Universidad de Valladolid

Escuela de Doctorado Universidad de Valladolid

PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA Y AMBIENTAL

TESIS DOCTORAL:

Innovative approaches for enhancing the cost-efficiency of biological methane abatement

Presentada por **Sara Cantera Ruiz de Pellón** para optar al grado de
Doctor/a por la Universidad de Valladolid

Dirigida por:
Dr. Raúl Muñoz Torre
Dr. Pedro Antonio García Encina

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SARA CANTERA RUIZ DE PELLÓN ha realizado bajo su dirección el trabajo "*Innovative approaches for enhancing the cost-efficiency of biological methane abatement*", en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, a _____ de_____ de 2018

Fdo. Raúl Muñoz Torre

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Resumen I

Abstract VI

List of publications XI

Contribution to the papers included in the thesis XII

Chapter 1. Introduction 1

1.1 Methane emissions: origin and environmental concerns 2

1.1.1 Methane sources and sinks: CH_4 budget 3

1.1.2 International initiatives for the reduction of methane emissions 4

1.2 End of the pipe solutions for methane abatement 5

1.2.1 Methane-oxidizing bacteria as the core of methane abatement biotechnologies 6

1.2.2 Biological technologies for CH_4 abatement 11

1.3 Current limitations in biological CH_4 treatment 12

1.3.1 Methane mass transfer in bioreactors 12

1.3.2 CH_4 biodegradation based on a waste-to-value approach 18

1.3.3 The need for a better understanding of methane-oxidizing bacteria 23

1.4 References 25

Chapter 2: Aims and Scope of the Thesis 34

2.1 Justification of the thesis 35

2.2 Main objectives 35

2.3 Development of the thesis 36

Chapter 3: Comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors for methane : Mass Transfer and Biological Considerations 38

Chapter 4: Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement. 61

Chapter 5: Continuous abatement of methane coupled with ectoine production by Methylomicrobium alcaliphilum 20Z in stirred tank reactors: a step further towards greenhouse gas biorefineries. 81

Chapter 6: Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-refineries into high added-value products. 106

Chapter 7: Multi-production of high added market value metabolites from methane emissions by an halotolerant methanotrophic consortia. 122

Chapter 8: Novel Alkalophilic and halophilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery **.** 141

Chapter 9: Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures. 165

Chapter 10: Conclusions and future work 186

Chapter 11: About the author 192

Resumen

El metano (CH_4), con un potencial de calentamiento global 25 veces mayor que el del CO_2 (en un horizonte temporal de 100 años) y unas emisiones a la atmósfera que representan el 18 % de las emisiones totales de gases de efecto invernadero en la UE-28, es el segundo gas de efecto invernadero más importante. La combustión de metano es energéticamente favorable a concentraciones superiores al 20 %, lo que posibilita que se pueda usar para la producción de energía en forma de calor. Sin embargo, más del 56 % de las emisiones antropogénicas contienen concentraciones de este gas inferiores al 5 %, por lo que no pueden ser destinadas a la producción de energía.

A pesar de la relevancia medioambiental del metano y de las cada vez más estrictas legislaciones para el control de sus emisiones, el desarrollo de tecnologías de tratamiento de CH_4 eficaces, económicas y respetuosas con el medio ambiente ha sido muy limitado. De hecho, incluso las tecnologías fisico/químicas más avanzadas son, a día de hoy, costosas e ineficaces para tratar las emisiones de las fuentes mayoritarias de CH_4 (gestión de residuos, ganadería intensiva, minería y combustión de carburantes). A este respecto, el tratamiento biológico de metano, basado en la acción de microorganismos capaces de degradar este gas, emerge como una alternativa más rentable y medioambientalmente respetuosa para el tratamiento de emisiones diluídas de metano. Las tecnologías biológicas han demostrado de forma concluyente ser un método de bajo coste, eficaz y de reducido impacto ambiental para el tratamiento de emisiones gaseosas odoríferas e industriales. Sin embargo, a pesar de que diferentes configuraciones de biorreactores se han implementado exitosamente para el tratamiento de compuestos orgánicos volátiles, el tratamiento de metano está limitado hoy en día por: 1) sus bajas tasas de transferencia desde la fase gas a la fase líquida donde se encuentran generalmente las comunidades microbianas encargadas de degradar el metano; 2) la falta de procesos rentables e industrialmente competitivos para degradar metano; 3) el limitado conocimiento del efecto de los parámetros ambientales sobre la estructura y las características de las comunidades metanotróficas encargadas de degradar el metano, así como de las cinéticas de biodegradación propias del tratamiento de emisiones diluidas de CH_4.

