treatment at 160°C reached 74% (Mendez et al., 2013; 2014). In the present study, the value attained after protease hydrolysis was ten percentual points higher (85%). This enhanced methane production clearly shows the benefit of using this protease previously to anaerobic digestion. The proteins in the soluble phase, as carbohydrates, were quite high and then bioavailable for anaerobic microorganisms. More specifically, some other studies that monitored macromolecular distribution upon pretreatments did not report enhanced protein solubilisation since the harsh thermochemical conditions employed presumably converted proteins solubilised in other polymers due to Maillard side-reactions (Mendez et al., 2013). The high selectivity and mild conditions (T and pH) employed during this enzymatic hydrolysis resulted in very promising results. Literature is really scarce on this topic. The most comparable result, given by Ometto et al., (2014) also showed COD hydrolysis ranging 35–45% when using an enzymatic cocktail composed of esterase and protease to treat *Chlorella sorokiniana*. This hydrolysis efficiency provided an enhancement in methane yield by (3-fold) compared to that attaine with raw biomass (273 mL $CH_4$ g VS in$^{-1}$). Likewise, Miao et al., (2013)

reported 37% methane yield improvement on blue algae when this substrate was stored for 15 days. This enhancement was attributed to the fact that protease activity increased during the natural storage of biomass. This period was used as a pre-fermentation step.

In the experiments with *C. reinhardtii,* the highest methane production was reached after proteases addition. The addition of Alcalase and its combination with Viscozyme improved methane production from 289±2.5 to 311.6±2.1 mL $CH_4$ g COD $in^{-1}$, respectively (Figure 4.11.B). These results confirmed the effectiveness of proteases on the cellulose-deficient cell wall of *C. reinhardtii*. Overall, the biodegradability of these pretreated biomass achieved 87%, and thus it increased the anaerobic biodegradability by 10 percentual points. Regarding other pretreatments employed prior to anaerobic digestion of this biomass, thermal application (140–170°C for 15 min) resulted in 21–27% biodegradability enhancement while ultrasound application (10,000–40,000 kJ kg $TS^{-1}$) only provided 5–9% biodegradability increase (Alzate et al., 2012). It can be concluded thus that enzymatic hydrolysis carried out with protease supported the highest anaerobic biodegradability of *C. reinhardtii* biomass (87%). Despite the enhancement on methane production obtained with the protease treatment, as for any other pretreatment, the extra cost of adding the enzyme should be economically justified.

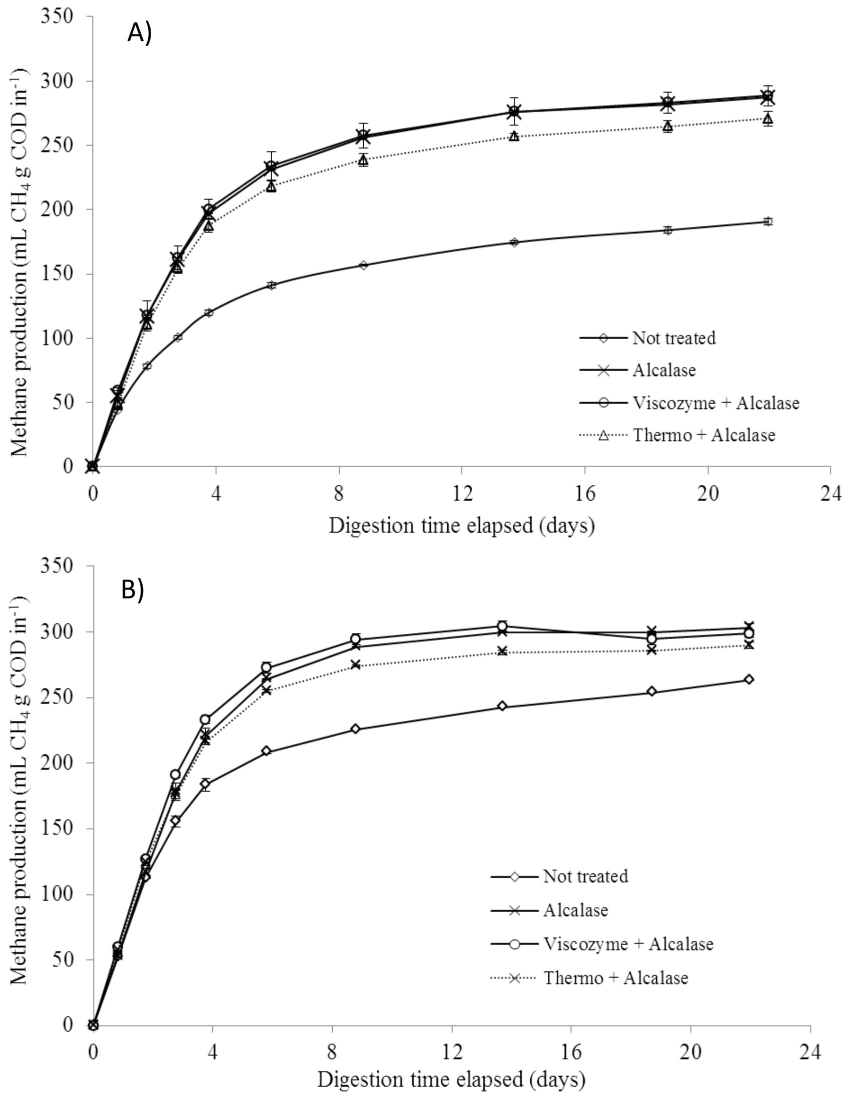



**Figure 4.11.** Cumulative methane yield achieved by protease addition and its combination with the carbohydrase applied to (A) *C. vulgaris* and (B) *C. reinhardtii*. Discontinuous lines refer to the trials in which thermal pretreatment was applied before enzymatic hydrolysis.

### 4.2.6. Protease addition optimization: enzymatic dosages and microalgae biomass loads

During the development of this thesis, the results have demonstrated that proteases (Alcalase) are very efficient agents for organic matter solubilisation and methane production enhancement. Protease addition before anaerobic digestion produced a significant increase in methane yield in all experiments with three microalgae strains (*C. vulgaris, Scenedesmus* sp., and *C. reinhardtii*). Those strains exhibited different cell wall composition and macromolecular profiles. Although low-cost commercial enzymes are available, the enzyme cost is a burden in microalgae pretreatment and hence, the enzyme dosage needs to be optimized. The present experiment focused on the optimization of protease treatment at different microalgae biomass loads to hydrolyze *C. vulgaris* biomass and solubilised their organic matter with the ultimate goal of enhancing methane yield.

#### 4.2.6.1. *C. vulgaris* biomass composition

The characterization of raw *C. vulgaris* biomass is shown in Table (4.5.). Organic matter accounted for 91% of dry biomass and hence, less than 10% of dry biomass was inorganic material (ash). This value is quite similar to *C. vulgaris* biomass characterized in previous section (Section 4.2.1). Protein fraction constituted the major macromolecule of the dry biomass weight, accounting approximately for 64%, followed by the carbohydrate fraction (19%).

**Table 4.5.** Chemical characterization of *Chlorella vulgaris* used in this experiment.

| Chemical parameters | Average (± standard deviation) |
|---|---|
| TS (g $L^{-1}$) | 16.7±0.1 |
| VS (g $L^{-1}$) | 10.4±2.1 |
| tCOD (g $L^{-1}$) | 23.2±0.4 |
| sCOD (g $L^{-1}$) | 0.78±0.3 |
| Protein (% mg g $DW^{-1}$) | 63.8±2.1 |
| Carbohydrate (% mg g $DW^{-1}$) | 18.7±2.7 |
| Lipids and others (% mg g $DW^{-1}$) | 7.9±1.3 |
| Ash (% mg g $DW^{-1}$) | 9.2±2.7 |

#### 4.2.6.2. Effect of enzymatic dosage on *C. vulgaris* organic matter solubilisation

Three protease dosages (0.146, 0.293 and 0.585 AU g $DW^{-1}$) were used in order to evaluate the effect of enzymatic loads on organic matter solubilisation. Figure 4.12. shows the sCOD released during the enzymatic hydrolysis pretreatment. In this study, only the solubilisation of total organic matter (sCOD) was explored since an in-depth study of macromolecules solubilisation may be found in Section 4.2.4. The initial sCOD of *C. vulgaris* accounted for approximately 3.4% of the total organic matter (Table 4.5. and 4.6.). Hydrolysis efficiency was enhanced at increasing protease dosage. The same pattern in terms of organic matter solubilisation was achieved in all trials. Most of the organic solubilisation took place within the first hour of hydrolysis. Increasing the pretreatment time to 3 h led to a hydrolysis yield increase of 5-8% of the initial tCOD. Highest hydrolysis sCOD yield (49.3%) was observed for *C. vulgaris* pretreated at the highest enzyme dosage. Results are in agreement with those obtained in Section 4.2.3, where slightly higher hydrolysis efficiency (52.2%) was observed using the same biocatalyst on *C. vulgaris* for 5 h.

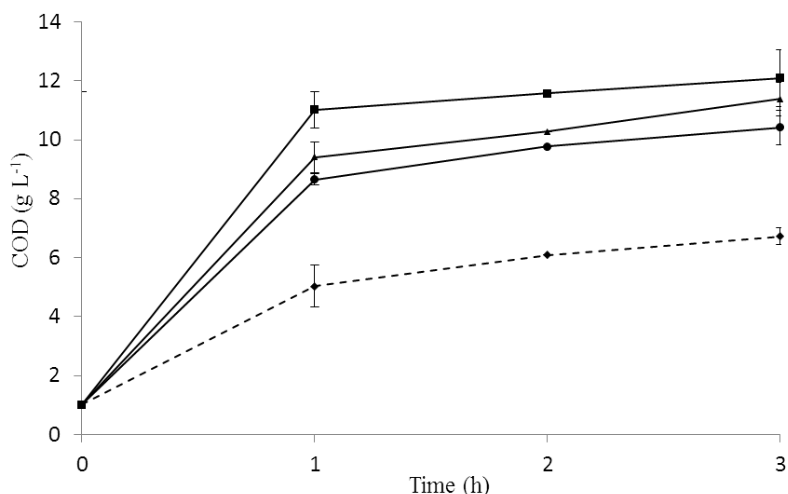



**Figure 4.12.** Time course organic matter solubilisation concentration upon the different enzymatic dosages (continuous lines where the circle symbol stands for 0.146 AU g $DW^{-1}$, triangle for 0.293 AU g $DW^{-1}$ and square for 0.585 AU g $DW^{-1}$) and control of incubation temperature without catalyst (discontinuous line) applied to *C. vulgaris*.

Slightly lower values were attained for the lower enzymes dosages studied. The hydrolysis efficiency was 45.2% and 41.2% for the enzymes dosages at 0.293 and 0.146 AU g $DW^{-1}$, respectively (Table 4.6.). Since proteins accounted for 63.8 % DW, the highest proteases dosage (0.585 AU g $DW^{-1}$) was shown to be more beneficial from an organic matter solubilisation point of view. Nevertheless, the same conclusion cannot be withdrawn for the overall process in which the final goal is methane production. Since the cost of the enzyme play a major role in determining the best pretreatment for methane production, and given that it has been demonstrated that there is not a direct link between organic matter solubilisation and methane production enhancement (Mendez et al., 2013), biomethane assays are required to evaluate if the potential increase in methane production would justify the use of high enzymes dosages.

To determine the effect of temperature incubation (50°C) on organic matter solubilisation, a control (without proteases) was set. Similarly to what it was observed for the enzymatically pretreated biomass, the main organic matter solubilisation (18.9%) took place during the first hour of thermal incubation (Figure 4.12.). After 3 h of thermal incubation, sCOD hydrolysis yield reached 24.8% (Table 4.6.). This result was markedly lower than that registered for enzymatic trials. Additionally, it should be stressed that the chemical composition of the organic matter released upon both treatments (thermal and enzymatic) may be different. Indeed, even under the same pretreatment methodology, the chemical composition of the organic matter released can change. González-Fernández et al., (2012c) observed similar COD solubilisation after pretreating *Scenedesmus obliquus* at 70 and 90°C for 3 h; however, the final anaerobic biodegradabilities of those pretreated biomass were quite different.

Hydrolysis yield (24.8%,6-fold increase) obtained in control experiment (50ºC incubation temperature without catalysts) is quite close to that obtained in Section 4.1.1.1 (16% of solubilisation for *Chlorella* sp. pretreated at 50ºC for 24 h). Passos et al., (2013) obtained 5-fold increase in soluble organic matter of *Chlamydomonas* sp. subjected to 55°C for 4 h. Therefore, the results presented herein showed that low temperatures affected slightly to the microalgae cells organic matter release. Even though the optimum temperature of pretreatment is strain specific, several authors have demonstrated that higher temperatures (from 75 to 120°C) are more effective for biomass disruption and subsequent methane production (Mendez et al., 2014, Passos et al., 2013 and González-Fernández et al., 2012c).

**Table 4.6**. Hydrolysis efficiencies of raw *Chlorella vulgaris* biomass and enzymatically pretreated biomass at different protease dosages and biomass loads.

| | | Hydrolysis efficiency (%) |
|---|---|---|
| Not treated | | 3.4 |
| Control temperature | | 24.8 |
| Enzyme dosage (AU g $DW^{-1}$) at 16 g $L^{-1}$ | 0.146 | 41.2 |
| | 0.293 | 45.2 |
| | 0.585 | 49.3 |
| Biomass loads (g $L^{-1}$) with enzyme dosage of 0.585 AU g $DW^{-1}$ | 16 | 51.4 |
| | 32 | 50 |
| | 65 | 51.9 |

#### 4.2.6.3. Effect of biomass concentration on enzymatic pretreatment of *C. vulgaris*

After determining the optimum enzymatic dosage in terms of hydrolysis efficiency, initial biomass concentration was studied for further optimization of the enzymatic pretreatment. Many factors can affect microalgae enzymatic hydrolysis, namely the substrate composition, substrate loading and final product tolerance (Romero-Garcia et al., 2012). Optimum biomass loading that can be treated without diminishing the hydrolysis efficiency is a key parameter that can greatly affect the economic feasibility of the process.

In this study, three biomass loads (16, 32 and 65 g $L^{-1}$) were tested at the optimum protease dosage (0.585 AU g $DW^{-1}$) determined previously (Figure 4.13.). Regardless the biomass load tested, the hydrolysis efficiency ranged 49.5±1.1% (Table 4.6.). Thus, it can be concluded that the increased viscosity of the higher biomass loads did not hinder hydrolytic efficiency at the enzymatic dosage tested. These results differ from those obtained by Romero-Garcia et al., (2012). These authors observed a concomitant decrease of hydrolysis yield at increasing biomass loads. More specifically, the hydrolysis efficiency dropped from 55% to 20% when hydrolyzing 200 g $L^{-1}$ and 350 g $L^{-1}$ of *Scenedesmus almeriensis*, respectively. Authors attributed this behavior to a limited mass transfer due to the increased viscosity of the solution. In the present study, the biomass loads tested were significantly lower than the ones studied by Romero-Garcia et al., (2012). In this manner, it can be concluded that 65 g $L^{-1}$ of *C. vulgaris* biomass is bioavailable to the enzymes and no reduction in hydrolysis efficiency was detected.

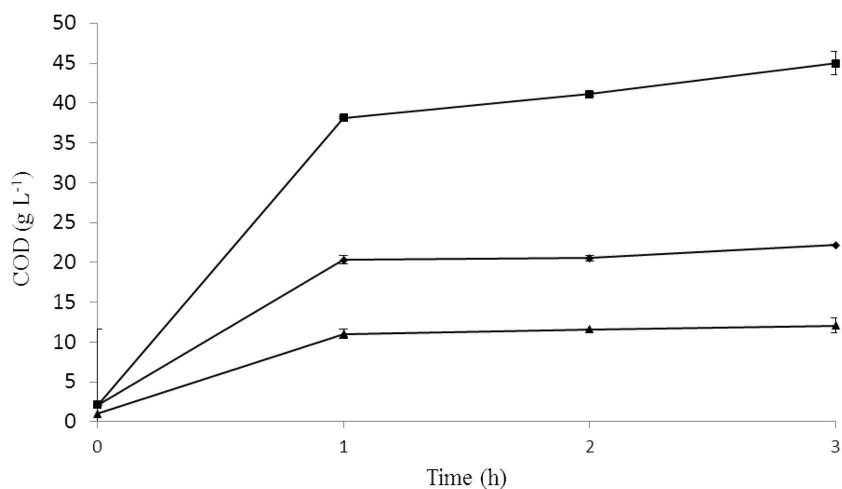



**Figure 4.13.** Time course organic matter solubilisation concentration during enzymatic hydrolysis of *C. vulgaris* at different biomass loads. The circle symbols correspond to 16 g $L^{-1}$, the triangles 32 g $L^{-1}$ and the squares 65 g $L^{-1}$.

#### 4.2.6.4. Methane production potential of pretreated and raw *C. vulgaris*

The digestibility of microalgae biomass mainly depends on an efficient cell wall disruption/hydrolysis method. Therefore, the selection of an efficient and cost-effective pretreatment to increase methane production rate and methane final yield is crucial. Figure 4.14. shows the cumulative $CH_4$ yield as a function of time obtained from the anaerobic digestion of *C. vulgaris* biomass treated with different protease dosages. Data from anaerobic digestion experiments with raw biomass and incubation temperature control are also included. Methane yield of fresh *C. vulgaris* ranged 147–160 mL $CH_4$ g COD in$^{-1}$, which corresponded to an anaerobic biodegradability of 42–45%. This value is in good agreement with some other studies dealing with anaerobically digested *C. vulgaris* (Ras et al., 2011; Mendez et al., 2013). For the enzymatically pretreated substrates, regardless the protease dosage applied, a rapid initial methane production was observed during the first days. From the observed results, it can be concluded that initial stage of anaerobic digestion, hydrolysis, was favored by the enzymatic pretreatment.

Similarly, the temperature incubation control sample also provided an enhanced methane production during the first days of the digestion. Nevertheless, the methane production profile attained with samples with or without biocatalyst was different, indicating different organic matter released in both processes in terms of chemical composition. The organic matter released during the thermal incubation (25%) resulted in higher methane productivity during the first days of the digestion. However, after 5 days, the methane production yield was pretty close to that observed for the raw material. As matter of fact, the control used to estimate the effect of the thermal incubation provided 14% methane yield enhancement. This value is in good agreement with the 10% methane production increase observed on *C. vulgaris* after thermal application of 50°C for 24 h (Section 4.1.1.3.1) and the 13% reported by Passos et al., (2013) pretreating *Chlamydomonas* sp. at 55°C. In this manner, it can be seen that despite the organic matter solubilisation registered, the methane production yield was not markedly increased.

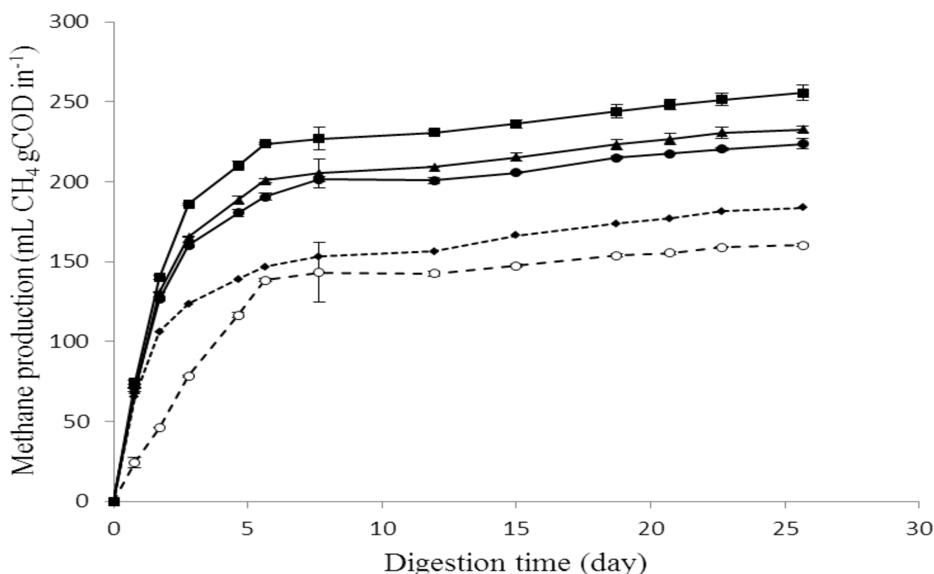



**Figure 4.14.** Cumulative methane yield achieved by the not treated *C. vulgaris* biomass (discontinuous line and open circle), incubation temperature control (discontinuous lines and closed circles) and protease pretreated biomass at different enzymatic dosages (continuous lines where the circle symbol stands for 0.146 AU g $DW^{-1}$, triangle for 0.293 AU g $DW^{-1}$ and square for 0.585 AU g $DW^{-1}$).

In comparison to the experiment without biocatalyst, enzymatically pretreated biomass exhibited much higher methane yields enhancements (Figure 4.14.). The different enzymatic dosages resulted in slightly different methane production yields. The highest protease dosage tested (0.585 AU g $DW^{-1}$) provided 256 mL $CH_4$ g COD $in^{-1}$ (Figure 4.14.), which corresponded to 73% anaerobic biodegradability (Table 4.7.). In comparison to the untreated biomass, methane production yield was enhanced by 60%. A similar result was reported in Section 4.1.2.3. Enzyme dosages of 0.293 and 0.146 AU g $DW^{-1}$ resulted in methane production of 232.5 and 224 mL $CH_4$ g COD $in^{-1}$, respectively (Figure 4.14.). Taking into consideration these data, it can be concluded that the enhancement of methane production was directly correlated with hydrolysis efficiency.

**Table 4.7.** Overview of different measured parameters obtained at the end of the anaerobic digestion of raw *Chlorella vulgaris* biomass and enzymatically pretreated biomass at different protease dosages.

| | Not treated | Control temperature | Enzyme dosage (AU g $DW^{-1}$) at 16 g $L^{-1}$ | | |
|---|---|---|---|---|---|
| | | | 0.146 | 0.293 | 0.585 |
| pH final | 7.3 | 7.4 | 7.3 | 7.3 | 7.4 |
| Methane production (mL $CH_4$ g COD $in^{-1}$) | 160.4±0.9 | 196.6±1.8 | 223.8±3.1 | 232.5±1.9 | 255.6±4.9 |
| Anaerobic biodegradability (%) | 45.8 | 52.5 | 63.9 | 66.5 | 73.0 |
| Methane content (%) | 69.8 | 69.9 | 69.9 | 70.0 | 70.3 |

Figure 4.15. shows cumulative methane yield production of raw and protease pretreated *C. vulgaris* at different biomass loads and fixed enzyme dosage (0.585 AU g $DW^{-1}$). Regarding the effect of the enzymatic hydrolysis on methane production at increasing biomass loads, the results displayed no differences. As observed for the COD hydrolysis efficiencies obtained for the different biomass concentrations studied, the enzymatically treated biomass provided no significant changes on methane production (Table 4.8.). These results confirmed that increasing amounts of microalgae biomass pretreated with proteases did not directly affect the activity of anaerobic microorganism in the digestion process. Concerning methane productivity, 90% of the potential methane production was achieved within the first 6 days of digestion for the enzymatically pretreated biomass. No differences in methane content of the biogas were observed among samples at different enzymatic dosages or biomass loads (Table 4.7. and 4.8.).