La principal limitación del tratamiento biológico de metano reside en la baja solubilidad de este gas en el medio acuoso donde se encuentra la biomasa encargada de degradarlo (constante de Henry (H) = 30 a 25 ºC), lo que supone mayores tiempos de residencia del gas y por ende mayores inversiones y costes de operación. Por ello, la implementación de un nuevo concepto de tratamiento biológico de emisiones gaseosas basado en un transporte directo gas-célula es indispensable tanto para optimizar el transporte como para mejorar el balance económico del proceso. El desarrollo de tecnologías innovadoras para el tratamiento biológico de metano centradas en la intensificación del transporte ha experimentado un fuerte crecimiento en los últimos diez años. Dentro de estas biotecnologías destacan los reactores bifásicos, los reactores de membrana, los biofiltros fúngicos y los reactores de flujo Taylor. Sin embargo, los estudios sobre el potencial de estas configuraciones para el tratamiento de metano son a día de hoy escasos y poco concluyentes.

En este contexto, una parte de la investigación planteada en esta tesis se centró en evaluar el potencial de una nueva generación de biorreactores bifásicos basados en el transporte directo de CH_4 *gas-célula*. Para ello se llevó a cabo el enriquecimiento y posterior caracterización de microorganismos metanótrofos hidrofóbicos, ensayos que permitieron determinar las condiciones óptimas de enriquecimiento (altas tasas de dilución y baja agitación). Además, se comparó el rendimiento de un biorreactor de tanque agitado convencional (medio mineral acuoso) con un sistema bifásico que contenía 60 % (vol.) de aceite de silicona y 40 % (vol.) de medio mineral acuoso, en el que los microorganismos metanótrofos crecían en la fase orgánica. Los resultados de este estudio no mostraron una mejora de la eliminación de metano en el reactor bifásico con los microorganismos embebidos en el aceite de silicona con respecto al reactor control. De hecho, se obtuvieron capacidades de eliminación de metano 3 veces mayores usando el reactor convencional bajo las mismas condiciones de operación. Este resultado se atribuyó a la baja afinidad del aceite de silicona por el metano (que es sólo 10 veces mayor que la del metano por el agua) en comparación con otros compuestos orgánicos volátiles, y a la posible limitación biológica por nutrientes y agua a la que están expuestos los organismos que crecen dentro de la fase orgánica.

En base a los resultados obtenidos en los biorreactores de partición de dos fases, se descartó esta alternativa, y la investigación posterior se centró en aumentar la rentabilidad del proceso de tratamiento de emisiones de metano en biorreactores de alto rendimiento mediante la producción de bioproductos de alto valor añadido. En este caso concreto, se evaluó la eliminación de metano acoplada a la producción de ectoína. La ectoína es un imino-ácido que producen distintos microorganismos para sobrevivir bajo altas concentraciones de sal, y es probablemente uno de los productos microbianos más valorados en la industria farmacéutica (US$1000 kg^{-1}), ya que es un importante estabilizador de enzimas, complejos ADN-proteína y ácidos nucleicos. A pesar de su potencial y alto valor, actualmente sólo se sintetiza comercialmente usando el microorganismo halófilo *Halomonas elongata* a través de un proceso industrial llamado *bio-milking*, el cual consiste en una fermentación en lote basada en choques hipo e híper osmóticos que promueven la producción de ectoína en el interior celular y su excreción al medio de cultivo. Sin embargo, este proceso está aún limitado por el alto coste del sustrato empleado en los fermentadores para el crecimiento de *H. Elongata* (habitualmente glucosa). En 1999, el equipo de Khmelenina y col. aislaron un nuevo metanótrofo alcalófilo y halotolerante (*Methylomicrobium alcaliphilum 20Z*) capaz de expresar los genes implicados en la producción de ectoína. Este estudio probó por primera vez la posibilidad de producir ectoína acoplada a la eliminación de metano. Sin embargo, no existía hasta la fecha ningún estudio de bioconversión en continuo de CH_4 a ectoína, además de haber una falta de conocimiento acerca de la influencia de los parámetros de operación tanto en el crecimiento de *M. alcaliphilum 20Z* como en la síntesis de este compuesto. Por ello, en la siguiente etapa de esta tesis doctoral se llevaron a cabo una serie de investigaciones destinadas a estudiar la transformación de metano en la sustancia de alto valor en el mercado, ectoína. En este contexto, se desarrolló un primer estudio para identificar aquellas condiciones de cultivo que proporcionaran máximas producciones de ectoína acopladas a la eliminación de metano. Los ensayos determinaron que concentraciones de 20 % CH_4, 6 % NaCl, 25 µM Cu^2 y temperaturas de 25 ºC promovían producciones máximas de ectoína intra-celular de hasta 67 ± 4 mg g $biomasa^{-1}$ usando *M. alcaliphilum* 20Z. Además, se observó que bajo altas concentraciones de cobre, esta bacteria era capaz de excretar de forma natural la ectoína al medio de cultivo. Lo que abría la posibilidad de extraer la