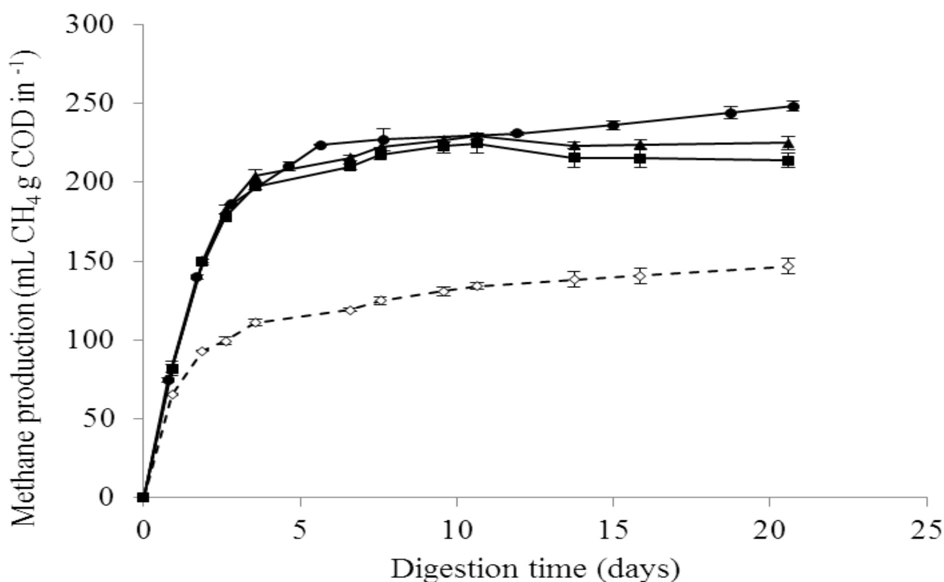



**Figure 4.15.** Cumulative methane yield achieved by raw *C. vulgaris* biomass (discontinuous line) and protease pretreated biomass at different biomass loads (16 g $L^{-1}$ represented by circles, 32 g $L^{-1}$ by triangles and 65 g $L^{-1}$ by square symbols and continuous lines).

**Table 4.8.** Overview of different measured parameters obtained at the end of the anaerobic digestion of raw *Chlorella vulgaris* biomass and enzymatically pretreated biomass at different biomass dosages.

| | Not treated | Biomass loads (g $L^{-1}$) with enzyme dosage of 0.585 AU g $DW^{-1}$ | | |
|---|---|---|---|---|
| | | 16 | 32 | 65 |
| pH final | 7.3 | 7.4 | 7.3 | 7.2 |
| Methane production (mL $CH_4$ g COD $in^{-1}$) | 147.4±4.9 | 255.6±4.9 | 213.7±4.6 | 224.9±4.4 |
| Anaerobic biodegradability (%) | 41.9 | 73.0 | 64.2 | 61.1 |
| Methane content (%) | 69.8 | 70.3 | 69.0 | 68.8 |

### 4.2.6.5. Nitrogen mineralization and ammonia inhibition

Proteases are enzymes that break down peptide bonds and releases amino acids. Considering that the main component of *C. vulgaris* is protein and proteases are the biocatalysts used in the pretreatment step, the nitrogen fate in the anaerobic digestion cannot be neglected. An efficient anaerobic digestion of a highly rich protein substrate, such is the case of microalgae, may result in high ammonium concentration released to the medium from the biological breakdown of nitrogenous matter in hydrolysis and acidogenesis phase. In the conversion of microalgae biomass into biogas, a high content of proteins degradation to ammonium would be expected. Nitrogen mineralization (organic nitrogen converted to ammonium) was calculated taking into consideration biomass protein content, substrate loading and final ammonium concentration in the aqueous phase after anaerobic digestion (Table 4.9.). Nitrogen mineralization ranged 85–88% in experiments with raw and incubated at 50°C *C. vulgaris* biomass (Table 4.9.). These values are higher than the ones reported in literature. For instance, Ras et al., (2011) calculated 68% nitrogen mineralization for a continuously operated anaerobic reactor fed with raw *C. vulgaris*. Even lower nitrogen mineralization (40–43%) was obtained after anaerobic digestion of raw and thermally pretreated *S. obliquus* (González-Fernández et al., 2011; 2013). However, it should be highlighted that *S. obliquus* is considerably much recalcitrant than *C. vulgaris* towards anaerobic digestion and therefore, nitrogen mineralization would be lower as well.

**Table 4.9**. Ammonium concentration and pH degree obtained at the end of the anaerobic digestion of raw and enzymatically pretreated *Chlorella vulgaris* biomass at different protease dosages and biomass loads.

| | | Ammonium concentration (mg $L^{-1}$) | Final pH |
|---|---|---|---|
| Not treated | | 204±10 | 7.3 |
| Control temperature | | 198±7 | 7.4 |
| Enzyme dosage (AU g $DW^{-1}$) at 16 g $L^{-1}$ | 0.146 | 240±18 | 7.3 |
| | 0.293 | 249±38 | 7.3 |
| | 0.585 | 237±18 | 7.4 |
| Biomass loads (g $L^{-1}$) with enzyme dosage of 0.585 AU g $DW^{-1}$ | 16 | 237±18 | 7.4 |
| | 32 | 119±38 | 7.3 |
| | 65 | 74±11 | 7.2 |

In experiments with enzymatically pretreated biomass, total nitrogen mineralization (100%) was attained regardless the protease dosage (Table 4.9.). In this manner, opposite to organic matter degradation that was dependent on protease dosage, the organic nitrogen was easily converted to ammonium during digestion at all enzyme dosages tested. In the case of the different biomass loads pretreated at the same protease dosage, decreasing nitrogen mineralization values were determined at increasing biomass loads (Table 4.9.). More specifically, organic nitrogen converted to ammonium were 51.5% and 32.0% for biomass loads of 32 and 65 g $L^{-1}$, respectively. Opposite to what it was observed for organic matter conversion to biogas, the different biomass loads pretreated at 0.585 AU g $DW^{-1}$ provided different nitrogen mineralization. This would mean that neither the protease addition nor the anaerobic digestion would be sufficient for total conversion of organic nitrogen to ammonium.

As mentioned above, ammonium is one of the intermediate compounds in anaerobic digestion process derived from hydrolysis and formed during the degradation of nitrogenous organic materials such as proteins. In nitrogen-rich substrates, such as microalgae, anaerobic digestion may be affected by excessive ammonium nitrogen. Reduced nitrogen in anaerobic digesters mainly exists as ammonium ion ($NH_4^+$) in aqueous phase and free ammonia ($NH_3$) in both aqueous and gas phase. As pH tends to be alkaline, the equilibrium ammonium/ammonia becomes unbalance and the ammonia form prevails. Under this circumstance, ammonia toxicity may affect dramatically anaerobic microorganisms' performance. Ammonia concentration for the biomethane potential assays was calculated taking into consideration the ammonium concentration measured at the end of the digestion process, effluent pHs and the acidic dissociation constant of ammonium/ammonia. As expected by the pH measured at the end of the digestion (close to neutrality, Table 4.9.), the results showed that the ammonia concentration was negligible. More specifically, these values ranged 2–3% of the total ammonium measured in solution. In this manner, the ammonium/ammonia concentration was far from toxicity threshold (Section 2.4.4.3). At this point it should be stressed out that this calculation was made in batch assays and

this should be further confirmed by operating a continuously fed reactor in which ammonium concentration can build up.

### 4.3. Protease pretreated *Chlorella vulgaris* biomass bioconversion to methane via semi-continuous anaerobic digestion

Methane potential batch assays are used to determine the amount of organic carbon in a given material that can be anaerobically converted to methane. Data from batch assays can provide guidance, but assessing the benefits of a pretreatment in a continuously fed reactor (CSTR) is highly required to study in-depth the performance of anaerobic microorganisms fed with pretreated microalgae biomass. Batch assays results could be extrapolated to CSTRs only when no inhibition occurs. The present study was designed to evaluate the performance of a CSTR fed semi-continuously with enzymatically pretreated *C. vulgaris* biomass. The pretreatment was conducted in accordance to previous results obtained during the optimization of *C. vulgaris* hydrolysis by protease addition (Section 4.2.6.). Organic matter removal, biogas quality, methane yield, and nitrogen mineralization achieved during anaerobic digestion of *C. vulgaris* biomass was assessed. Additionally, ammonium and volatile fatty acids concentration of the effluents were monitored in order to discard the most common inhibitions related to protein rich substrates and organic overloading, respectively.

#### 4.3.1. Substrate characterization: raw and enzymatically pretreated *C. vulgaris*

Both, raw and enzymatic pretreated *C. vulgaris,* were chemically characterized since the different organic matter composition of the substrate used in anaerobic digestion may affect the methane yield achievable. Organic matter content of *C. vulgaris* was higher than 90% while ash content (inorganic matter) ranged 6–10% (Table 4.10.). The evaluated biomass of *C. vulgaris* was mainly composed of proteins (60–65% DW) and carbohydrates (22% DW). Due to the high protein content, the total COD/TKN ratio was 13.7. This is in agreement with protein rich substrates. This is the case for instance of microalgae biomass characterized by a low C/N ratio (5–6) (Mahdy et al.,

2015). Raw *C. vulgaris* biomass displayed low sCOD/tCOD ratio (2.5%) and low $NH_4^+$/TKN (10%), which confirmed that the cell walls were mostly intact.

After the treatment with protease biocatalyst, *C. vulgaris* organic matter solubilisation was drastically increased (Figure 4.16.). The organic matter in the soluble fraction rose from 2.5% in raw biomass to approximately 45% in the pretreated substrate. This result was in good agreement with previous hydrolysis efficiencies registered when evaluating the same biomass (*C. vulgaris*) and biocatalyst (Section 4.2.6). The effect of protease biocatalyst was also reflected in the release of ammonium. The $NH_4^+$/TKN ratio was raised to 44% (Table 4.10.).

**Table 4.10.** Biomass characteristics of enzymatically pretreated *C. vulgaris* prior to anaerobic digestion (CSTR and BMP).

| | Average | Standard deviation |
|---|---|---|
| TS (g $L^{-1}$) | 63.4 | 4.5 |
| VS (g $L^{-1}$) | 58.3 | 3.9 |
| tCOD (g $L^{-1}$) | 89.3 | 5.5 |
| sCOD (g $L^{-1}$) | 40.8 | 5.3 |
| Protein (% mg g $DW^{-1}$) | 64.5 | 2.6 |
| Carbohydrate (% mg g $DW^{-1}$) | 22.0 | 1.5 |
| Lipid and others (% mg g $DW^{-1}$) | 5.4 | 1.4 |
| Ash (% mg g $DW^{-1}$) | 8.1 | 1.1 |
| TKN (g $L^{-1}$) | 6.7 | 0.1 |
| Ammonium (g N-$NH_4^+$ $L^{-1}$) | 2.9 | 0.1 |

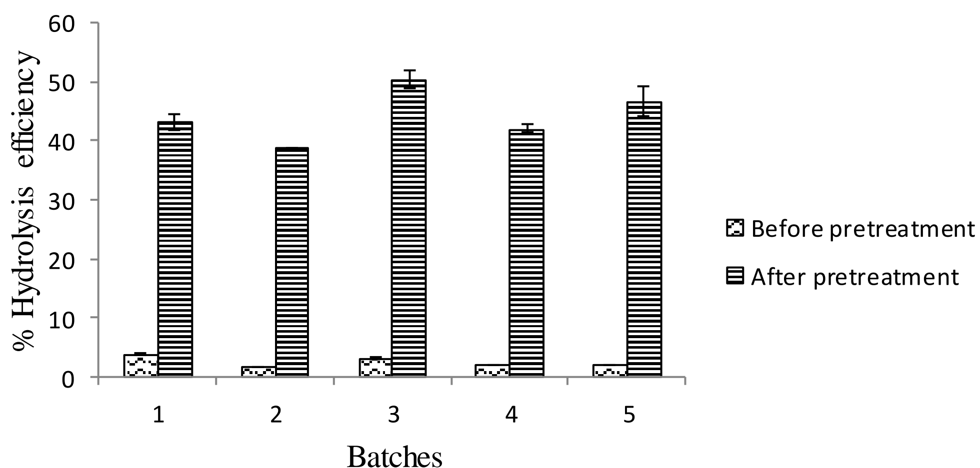



**Figure 4.16.** Hydrolysis efficiency enhancement achieved with protease addition on the different batches employed to feed the CSTR after enzymatic pretreatment of *C. vulgaris*.

#### 4.3.2. Semi-continuous anaerobic digestion of raw *C. vulgaris*

The daily biogas production measured on the CSTR fed with untreated *C. vulgaris* and HRT of 15 days is illustrated in Figure 4.17. Since the methane content in the biogas was around 73%, the methane yield calculated was 50 mL $CH_4$ g COD $in^{-1}$. Only few investigations have focused on microalgae digestion under semicontinuous feeding operation and, therefore, studies for comparison purposes are limited. In the case of raw *Scenedesmus obliquus*, the methane yield reported was 33 mL $CH_4$ COD $in^{-1}$ when using an OLR of 1 g tCOD $L^{-1}$ $day^{-1}$ (González-Fernández et al., 2013). Digesting a mixture of microalgal biomass resulted in 90 mL $CH_4$ COD $in^{-1}$ when using an OLR of 1.5 g tCOD $L^{-1}$ $day^{-1}$ (Passos et al., 2014). These CSTRs were operated under the same HRT; however, it was shown that the different microalgae species displayed different behavior toward anaerobic digestion. One possible reason of this variability is the biomass composition and the different cell wall hardness among microalgae strains. In this context, it has been previously shown that *C. vulgaris* and *Scenedesmus* sp. belong to the group of algae most difficult to digest anaerobically (González-Fernández et al., 2013; Mussgnug et al., 2010). Given the low values attained under this scenario, no

further description on this CSTR performance was included herein. At this point it should be stressed out that the HRT of the operated CSTR was set at 15 days accordingly to the values attained in batch mode anaerobic digestion (Figure 4.15. of Section 4.2.6.3). BMP conducted with raw *C. vulgaris* evidenced that more than 90% of the total methane yield was produced within the first 15 days. In the particular case of *C. vulgaris*, Ras et al., (2011) found an increasing COD removal trend when elongating the HRT from 16 to 28 days. Therefore, in an attempt to improve *C. vulgaris* anaerobic digestion, microalgae biomass was subjected to enzymatic pretreatment using protease as biocatalyst previously to anaerobic digestion and elongating the HRT to 20 days.

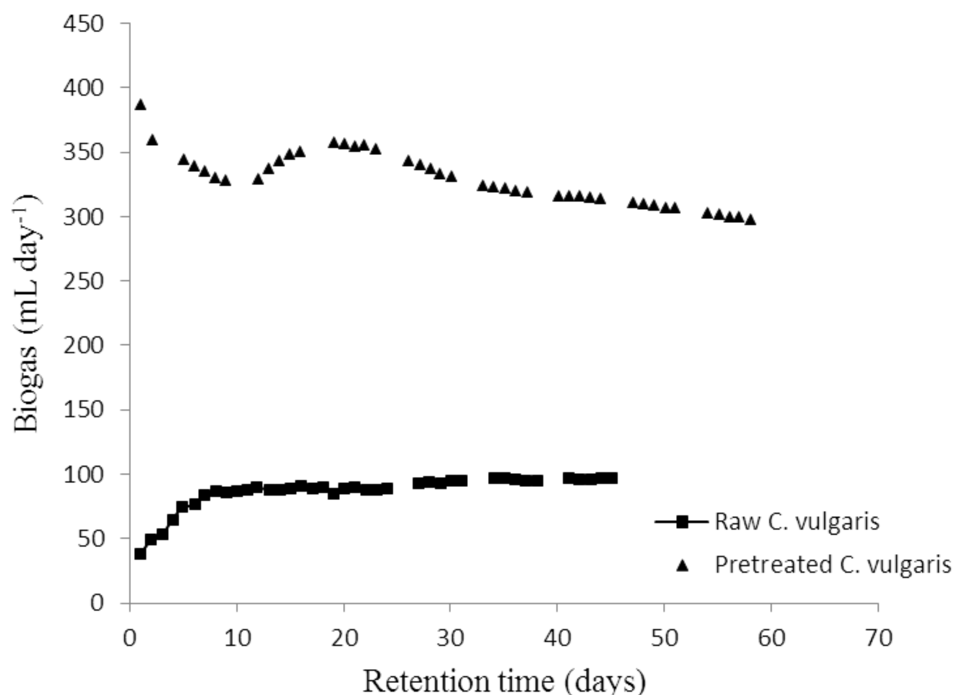



**Figure 4.17.** Biogas production rate achieved by the CSTR operated at 15 day HRT fed with raw C. vulgaris and CSTR operated at 20 days HRT fed with pretreated *C. vulgaris.*

### 4.3.3. Semi-continuous anaerobic digestion of enzymatically treated *C. vulgaris*

#### 4.3.3.1. Methane production and organic matter removal

In comparison with the CSTR fed with not treated *C. vulgaris* operated at 15 days HRT, the CSTR operated with the pretreated biomass increased methane yield by 2.6-fold (128.4 mL $CH_4$ g COD $in^{-1}$, Figure 4.17., Table 4.11.). This enhancement was similar to the values obtained for others CSTRs fed with thermally pretreated *Scenedesmus* sp. (2.9-fold; González-Fernández et al., 2013) and higher than that attained with a CSTR fed with microwave pretreated microalgae biomass (1.6-fold; Passos et al., 2014). Even though, the methane yield enhancement registered for the CSTR (2.6-fold) fed with enzymatically pretreated biomass was higher than the one obtained in BMP (1.8-fold; Section 4.2.6), the absolute methane yield values were lower. The methane yield attained in batch mode for the pretreated *C. vulgaris* was 224.5 mL $CH_4$ g COD $in^{-1}$, which was 41% higher than the value obtained in the CSTR. This lower methane yield observed under CSTR operation mode may be due to several reasons. In order to elucidate this fact, a closer look into organic matter removal and common anaerobic digestion inhibitors (VFAs and ammonia concentration) was required.

**Table 4.11.** Overview of different measured parameters obtained in anaerobic digestion (CSTR) of enzymatically pretreated *C. vulgaris* at 20 days HRT.

| | Average | Standard deviation |
|---|---|---|
| Methane production rate (mL $CH_4$ $day^{-1}$) | 226.9 | 12.3 |
| Methane yield (mL $CH_4$ g COD $in^{-1}$) | 128.4 | 15.3 |
| % $CH_4$ content | 70.5 | 1.2 |
| % TS removed | 48.7 | 3.5 |
| % VS removed | 55.7 | 1.9 |
| % tCOD removed | 55.7 | 1.5 |
| % sCOD removed | 94.4 | 0.9 |
| % N mineralization | 77.3 | 7.5 |
| Free $NH_3$ (mg $L^{-1}$) | 69.7 | 7.7 |

Total COD and VS removal of pretreated microalgae was 56% (Figure 4.18, Table 4.11.). This value is in good agreement with the values attained for methane production. Based on an optimum conversion of 350 mL $CH_4$ produced per g COD introduced, the methane yield obtained corresponded to an anaerobic biodegradability of approximately 37%. Taking into consideration that methane content in the biogas was 70%, this meant that anaerobic biodegradability on biogas basis corresponded to 52%. The value measured in the present study was comparably higher to the values reported with other pretreated microalgae biomass. For instance, Passos et al., (2014) obtained 38% VS removal applying microwave treatment for a mixture of microalgae while González-Fernández et al., (2013) achieved 30% total COD removal when pretreating thermally at 90°C *Scenedesmus* sp. biomass. It is also worth to mention that the pretreatments (microwave and thermal application) of those investigations were applied on diluted biomass (2.2 and 0.8% TS, respectively) compared to 6.5% biomass concentration used in this study. Application of pretreatments on diluted biomass can be highly efficient but the overall costs can compromise their feasibility. In the case of enzymatic pretreatment using protease as biocatalyst, the results have shown than even

working at high biomass loads, the efficiency of the pretreatment is not diminished (Section 4.2.6.2.). In this context, the high efficiency of this enzymatic hydrolysis prior to anaerobic digestion was also confirmed on semicontinuously fed CSTR. Enzymatic pretreatment (0.585 AU g $DW^{-1}$) applied to 6.5% TS resulted in 50% total COD converted to soluble organic matter. As it can be seen in Table 4.11. and Figure 4.18., this soluble COD was easily biodegradable and almost all organic matter available in soluble phase was removed (94.4%). The soluble COD in the effluent averaged 745 mg $L^{-1}$ and started increasing at 55 days of digestion).

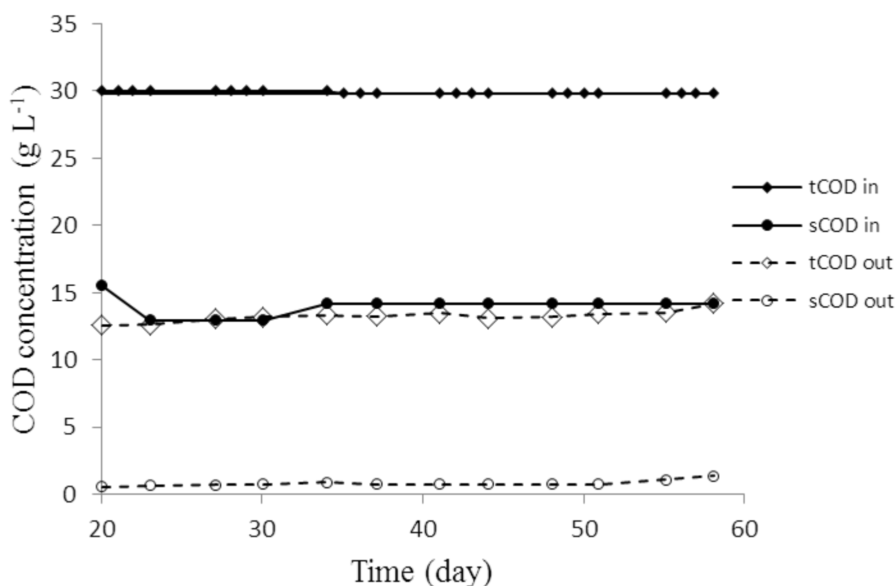



**Figure 4.18.** Inlet and outlet total and soluble organic matter concentrations obtained for the CSTR at 20 days HRT.

#### 4.3.3.2. Potential inhibitors: VFAs and $NH_3$

VFAs are one of the intermediates produced during organic matter degradation in the anaerobic digestion process. When acidogenesis rate is higher than methanogenesis, VFAs accumulation takes place. Organic overload is the main contributor to VFA accumulation. This accumulation is normally leading to pH decrease in the digester, which ultimately results in methanogenic flora inhibition. In the present study, VFAs were analyzed to discard organic matter overdose. As shown in Figure 4.19., VFAs concentration in the CSTR effluent fed with the pretreated biomass were very low or even negligible in the second retention time. This low VFAs concentration confirmed that the activity of acidogens, acetogens and methanogens was balanced. During the third retention time (last 20 days of digestion), the VFAs started to accumulate gradually from 57.1 mg $L^{-1}$ until 615.1 mg $L^{-1}$ reached at the end of experimental time. The last sampling points resulted in 274 and 700 mg COD (VFA) $L^{-1}$ for days 55 and 58, respectively. Thus, it can be concluded that almost all the soluble COD registered at the end of the experiment (Figure 4.16.) corresponded to the raise of VFA concentration. In fact, pH remained at 7.5 throughout the experimental time.