ecotína intra-celular modificando simplemente las condiciones de cultivo, reduciendo así los costes del proceso y mejorando notablemente el balance económico global. Un segundo estudio en la misma temática llevado a cabo en biorreactores de tanque agitado usando *M. alcaliphilum* 20Z demostró que esta cepa era capaz de producir de manera continua concentraciones de ectoína de 37 ± 4 mg g biomasa^{-1} operando el proceso de biodegradación de CH_4 con una concentración de NaCl del 6 %. Sin embargo, este estudio también demostró que el uso de altas velocidades de agitación en el proceso (600 rpm) dañaba la estructura celular de *M. alcaliphilum* 20Z, con la consiguiente diminución de la concentración de ectoína y de la capacidad del cultivo para eliminar CH_4.

Esta línea de investigación de la tesis se completó con un estudio de producción de ectoína mediante el proceso denominado *bio-milking*, con una fermentación por lotes inicial y una evaluación posterior del proceso en reactores en continuo. Los resultados obtenidos mostraron que, independientemente del proceso, *M. alcaliphilum 20Z* exhibía una respuesta rápida durante los choques osmóticos con la consiguiente acumulación de ectoína a altas salinidades y su excreción al inducir choques hiposmóticos. De hecho, concentraciones intra-celulares medias de ectoína de 70 ± 14 mg g biomasa^{-1} fueron registradas en este estudio, demostrándose así por primera vez que era factible el uso de *M. alcaliphilum 20Z* para producir ectoína a través del proceso *bio-milking*, lo que sugiere la posibilidad de implementar el tratamiento de este gas de efecto invernadero a mayor escala.

Sin embargo, a pesar de la producción en continuo de altas concentraciones de ectoína, *M. alcaliphilum 20Z* mostraba bajas tasas de crecimiento y fue muy sensible al esfuerzo cortante, lo que obligaba a operar los biorreactores a bajas velocidades de agitación y conllevaba bajas productividades de biomasa en comparación con sus homólogos heterótrofos en sustratos solubles. De acuerdo a estos resultados se reorientó la investigación doctoral con el objetivo de conseguir: i) una producción simultánea de múltiples bioproductos de alto valor usando metano como única fuente de carbono y energía; ii) el aislamiento de nuevos microorganismos metanótrofos capaces de producir ectoína a partir de la degradación de metano, lo que permitiría encontrar cepas más eficientes en la producción de ectoína y degradación de metano; iii) la determinación de las condiciones de cultivo óptimas para mejorar las tasas de degradación de CH_4 de comunidades metanótrofas.

Para ello se diseñó, implementó y evaluó un biorreactor de columna por burbujeo para el tratamiento de CH_4 que permitiera la producción simultánea de varios bioproductos de alto valor agregado usando un consorcio metanótrofo haloalcalófilo enriquecido, conformado principalmente por los géneros *Halomonas*, *Marinobacter*, *Methylophaga* y *Methylomicrobium*. Este consorcio fue capaz de acumular en continuo la misma concentración de ectoína que *M. alcaliphilum* (80 mg ectoína g biomasa^{-1}) y co-producir además hidroxiectoína (13 mg hidroxiectoina g biomasa^{-1}) y exopolisacáridos (hasta 2.6 g EPS g biomasa^{-1}). Desafortunadamente, este consorcio no fue capaza de sintetizar bioplásticos bajo estas condiciones de operación, ya que su síntesis requiere de la limitación de nutrientes lo que conllevaría una diminución de la producción del resto de bioproductos. Este trabajo constituyó el primer estudio de viabilidad de la co-producción de bioproductos comerciales a partir de metano, lo que permitiría mejorar el balance económico de los tratamientos biológicos actuales gracias a la comercialización de los bioproductos obtenidos.