VFAs may be used as an indicator for appropriate equilibrium between acidogenesis/acetogenesis and methanogenesis. In this particular case, it seems that after feeding semicontinuously the CSTRs, the effective organic matter hydrolysis mediated by enzymatic pretreatment led to a slight organic matter overload during the last days of digestion. Soluble organic matter (VFAs) was available but methanogens were not fast enough or were inhibited to convert this substrate to biogas. It can be suggested that digestion of this hydrolyzed substrate using longer HRT could help decreasing VFAs concentration and thus increase methane yield.

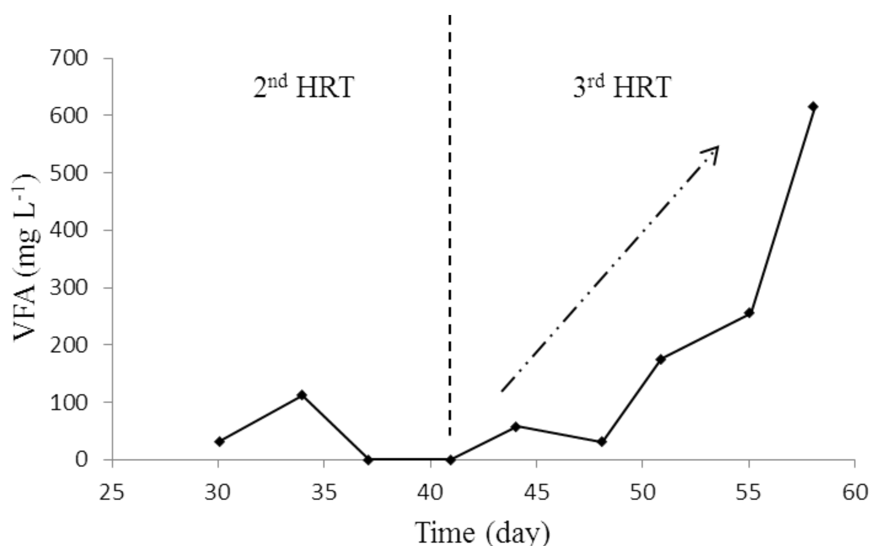



**Figure 4.19.** Time course of VFAs concentration in the CSTR fed with enzymatically pretreated *C. vulgaris* operated at 20 days HRT.

During anaerobic digestion, proteins are degraded and the main by-product is ammonium. Since the predominant macromolecule of *C. vulgaris* was the protein fraction, it was important to study the nitrogen fate in order to avoid potential ammonia inhibitions. Nitrogen mineralization calculated for the CSTR fed with enzymatically pretreated biomass was 77% (Figure 4.20. and Table 4.11.). This result indicated that the pretreatment used herein was appropriate for organic nitrogen conversion. In comparison to other studies, González-Fernández et al., (2013) presented 43.5% nitrogen mineralization for a CSTR fed with thermally pretreated *Scenedesmus* sp. at 90°C. The higher value obtained in this work may be attributed to the pretreatment method since protease addition was targeted at the hydrolysis of proteins (main nitrogen source). On the other hand, thermal pretreatment, such is the case of González-Fernández et al., (2013) prevails carbohydrate solubilisation over proteins (Mendez et al., 2013) and thus, nitrogen mineralization was lower.

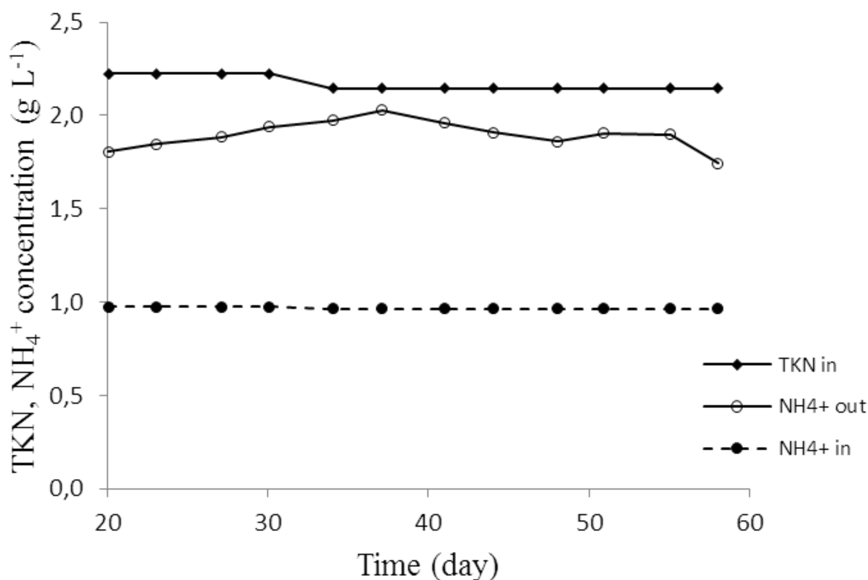



**Figure 4.20.** Inlet and outlet TKN and $NH_4^+$ concentrations obtained for the CSTR at 20 days HRT.

The effluent ammonium concentration was around 1895.9±76.5 mg $L^{-1}$. This value was much higher than previously reported in other CSTRs digesting microalgae biomass (González-Fernández et al., 2013; Ras et al., 2011). The toxic threshold limit depends on the substrate, inoculum, retention time and environmental conditions. Ammonium concentration of around 1700-1800 mg $L^{-1}$ was considered inhibitory for un-acclimated inoculum (Yenigün and Demirel, 2013) while this value can be raised up to 4000-6000 mg $L^{-1}$ when using adapted inoculum (Koster and Lettinga, 1988).

Depending on the pH, ammonia is another form of inorganic nitrogen that may exist in anaerobic digestion processes. Therefore, ammonia concentration was calculated in accordance to the pH and ammonium concentration measured in the CSTR effluents. As expected by the pHs measured throughout the experiment (approximately 7.5), the predominant form of inorganic nitrogen was ammonium. The ammonia concentration was 69.7 mg $L^{-1}$. Ammonia toxic threshold concentration is much lower than ammonium. As a matter of fact, values of around 300 mg $L^{-1}$ have been reported inhibitory for methanogenic activity (Braun et al., 1981). In the present study the ammonia

concentration was not too high, but a clear inhibition effect was observed probably due to the high ammonium concentration measured in the effluents. VFAs accumulation is a symptom of methanogenic activity inhibition due to high ammonium concentration (Yenigün and Demirel 2013). As a matter of fact, VFAs started to accumulate during the last stage of the experimentation and, therefore, a slight inhibition of the methanogenic microorganisms could be inferred. To the best of the author's knowledge, this is the first study reporting ammonium inhibition on CSTR fed with microalgae biomass. This inhibition was mediated by the high protein content of the evaluated biomass and the proteolytic pretreatment employed prior to anaerobic digestion. Strategies for controlling ammonium/ammonia inhibition include substrate input dilution, longer HRT, immobilization of microorganisms to allow a longer retention time or C/N ratio adjustment through codigestion with carbon rich substrates. In the particular case of microalgae biomass, one possible strategy would also be to decrease the protein content of this substrate by prevailing the accumulation of other macromolecules (lipids or carbohydrates).

#### 4.3.4. Batch anaerobic digestion using the inoculum from the CSTR

Despite the common assumption that carbohydrates are responsible for the low digestibility of microalgae, it has been shown that proteins were the main polymers hampering anaerobic digestion (Section 4.1.2.3.). Those results were obtained in BMPs assays using sludge collected at wastewater treatment plants. In this manner, microbial population is adapted to degrade different substrates, namely activated and primary sludge. Using anaerobic sludge from wastewater treatment plants (WWTP), the hydrolysis with proteases has revealed an increase in anaerobic biodegradability from around 40% to 71% and 62% for *C. vulgaris* and *Scenedesmus* sp., respectively (Section 4.1.2.3.). As it can be seen in the dashed line of Figure 4.21., the methane yield plateau was achieved after 10 days of digestion. When using WWTP sludge, methane productivity ranged 25 mL $day^{-1}$ the first days of digestion and then lowered down to 1 mL $day^{-1}$ around day 10. From that day onwards, methane yield remained around 223 mL $CH_4$ g COD $in^{-1}$.

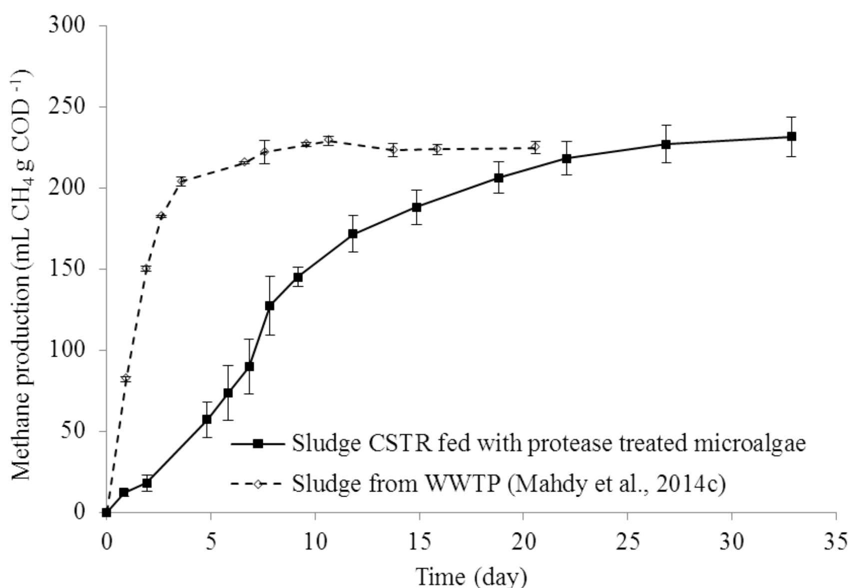



**Figure 4.21.** Cumulative methane production of enzymatically pretreated *C. vulgaris* during batch assays.

In the case of the BMP inoculated with sludge collected from the CSTR fed with protease hydrolyzed microalgae, a different trend in methane productivity was observed. Methane production rate was much lower than observed with the other inoculum, approximately 3 mL $day^{-1}$ (continuous line in Figure 4.21.). Even though the final methane yield was similar to the one obtained with the sludge coming from the WWTP, 223 mL $CH_4$ g COD $in^{-1}$ was reached after 25 days. It seems likely that the microbial population developed during the operation of the CSTR could not handle appropriately the hydrolyzed microalgae biomass and required longer retention time to attain maximum methane production. Methane content measured in the biogas was around 69% for both inocula used. Nevertheless, the methane production rate profile attained during the first days of digestion was quiet different (Figure 4.21.).

The process of anaerobic digestion usually includes four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis, in which hydrolytic, fermentative bacteria, acetogens and methanogens play different roles. At this point it should be stressed out that, even though anaerobic digestion of complex biomass such microalgae is limited by the first hydrolysis stage; this

was not the case as observed in Figure 4.21. In this experiment, methane was produced with the anaerobic inoculum collected at the WWTP right away after inoculation. However, the BMP assay using anaerobic inoculum from the CSTR fed with protease pretreated *C. vulgaris* revealed that methane production was somehow delayed the first days of digestion. As explained in the previous Section 4.3.3.2., organic overdose can lead to VFA accumulation. The VFAs accumulation detected during the CSTR operation revealed an unbalanced activity of the different microorganisms involved in anaerobic digestion. This unbalance can be probably overcome by using longer HRT. In order to fully elucidate the reason for a delayed methane production, it is crucial to study the population dynamics developed upon the CSTR operation. Insights on microbial populations could help identifying the microorganisms' shifts and consequently, further studies should be focussed on the metabolic interactions and activities of the archaeal and bacterial consortia developed in anaerobic reactors digesting this new substrate (microalgae).

## 4.4. CSTR fed with *C. vulgaris* grown in urban wastewater as a sole nutrient source

As it has been reported previously in CSTR digestion, the combination of the pretreatment together with the high protein content of microalgae resulted in high ammonium concentration. This high ammonium concentration mediated a stepwise volatile fatty acids accumulation that reduced concomitantly the biogas production. To circumvent this issue, a reduction of biomass protein content or the use of other biocatalyst different from proteases devoted to hydrolyze another macromolecule (carbohydrates or lipids) rather than proteins can be envisaged. The present study evaluated both options. In the first stage, microalgae were grown in wastewater with the aim at reducing biomass protein content and assess the feasibility of coupling wastewater bioremediation with energy production (biogas). Secondly, the effect of protease and carbohydrase hydrolysis of the harvested biomass was compared in terms of biogas production. For such a goal, the performance of two CSTRs fed semi-continuously was evaluated. Organic matter removal, biogas production rate, methane yield, and nitrogen mineralization achieved during anaerobic digestion of these substrates was determined.

### 4.4.1. Biomass characterization of *C. vulgaris* grown in wastewater

Table 4.12. shows composition of *C. vulgaris* biomass grown on wastewater. The macromolecular profile attained was markedly different than those measured when this microalga was grown on synthetic media (MBBM, Section 4.3.1.). In synthetic media, the microalgae organic matter was mainly composed by proteins while growing the same strain on urban wastewater resulted in a macromolecular profile shift towards carbohydrate accumulation (current study and previous results, Section 4.3.1.). More specifically, the macromolecular profile resulted in 64% proteins and 22% carbohydrates when growing *C. vulgaris* in synthetic media (Section 4.3.1). On the contrary, when growing in urban wastewater, the carbohydrate fraction was doubled (40% on VSS basis) while the protein content was reduced to half (33.3%, Table 4.12.). This marked diminishment in protein content observed in the biomass grown in urban wastewater was attributed to the low ammonium

content in the growing medium. In this sense, the ammonium concentration of MBBM ranged 480 mg $L^{-1}$ while that of urban wastewater was 13.2-fold lower (Table 3.2.). The lower nitrogen available for microalgae assimilation affected the protein content of *C. vulgaris* and, therefore, its macromolecular composition. In this context, the nitrogen available for microalgae uptake in urban wastewater was limited. This nitrogen limitation resulted in arrested protein synthesis and nutrients uptake were mostly allocated as carbohydrates. Under this scenario of nitrogen limitation, previous investigations have shown similar microalgae behavior. Nitrogen limitation triggered the enhancement of lipids or carbohydrates depending, not only on the operational conditions during cultivation, but also on the microalgae strain subjected to this limitation (Dragone et al., 2011, Ho et al., 2012 and Siaut et al., 2011). In the particular case of *C. vulgaris*, nitrogen limitation has been previously reported to increase carbohydrate content of this biomass (Ho et al., 2013 and Dragone et al., 2011).

In addition, using wastewater as nutrient source for microalgae cultivation (instead of synthetic media), resulted in biomass flocs formation. This fact may be attributed to the inherent presence of bacteria in wastewater since non aseptic conditions were used during the cultivation process. More information on this interaction can be found elsewhere (González-Fernández and Ballesteros, 2013). Therefore, besides inducing microalgae to accumulate targeted macromolecules and decreasing the cultivation cost, wastewater grown microalgae presented an additional benefit in terms of the cost reduction associated to biomass harvesting. At this point it should be stressed that, despite of the different size distribution observed depending on the growth medium, the hydrolysis efficiency of the different biocatalysts was not affected (as discussed later).

**Table 4.12.** Biomass characteristics of raw *C. vulgaris* cultivated in wastewater and subjected to enzymatic pretreatment prior to anaerobic digestion.

| | Average | Standard deviation |
|---|---|---|
| TS (g $L^{-1}$) | 29.5 | 3.8 |
| VS (g $L^{-1}$) | 24.0 | 2.7 |
| tCOD (g $L^{-1}$) | 42.4 | 4.1 |
| sCOD (g $L^{-1}$) | 1.1 | 4.5 |
| Protein (% mg g $VSS^{-1}$) | 33.3 | 1.5 |
| Carbohydrate (% mg g $VSS^{-1}$) | 39.6 | 2.1 |
| Lipid and others (% mg g $VSS^{-1}$) | 15.7 | 6.7 |
| Ash (% $TSS^{-1}$) | 11.3 | 5.1 |

**4.4.2. Enzymatic pretreatment**

**4.4.2.1. Organic matter solubilisation**

Raw *C. vulgaris* biomass displayed the lowest organic matter available in soluble phase, which confirmed that the cell walls were mostly intact (Table 4.12.). With the aim of preteating the microalgae cell walls and increase the availability of organic matter for anaerobic digestion, two biocatalysts hydrolyzing different macromolecules (proteins and carbohydrates) were tested. Previous experiments have shown that hydrolysis efficiency is higher when *C. vulgaris* grown on synthetic media was pretreated with proteases than with carbohydrases (Sections 4.1.2.2. and 4.2.3.). However, the same conclusion cannot be assumed in these experiments since the current biomass, grown in urban wastewater, was rich in carbohydrates and not in proteins such is the case of the later studies. Therefore, in order to check whether the macromolecular composition of microalgae affects the hydrolysis efficiency of the biocatalysts, *C. vulgaris* was subjected to the action of both carbohydrase and protease.

To evaluate the hydrolysis efficiency of the enzymatic pretreatment, the increase in sCOD was determined after the pretreatment and compared to the sCOD in raw biomass. Both biocatalysts exhibited a great influence on organic matter solubilisation. Compared to the fresh *C. vulgaris,* where only 2% of the tCOD was soluble (Table 4.12.), the hydrolysis conducted with both enzymes revealed an organic matter hydrolysis efficiency of 28.4% and 54.7% for samples pretreated with Viscozyme (carbohydrase) and Alcalase (protease), respectively. The values are quiet similar to that attained in previous sections. The hydrolysis efficiency attained with Viscozyme and Alcalase ranged 25-29% and 47-57%, respectively (Sections 4.1.2.2.1; 4.2.3 and 4.2.6.2). Likewise, hydrolysis efficiency attained with protease was similar to that provided by Romero-Garcia et al., (2012).

Despite of the different size distribution observed depending on the growth media, the hydrolysis efficiency of the biocatalysts was not affected. The difference in macromolecular distributions between microalgae used in this study and that used in previous studies dealing with microalgae grown in synthetic media did not display any significant difference in hydrolysis efficiency between protease and carbohydrase hydrolysates. In this context, in Section 4.2.3., organic matter hydrolysis efficiency of 29% and 57% were obtained for carbohydrase and protease pretreated biomass, respectively, when subjecting protein-rich biomass (63.3% DW) to enzymatic pretreatment. One option could be that there was no difference between cell wall composition in microalgae grown on synthetic media and wastewater. The second option could be the glycoproteins content of microalgae cell walls. Glycoproteins are proteins that contain oligosaccharide chains (glycans) attached to polypeptide side-chains. In fact, Cheng et al., (2011) demonstrated that aminosugars, commonly found in glycoproteins, represents almost 31.3% of the total carbohydrate of *C. vulgaris* cell wall. Interestingly, glycoproteins of red microalga have been identified as a site-recognition to prevent prey attack (Ucko et al., 1999) and therefore this feature could be linked as well with the fact that microalgae proteins is the fraction responsible for the low anaerobic biodegradability of this substrate. Further research on microalgae cell wall composition is highly required to confirm this hypothesis.

#### 4.4.2.2. Cell morphology observation before and after biocatalysts pretreatment

The differences among raw and pretreated biomass were also observed by using transition electron microscopy (TEM). Starch granules were accumulated into the cells. Starch content increased drastically from approximately 5% in *C. vulgaris* grown on synthetic media to 33.4±1.5 on VSS basis when cultivating *C. vulgaris* in wastewater (Table 4.12.).

With regard to the biomass pretreated with the biocatalysts, protease pretreated biomass resulted in cell fragmentation and complete degradation (Figure 4.22.) while in the case of biomass hydrolyzed with the carbohydrase, the cell wall was slightly affected and a significant amount on organic matter was still inside the cell. Likewise, these images also showed that in the case of the protease pretreated *C. vulgaris,* the hydrolysis was not confined to wall constituents but also progressed to internal components. The differences observed in TEM images supported also the fact that the hydrolysis efficiency achieved by the biomass treated with the protease was almost 2-fold higher than that of the biomass treated with carbohydrases. At this point, it should be also highlighted that the hydrolysis was not selective to wall constituents but internal and wall constituents mediated this enhanced hydrolysis efficiency. Different TEM images can be found in the literature after microalgae pretreatment. For instance, Passos and Ferrer (2015) demonstrated a break in the outer structure of the cell wall and damages in the intracellular structure after thermal pretreatment of *Oocystis* sp. Wang et al., (2015) showed a break down in the cell wall and leakage of intracellular material of *Neochloris oleoabundans* after combined mechanical-enzymatic pretreatment.

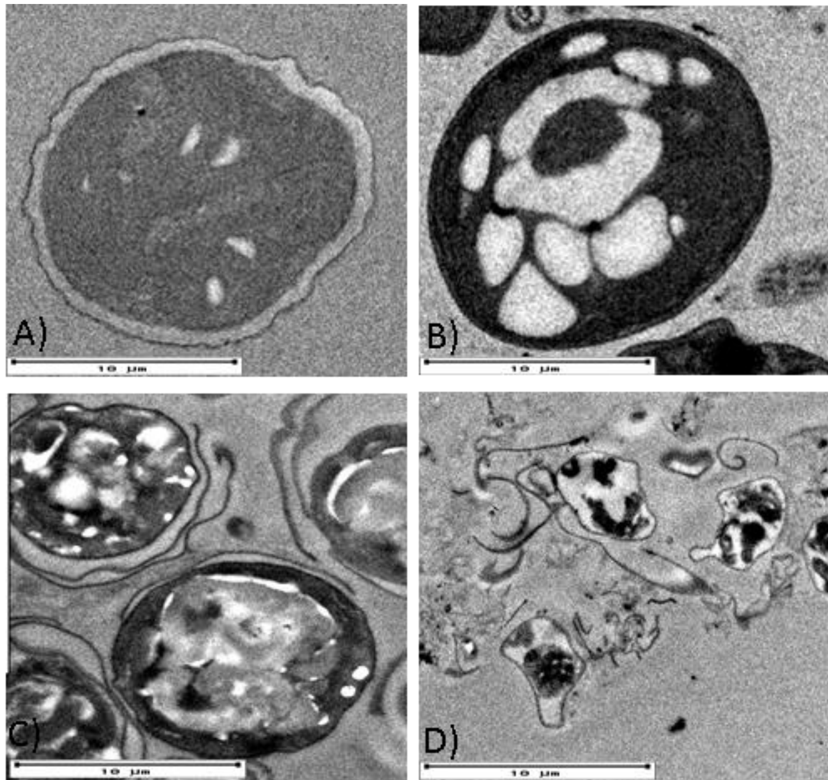



**Figure 4.22.** TEM images represent raw *C. vulgaris* grown in synthetic media (A) and urban wastewater (B) while (C) and (D) represented the pretreated microalgae grown in wastewater with carbohydrases and proteases, respectively.