Por otro lado, el enriquecimiento de nuevos microorganismos metanótrofos halotolerantes permitió el aislamiento de dos nuevas especies capaces de oxidar metano a alta salinidad: *Alishewanella* sp. strain RM y *Halomonas* sp. strain PGE (géneros no identificados hasta el momento como degradadores de metano). *Halomonas* sp. strain PGE fue capaz de sintetizar ectoína (70-92 mg ectoína g biomasa^{-1}) a mayores concentraciones que los metanótrofos productores de ectoína identificados hasta el momento (entre 37.4 y 70.0 mg ectoína g biomasa^{-1}). Sin embargo, su crecimiento se veía probablemente inhibido por la producción de un metabolito tóxico en el proceso de degradación de metano, lo que obligaba a aumentar la tasa de dilución del medio mineral para mantener la capacidad de degradación de CH_4.

Finalmente, se evaluó el efecto del cobre, de la concentración de CH_4 y de la velocidad de transferencia durante el enriquecimiento de microorganisms metanótrofos en sistemas en lote. Los resultados obtenidos demostraron que un incremento en la concentración de cobre de 0.05 a 25 μM aumentaba la q_{max} y K_s de las comunidades enriquecidas en un factor de 3. Además, se observó por primera vez que altas concentraciones de cobre promovían el crecimiento de poblaciones más especializadas y menos diversas. Aunque la concentración de CH_4 no tuvo un efecto significativo sobre la estructura microbiana o su cinética de

degradación de CH_4 a las dos concentraciones estudiadas (4 y 8%), este estudio constituyó una prueba empírica sólida de la importancia de las condiciones de enriquecimiento en las características de las comunidades metanótrofas, identificándose el cobre como un factor crucial para mantener una alta eficiencia y resiliencia en la degradación de metano.

Los resultados de esta tesis constituyen una primera aproximación al tratamiento de gases basado en el concepto de biorrefinería, y abren una nueva puerta al tratamiento de metano competitivo y eficiente mediante su transformación en bioproductos de valor agregado como la ectoína.

Abstract

Methane (CH_4), which is considered the second most important greenhouse gas (GHG), represents nowadays 18 % of the total EU-28 GHG emissions and possesses a global warming potential 25 times higher than that of CO_2 (in a 100 yr horizon). The methane produced during the anaerobic digestion of organic waste (wastewater and organic solid waste) can be collected and combusted for the generation of electricity and/or heat, thus replacing fossil fuels. However, methane can be only used for energy generation when its concentration in the emission is higher than 20 %. Unfortunately, more than 56 % of the anthropogenic CH_4 emissions contain CH_4 concentrations below 5 %, which significantly limits the implementation of energy recovery-based treatment technologies. Therefore, cost-efficient abatement methods to mitigate the pernicious environmental effects of residual CH_4 emissions are required.

In this regard, despite the environmental relevance of CH_4 and forthcoming stricter regulations, the development of cost-efficient and environmentally friendly GHG treatment technologies is nowadays very limited. Hence, even state-of-the-art physical/chemical technologies are either costly or inefficient when treating the emissions of the largest sources of CH_4 (waste treatment, livestock farming, mining, fuel combustion). In this context, despite biological technologies have been consistently proven as a low-cost, efficient, and environmentally friendly method for the treatment of gas emissions containing malodours or industrial volatile organic compounds, the application of conventional biotechnologies for the abatement of this GHG is nowadays severely limited by: 1) its poor mass transport from the gas to the microbial community as a result of its poor aqueous solubility 2) the lack of cost-competitive abatement methods for the treatment of diluted CH_4 emissions 3) the limited knowledge of the microbial communities and biodegradation kinetics of methane at the trace level concentrations typically encountered during the treatment of diluted CH_4 emissions.