### 4.4.3. Semi-continuous anaerobic digestion of raw and enzymatically pretreated *C. vulgaris*

#### 4.4.3.1. Biogas production

CSTRs fed with both raw and enzymatically pretreated *C. vulgaris* were monitored for biogas production over 60 days (3×HRT). The daily biogas production for the raw *C. vulgaris* biomass ranged 56 mL biogas L reactor$^{-1}$ day$^{-1}$. This value was lower than that obtained in Section 4.3.2 when digesting the same microalgae strain under similar anaerobic conditions. The daily biogas production of the later study (using 15 days HRT and fed with protein-rich *C. vulgaris*) was 1.2-fold higher than the one observed in the current study. This fact was attributed to the floc formation of the biomass when growing on wastewater. Floc formation would also affect the methane yield achievable using this substrate by hampering the organic matter accessibility. Overall, the methane yield reached by the raw biomass was low and the need of pretreatment before anaerobic digestion was evidenced once again.

Figure 4.23. shows biogas production kinetics of both raw and enzymatically pretreated *C. vulgaris*. Daily biogas production obtained from the biomass pretreated with carbohydrases resulted in 150 mL L$^{-1}$ day$^{-1}$ (3-fold higher than the CSTR fed with raw biomass). This enhancement was a result of the organic matter hydrolysis (approx. 30%) registered when treating *C. vulgaris* with carbohydrases. Despite of this enhancement, anaerobic biodegradability was low and only 40% of tCOD was removed while methane yield ranged 65 mL $CH_4$ g COD in$^{-1}$ (Table 4.13.). CSTRs fed with carbohydrase pretreated biomass in this work displayed similar values than other reported in literature. For instance, Passos et al., (2014) and González-Fernández et al., (2013) reported 38% VS removal and 30% tCOD removal when operating CSTRs fed with thermally pretreated microalgae biomass, respectively. At this point, it has to be stressed out that carbohydrase pretreated biomass reported similar values to previous ones but far below those obtained in the CSTR fed with protease pretreated biomass. In accordance to the higher hydrolysis efficiency registered for the biomass pretreated with the proteases, the CSTR fed with this biomass evidenced better performance. More specifically, the total COD removal ranged 52% and the methane yield reached 137 mL $CH_4$ g COD in$^{-1}$.

In this case, methane yield was 6-fold higher than the CSTR fed with raw biomass. The organic matter solubilized during both pretreatments was easily degradable and almost all organic matter available in soluble phase was removed (Table 4.13.). Regarding methane content of the biogas produced in the CSTRs, no significant differences were observed. This percentage ranged 64–70% along the experimental time.

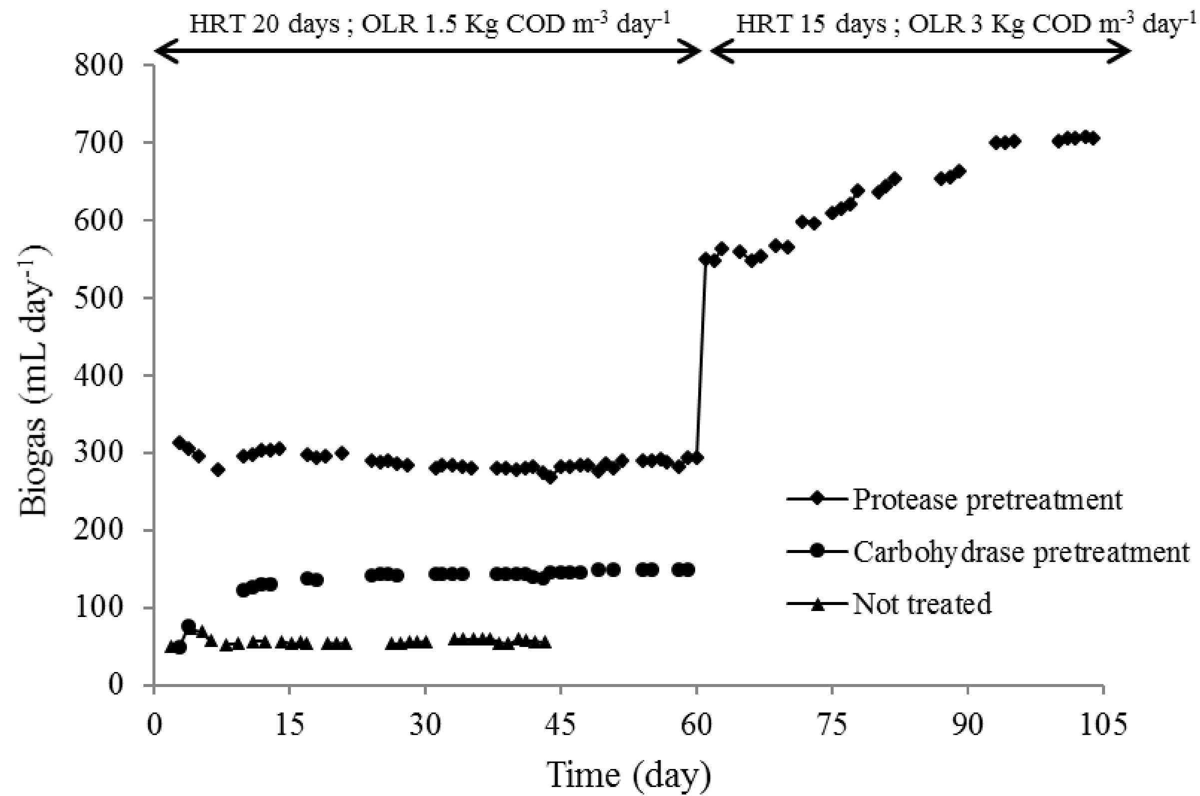



**Figure 4.23.** Biogas production rate achieved by the CSTRs fed with raw and enzymatically pretreated *C. vulgaris*.

**Table 4.13.** Overview of different measured parameters obtained in anaerobic digestion (CSTR) of enzymatically pretreated *C. vulgaris.*

| | Carbohydrase pretreated biomass | | Protease pretreated biomass | |
|---|---|---|---|---|
| | Average | STD | Average | STD |
| Methane production rate (mL $CH_4$ $day^{-1}$) | 86.6 | 20.2 | 200.8 | 4.5 |
| Methane yield (mL $CH_4$ g COD $in^{-1}$) | 64.9 | 14.5 | 136.9 | 19.2 |
| % Methane content | 64.6 | 0.8 | 70.1 | 1.2 |
| % VS removed | 39.7 | 4.3 | 53.4 | 3.6 |
| % tCOD removed | 39.7 | 2.2 | 51.9 | 2.5 |
| % sCOD removed | 95.3 | 1.1 | 93.9 | 0.8 |
| % N mineralization | 39.6 | 5.5 | 68.5 | 14.0 |
| Free $NH_3$ (mg $L^{-1}$) | 6.3 | 0.8 | 28.2 | 4.6 |
| pH (effluent) | 7.1 | 0.0 | 7.5 | 0.1 |

It is important to highlight that regardless the microalgae protein content and the use of protease pretreatment, the results obtained during the digestion in the CSTRs were similar to that obtained in previous experiments. In this context, previous results in Section 4.3.3 showed methane production rate up to 226.9±12.3 mL $CH_4$ $L^{-1}$ $day^{-1}$ with the CSTR fed with protein-rich *C. vulgaris* pretreated with protease. Carbohydrate-rich biomass pretreated with proteases used in these experiments resulted in 200.9±4.5 mL $CH_4$ $L^{-1}$ $day^{-1}$ (Table 4.13.). Opposite to the CSTR performance fed with protein rich biomass (Section 4.3.3.), the CSTR fed with carbohydrate-rich *C. vulgaris* (current study) displayed a steady methane production rate along digestion.

The reduction in biomethane rate of the former study was attributed to an ammonium inhibition that took place as a consequence of the high protein content of the substrate and the pretreatment with protease that resulted in high nitrogen mineralization.

Most of the studies available on CSTR anaerobic digestion of microalgae biomass have been carried out at low organic loading rate (OLR) (Kinnunen et al., 2014, Ras et al., 2011, Passos et al., 2014 and González-Fernández et al., 2013). Under high OLR, the system can fail due to organic overloading while, under low OLR, the anaerobic digester can run inefficiently due to underestimated activity of anaerobes. Given the good results and the stable methane production recorded for the CSTR fed with protease pretreated biomass, the OLR was doubled (3 kg COD $m^{-3}$ $day^{-1}$) to verify stability and results reproducibility. Moreover, in order to further testing the system, in addition to the OLR increase, the HRT was shortened to 15 days. Surprisingly, the CSTR fed with protease pretreated *C. vulgaris* performed even better using these later operational conditions. When protease pretreated microalgae was digested in the semi-continuous system at higher OLR, methane production was increased from 136.9±19.2 mL $CH_4$ g tCOD $in^{-1}$ registered at 1.5 kg tCOD $m^{-3}$ $day^{-1}$ to 173.2±10.7 mL $CH_4$ g tCOD $in^{-1}$ at 3 kg tCOD $m^{-3}day^{-1}$. As it can be observed in Figure 4.23., daily biogas production rate reached 671.3±31.6 mL $L^{-1}$ $day^{-1}$ while the methane content of the biogas remained in the same range (70%). When compared to the untreated biomass, the methane yield increased 5- and 6.3-fold at OLR of 1.5 and 3 kg tCOD $m^{-3}$ $day^{-1}$, respectively. Under this anaerobic digestion conditions, tCOD removed was approximately 57.8±0.6%. The higher methane yield exhibited during the highest OLR denoted an underestimated activity of anaerobic microorganism at the lowest OLR. Additionally, this enhancement could be also attributed to the adaptation of the inoculum to protease *C. vulgaris* hydrolysates since the anaerobic inoculum used for the second OLR was recovered from the CSTR operating at the lower OLR.

At this point it should be stressed out that in large scale application, for economic reasons, it is preferably to achieve the highest biogas production at shorter hydraulic retention time and higher organic loads. Since increasing the

OLR in the CSTR fed with protease pretreated carbohydrate-rich microalgae did not display any reduction in biogas production along the digestion, the present finding suggested that using carbohydrate-rich microalgae grown on wastewater treated with proteases would provide tremendous benefits in the anaerobic digestion of this substrate. Overall, the organic matter fate revealed that higher organic loading rate and shorter retention time did not provoke operational instabilities since methane yield was not affected negatively. In this sense, no microbial imbalances were evidenced but to further prove this, common inhibitors were followed up during digestion.

#### 4.4.3.2. Potential inhibitors

During the organic matter degradation into biogas, many intermediate products are produced. In the particular case of microalgae, ammonium release during nitrogen mineralization has been pointed out to inhibit anaerobes. Likewise, when increasing the OLR, the organic overload can cause digester failure by volatile fatty acids built up. Therefore, monitoring these intermediate products and their effects along the experiment could be helpful for avoiding potential inhibitions.

Inhibition of anaerobes caused by ammonium concentration in the digester of protein rich substrates is directly linked to the nitrogen mineralization. 68.6±14.4% of nitrogen mineralization was obtained in the CSTR fed with protease pretreated biomass (Table 4.13., Figure 4.24.). Nitrogen mineralization in the CSTR fed with carbohydrases pretreated *C. vulgaris* was 39.6±5.5%. This result was similar to that obtained by González-Fernández et al., (2013) who demonstrated 43.5% nitrogen mineralization when operating a CSTR fed with thermally pretreated *Scenedesmus* sp. When focusing on the same microalgae strain, *C. vulgaris*, Ras et al., (2011) showed nitrogen mineralization of around 19% when raw biomass was digested in a CSTR operating at OLR of 1.5 kg COD $m^{-3}$ $day^{-1}$. This value was considerably lower than the obtained herein; unfortunately, no literature on nitrogen mineralization attained in CSTRs digesting pretreated *C. vulgaris* is available.