The most important limitation of biological methane abatement is caused by the low aqueous solubility of this GHG (Henry's law constant (H) = 30 at 25ºC). This limited mass transport entails process operation at high empty bed gas residence times, which significantly increases both the investment and operating costs of methane treatment

biotechnologies. In this regard, the implementation of a new concept of biological gas treatment based on the direct *gas-cell* transport of CH_4 could enhance the mass transport of this hydrophobic GHG. Multiple innovative and high-performance technologies for biological gas treatment have emerged in the last 10 years: two-phase partitioning bioreactors (TPPB), membrane bioreactors, fungal-based biofilters and Taylor flow bioreactors. Unfortunately, the number of studies assessing the potential of these bioreactors for the treatment of CH_4 is still scarce.

In this regard, a part of the research carried out on the present thesis focused on the comparative performance evaluation of conventional and two-phase partitioning stirred tank reactors for methane abatement. This research demonstrated for the first time the capability of methanotrophs to grow inside the non-aqueous phase (NAP) and identified the optimum cultivation conditions for enrichment of hydrophobic methanotrophs (high dilution rates and low agitation rates). The potential of the hydrophobic methanotrophs enriched was assessed in a single-phase stirred tank reactor and in a two-phase stirred tank reactor containing 60 % of silicone oil. Contrary to what it was expected, the results showed that the single-phase stirred tank reactor achieved higher elimination capacities (EC up to ≈3 times) than the TPPB. This might be due to the low affinity of silicone oil for methane, which is only 10 times higher than that of CH_4 for water, as well as to the limitation in nutrients or water activity inside the NAP, which can cause limitations in biological activity.

Based on the above results, alternative biotechnologies were developed, implemented and evaluated along this thesis, with a focus on biological methods that could make this process more environmentally friendly and cost-competitive. More specifically, the present thesis focused on the valorization of dilute methane emissions via CH_4 bio-conversion into ectoine, which is a cyclic imino acid used in the pharmaceutical industry due to its high effectiveness as stabilizer of enzymes, DNA-protein complexes and nucleic acids, with a retail value of approximately US$1000 kg^{-1}. Despite its potential, ectoine is only currently produced biotechnologically by *Halomonas elongate* through a long fed-batch fermentation process called *bio-milking*, which consists of sequential hypo and hyper osmotic shocks. However, this process is still inefficient due to the high cost of the sugar-based substrate used. In 1999, the team of Khemelenina and co-workers described a new species of

haloalkalophilic bacterium, *Methylomicrobium alcaliphilum 20Z*, capable of synthesizing ectoine from methane. However, little was known about the influence of environmental conditions on the bioproduction of this secondary metabolite when combined with the abatement of diluted CH_4 emissions. In addition, no study addressing the continuous production of ectoine by *M. alcaliphilum 20Z* has been carried out to date using methane as the sole carbon and energy source.

In this context, a first attempt assessing the influence of operational conditions on the bio-conversion of CH_4 into ectoine by the methanotrophic ectoine-producing strain *M. alcaliphilum 20 Z* was carried out in this thesis. The results obtained demonstrated that a proper selection of the environmental parameters (temperature, CH_4, Cu^{2+} and NaCl concentration) during *M. alcaliphilum 20Z* cultivation was crucial to simultaneously maximize both, the intra-cellular production and excretion of ectoine and CH_4 abatement. Hence, concentrations of 20 % CH_4, 6 % NaCl, 25 µM Cu^2 and a temperature of 25 ºC supported a maximum intra-cellular ectoine production yield of 67 ± 4 mg g biomass^{-1}. On the other hand, extra-cellular ectoine concentrations were detected at high Cu^{2+} concentrations despite this methanotroph has not been previously classified as an ectoine-excreting strain. These promising results supported further research in order to implement the bio-conversion of CH_4 into ectoine in a continuous system capable of creating value out of GHG mitigation. In this regard, a study of ectoine production during the continuous abatement of diluted emissions of CH_4 by *M. alcaliphilum 20Z* in stirred tank reactors under non-sterile conditions was carried out during the development of this thesis. In this research, NaCl concentration was identified as the main factor influencing the accumulation of intra-cellular ectoine in continuous mode, with high salt concentrations of 6% NaCl inducing average intra-cellular ectoine yields of 37 ± 4 mg g biomass^{-1}. Moreover, it was observed that process operation at high agitation rates (600 rpm) damaged cell integrity, with a subsequent decrease in both CH_4 removals and ectoine yields. Thus, this study demonstrated the feasibility of producing ectoine in continuous bioreactors during methane treatment and identified the best operational conditions of the process. These results encouraged further research in order to implement CH_4 biorefineries for the production of ectoine in a fed-batch fermentation using a *bio-milking* approach. The results demonstrated that *M. alcaliphilum 20Z* exhibited a rapid response to osmotic shocks in batch and