Despite of the difference in protein content of the substrate used in experiments in Section 4.3.3., there was no difference in nitrogen

mineralization. In this line, previous trials (Section 4.3.3.2.) showed 77% nitrogen mineralization in the CSTR fed with protease pretreated protein-rich biomass (63% DW protein). Even though the values attained for nitrogen mineralization were similar to those in Section 4.3.3.2., the effluent ammonium concentration was quite different. More specifically, the ammonium concentration in the current study was 856±103 mg $L^{-1}$ (Figure 4.24.B) while in the former study; ammonium concentration reached 1895±77 mg $L^{-1}$. The lower value obtained in this investigation was attributed to the lower protein content of microalgae biomass used in the study herein. Even though both studies entailed protease addition, the amount of organic nitrogen (amino acids) available to be easily mineralized during anaerobic digestion was higher in the nitrogen rich substrate (protein rich) grown on synthetic media. In the present study, protein content was lower and, even though, protease addition was targeted at the hydrolysis of proteins, the amount of organic nitrogen in the inlet of the reactor was lower. Despite of the high nitrogen mineralization registered during the anaerobic digestion process, the low protein content of the microalgae used in the present study resulted in low effluent ammonium concentration.

pH values were quite stable and close to neutrality (Table 4.13.). Ammonia concentration averaged 28.2±4.6 mg $L^{-1}$ in the CSTR fed with protease pretreated *C. vulgaris* and even lower (6.3±0.8 mg $L^{-1}$) for the CSTR fed with carbohydrase pretreated biomass. In this manner, both ammonia concentrations were far below the threshold values for hampering methanogenic bacteria activity (Braun et al., 1981). One of the most common features upon ammonium/ammonia inhibition of anaerobes is VFAs accumulation. Thus, VFAs were also measured in order to evaluate organic overloading of the process. VFA levels were negligible throughout the experiment. The VFA absence and pH levels (7.1–7.5, Table 4.13.) indicated that organic overload did not occur.

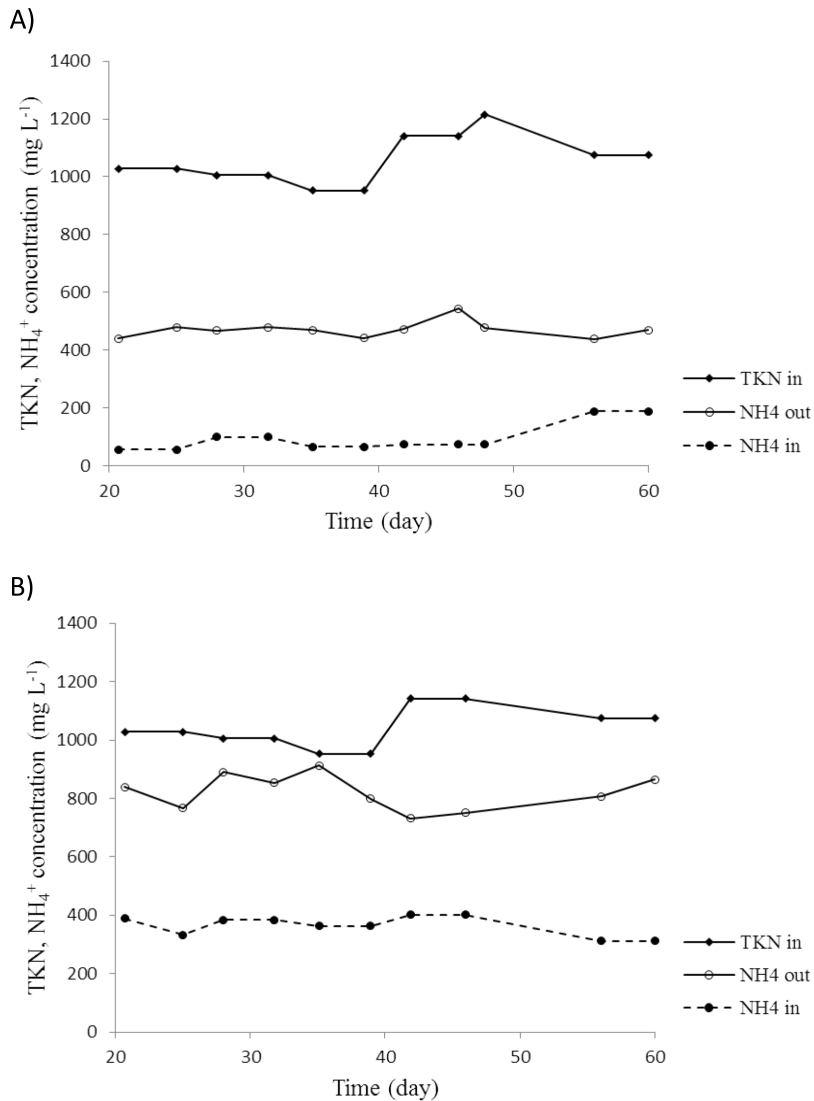



**Figure 4.24.** Inlet and outlet total nitrogen and ammonium obtained after *C. vulgaris* protease pretreatment: (A) corresponds to carbohydrase pretreated and (B) corresponds to protease pretreated *C. vulgaris* for the CSTRs operated at 20 days HRT.

In summary, the follow up of common inhibitors revealed that ammonium/ammonia inhibition can be avoided by using carbohydrate-rich *C. vulgaris*. Opposite to what it was observed before for protein rich *C. vulgaris*, the pretreatment with proteases did not result in high ammonium concentration in the effluent and thus VFAs accumulation was avoided. As a consequence, biogas production was steady along the digestion time.

# CHAPTER 5

# CONCLUSIONS AND FUTURE RESEARCH

# 5. Conclusions and future research

## 5.1. Conclusions

This thesis focused on the effect of different microalgae biomass pretreatment (autohydrolysis and enzymatic) on organic matter solubilisation and performance of anaerobic digester fed with those biomass. In this later point, methane yields enhancement and common anaerobic digestion inhibitor were investigated. The conclusions presented below are withdrawn according to the specific objectives set out in chapter 1:

### 1. Conclusions of the first objective

Although autohydrolysis at 50ºC evidenced organic matter and carbohydrates solubilisation, anaerobic digestion of pretreated biomass revealed only a slight increase on methane yield (10%) in the case of *Chlorella vulgaris* and negligible in the case of *Scenedesmus* sp.. This slight enhancement may be attributed to the exopolymers released rather than intracellular material. Therefore, the biological response of these microorganisms induced by heat was not enough to increase significantly methane yields.

### 2. Conclusions of the second objective

a. Regardless of the microalgae species targeted, macromolecular composition and microalgae cultivation media (synthetic MBB medium and urban wastewater), proteases (Alcalase 2.5L) are more effective hydrolyzing agents than carbohydrases (Celluclast 1.5, Viscozyme and Pectinase) in terms of organic matter (COD) solubilisation and methane production enhancement. Thus, despite the common assumption that carbohydrates are responsible for the low digestibility of microalgae, this study showed that proteins were the main polymer hampering anaerobic digestion. The application of protease prior to anaerobic digestion of microalgae biomass can be envisaged as a promising approach to decrease the energetic input required for cell wall disruption while enhancing markedly methane yield.

b. Out of the carbohydrases tested, Viscozyme was the most effective regarding carbohydrate solubilisation. With this agent, almost all carbohydrates were released in the soluble fraction, even though the methane yield enhancement remained low (14-16% with *C. vulgaris* and *Scenedesmus* sp.) or even negligible (with *C. reinhardtii*). These results supported the fact that carbohydrates are not responsible for the low methane yield.
c. The optimal hydrolysis time for all enzymes ranged 2-3 h. Longer reaction did not provide any further enhancement, exception made for *Scenedesmus* sp. pretreated with protease in which the highest hydrolysis efficiency was attained after 8 h.
d. Proteases and carbohydrases combination provided slightly higher hydrolysis efficiency and methane yields compared to that attained with proteases alone. Nevertheless, this low enhancement does not compensate the economic cost associated with longer hydrolysis time and enzyme addition.
e. Thermal application (75ºC for 30 min) prior enzymatic pretreatment did not have an additional effect on hydrolysis efficiency showing that there is not any structural impediment that hinders the access of enzymes to microalgae biomass.
f. No differences in organic matter hydrolysis efficiency (50%) or methane yields enhancement (1.55- fold) were observed when pretreating *C. vulgaris* at different biomass loads (16, 32 and 65 g $L^{-1}$) with protease at enzymatic dosage 0.585 AU g $DW^{-1}$. Thus, the increase of viscosity caused by the increasing biomass loads did not affect the protease hydrolytic efficiency.
g. Decreasing the enzymatic dosages (0.585, 0.293 and 0.146 AU g $DW^{-1}$) concomitantly decreased the organic matter hydrolysis efficiency (from 50 to 41%) of *C. vulgaris* biomass and accordingly, a reduction in methane production enhancement was attained (from 1.73 to 1.51 fold).

## 3. Conclusions of the third objective

a. Complete nitrogen mineralization was achieved in batch biomethane assays due to the combination of the protease pretreatment and organic nitrogen degradation during anaerobic digestion of the protein-rich microalgae. Nitrogen mineralization decreased when pretreating *C. vulgaris* at increasing biomass loads with 0.585 AU g $DW^{-1}$. Opposite to organic matter degradation that was protease dosage dependent, nitrogen mineralization was not.
b. Despite of the lower nitrogen mineralization (77%) reached in semicontinuous feeding mode conducted in CSTRs, the high ammonium concentration (≈ 1900 mg $L^{-1}$) mediated a stepwise volatile fatty acids accumulation that reduced concomitantly biogas production.
c. Ammonia toxic threshold concentration was never inhibitory since the pH levels remained almost neutral in all tested scenarios (different digestion mode or different biocatalysts).
d. Cultivation of *C. vulgaris* in wastewater as a sole nutrient source decreased the protein content of the biomass and hence, carbohydrate-rich *C. vulgaris* biomass was produced.
e. Despite of the different macromolecules distribution attained, hydrolysis efficiency by carbohydrase (≈ 29%) or protease (47-55%) of carbohydrate-rich *C. vulgaris* biomass was similar to that achieved with protein-rich *C. vulgaris* and consequently, the methane yields during the digestion in semi-continuous mode was similar (≈ 130 mL $CH_4$ g COD $in^{-1}$, 3 to 5-fold enhancement).
f. Opposite to the digestion of protein-rich *C. vulgaris*, the ammonium concentration in CSTR fed with carbohydrate-rich *C. vulgaris* pretreated with proteases was far below the inhibition threshold and a steady methane production rate along digestion was achieved. Thus, carbohydrate-rich *C. vulgaris* resulting from their cultivation in urban wastewater could circumvent ammonium inhibition taking place during anaerobic digestion.
g. Hydraulic retention times of 15 days and organic loading rates of 3 add units in semi-continuous mode digestion could provide significant

benefits in methane yield (6.3-fold enhancement) when feeding carbohydrate-rich *C. vulgaris* pretreated with proteases.

### 5.2. Future research

Future works about economic aspects of pretreatment process as well as reduction of ammonium inhibition effects on anaerobic digestion process to minor extent have to be considered in order to make the entire process economically-viable. In this manner, future research should focus on:

1) Biocatalysts cost

   The high cost of enzymes is considered as the main hurdle of applying this approach at industrial scale. Some options that could be proposed for investigation in order to overcome this problem include:

   - Producing enzymes *in situ* as there are many bacteria and fungi that might produce the required enzymes.
   - Another potential strategy would be anaerobic sludge bioaugmentation. This strategy involves sludge enrichment with the identified microorganisms able to release the most suitable enzyme for microalgae cell wall disruption.

2) Assessing different strategies to avoid ammonium inhibition in anaerobic digestion such as:

   - Codiestion of microalgae biomass with high C/N ratio substrates.
   - Bioaugmenation with ammonia tolerant methanogenic consortia.
   - Adaptation of anaerobic consortia to high ammonium levels.

Finally, a life cycle assessment of integrated process, taking into consideration microalgae cultivation, harvesting, pretreatment and anaerobic degradation processes, is highly required.

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