continuous mode, which resulted in the release of the accumulated ectoine under hyposmotic shocks and the immediate uptake of the previously excreted ectoine during hyperosmotic shocks. The intra-cellular ectoine yield obtained in this study under optimal operational conditions accounted for 70 ± 14 mg g biomass^{-1} under steady state, and constituted the first proof of concept of ectoine *bio-milking* coupled to CH_4 abatement from diluted emissions.

Although at this point, the perspective of a future methane-based biorefinery was promising, the implementation of these CH_4 bio-conversion processes was still limited by physical and biological limitations. Thus, the low growth rate of *M. alcaliphilum 20Z and* its fragility against mechanical stress hampered the process productivities obtained. These findings lead to explore the enhancement of the cost-effectiveness of the bio-conversion of methane into ectoine by i) co-producing multiple metabolites using methane as the sole carbon and energy source, ii) isolating new haloalkaliphilic methanotrophs able to produce ectoine and iii) determining the best culture conditions to obtain high bio-conversion rates of methane.

In this context, a bubble column bioreactor configuration was used to assess the co-production of multiple metabolites of high-added market value during CH_4 mitigation using an enriched methanotrophic consortium (consisted mainly of the genera *Halomonas*, *Marinobacter*, *Methylophaga* and *Methylomicrobium*). This consortium was capable of accumulating the same concentration of ectoine than *M. alcaliphilum* (80 mg ectoine g biomass^{-1}) with the concomitant synthesis of hydroxyectoine (up to 13 mg hydroxyectoine g biomass^{-1}) and exopolysaccharides (up to 2.6 g EPS g biomass^{-1}). Unfortunately, bioplastics were not accumulated inside the methanotrophic consortium based on the absence of a fasting period that allowed the bacterial synthesis of PHAs. This research represented the first proof of concept of a methane biorefinery based on the multi-production of high profit margin substances using methane as the sole carbon and energy source. Additionally, this thesis also focused on the enrichment and isolation of novel haloalcaliphilic methanotrophic strains able to support high productivities of ectoine during CH_4 abatement, which resulted in the discovery of two novel methane oxidizing bacterial species that were not previously identified as methanotrophic bacteria (*Alishewanella* and *Halomonas*). Based on a dissimilarity of at least 98 % in the 16SrDNA sequences with their

closest relatives, these bacteria were described as two novel strains (representing novel species): *Alishewanella sp.* strain RM1 and *Halomonas sp.* strain PGE1. *Halomonas sp.* strain PGE1 presented higher ectoine yields (70-92 mg ectoine g biomass^{-1}) than those generally obtained in *M. alcaliphilum* 20Z (37.4 to 70.0 mg ectoine g biomass^{-1}), although the growth of *Halomonas sp*. strain PGE1 was likely inhibited by the production of a toxic metabolite.

Finally, the influence of Cu^{2+} and CH_4 concentrations and CH_4 mass transfer rate during culture enrichment on the community structure and the CH_4 biodegradation kinetics was studied in this thesis in order to standardize the enrichment conditions for highly efficient methanotrophs. The results obtained demonstrated that an increase in Cu^{2+} concentration from 0.05 to 25 µM increased the q_{max} and K_s of the communities enriched by a factor of ≈ 3. In addition, high Cu^{2+} concentrations supported the growth of more adapted methanotrophs and less diverse bacterial communities. However, no clear effect of CH_4 concentration on the population structure or on the biodegradation kinetics of the communities enriched was recorded at the two CH_4 concentrations studied (4 and 8%). This study showed the key role of enrichment conditions to develop microbial communities capable of maintaining a high efficiency and robustness, overcoming some of the current limitations of biotechnologies and improving methane abatement performance.

The results obtained in the present thesis open up a new door of possibilities towards a more cost-effective methane treatment based on the bio-conversion of this GHG into products with a high profit margin, which could completely switch the current conception of GHG abatement technologies.

List of publications

The following publications are presented as part of the present thesis. Five of them are published in international journals indexed in ISI web of knowledge (Papers I to V). Paper VI has been submitted for publication and Paper VII remains to date as an unpublished manuscript.

Paper I. Cantera S, Estrada J.M, Lebrero R, García-Encina P, Muñoz R (2015) *Comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors for methane abatement: Mass Transfer and Biological Considerations.* Biotechnology and Bioengineering 113(6): 1203-1212.

Paper II. Cantera S, Lebrero R, García-Encina P.A, Muñoz R. (2016) *Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures*. Journal of Environmental Management 171 (15): 11-20.

Paper III. Cantera S, Sadornil L, Lebrero R, García-Encina P, Muñoz R. (2016***).*** *Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement*. Journal of Environmental Management 182: 160-165.

Paper IV. Cantera S, Lebrero R, Rodríguez E, García-Encina P, Muñoz R (2017). *Continuous abatement of methane coupled with ectoine production by Methylomicrobium alcaliphilum 20Z in stirred tank reactors: a step further towards greenhouse gas biorefineries.* Journal of Cleaner Production 152:134-151.

Paper V. Cantera S, Lebrero R, Rodríguez S, García-Encina P, Muñoz R (2017). Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-refineries into high added-value products. Chemical Engineering Journal 328: 44-48.

Paper VI. Cantera S, Sánchez-Andrea I, García-Encina P.A, Stams S.J.M, Muñoz R**.** *Novel halo-alkalophilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery* Waste Management submitted for publication.

Paper VII. Cantera S, Sánchez-Andrea I, Lebrero R, García-Encina P.A, Muñoz R (2018). *Multi-productionof high added market value metabolites from methane emissions by an halotolerant methanotrophic consortia*. Unpublished manuscript.

Contribution to the papers included in the thesis

Paper I. In this work, I was responsible for the design, start-up and operation of the experimental set-up in collaboration with Dr. José Estrada and under the supervision of Dr. Raúl Muñoz and Dr. Pedro A. García-Encina. Moreover, I was responsible for the microbiological characterization, data analysis and phylogenetical tree construction. I was in charge also of the manuscript writing under the supervision of Dr. Raquel Lebrero and Dr. Raúl Muñoz.

Paper II. During the execution of this work, I was responsible for the design, start-up and operation of the experimental set-up, results evaluation and manuscript writing with the collaboration of Dr. Raquel Lebrero and under the supervision of Dr. Raúl Muñoz. and Dr. Pedro A. García-Encina. Moreover, I was responsible of the microbiological characterization, data analysis and discussion.

Paper III. In this work, I was responsible for the design, start-up and operation of the experimental set-up and results evaluation in collaboration with Miss. Lidia J. Sadornil. I prepared the manuscript with the collaboration of Dr. Raquel Lebrero under the supervision of Dr. Raúl Muñoz and Dr. Pedro A. García-Encina

Paper IV. In this work, I was responsible for the design, start-up and operation of the experimental set-up and results evaluation with the collaboration of MEng. Suní Rodríguez and under the supervision of Dr. Raquel Lebrero and Dr. Raúl Muñoz. I prepared the manuscript under the supervision of Dr. Pedro A. García-Encina and Dr. Raúl Muñoz. Dr. Elisa Rodríguez was responsible of the microbiological characterization, where I contributed in the data analysis and discussion.

Paper V. During this research, I was in charge for the design, start-up and operation of the experimental set-up and results evaluation in collaboration with MEng. Suní Rodríguez and under the supervision of Dr. Raquel Lebrero and Dr. Raúl Muñoz. I prepared the manuscript under the supervision of Dr. Pedro A. García-Encina and Dr. Raúl Muñoz.

Paper VI. During this research, I was in charge of the experimental design, isolation development and results under the supervision of Dr. Irene Sánchez-Andrea (Wageningen University) and Raúl Muñoz. I prepared the manuscript under the supervision of Dr. Pedro

A. García-Encina and Dr. Raúl Muñoz. Dr. Irene Sánchez-Andrea was responsible of the phylogenetical analysis, where I contributed in the discussion section. Part of this work was carried out in the Laboratory of Microbiology of Wageningen University (The Netherlands).

Paper VII. In this work, I was responsible for the experimental methodology design, implementation and results evaluation under the supervision of Dr. Raúl Muñoz. Dr. Irene Sánchez-Andrea was responsible of the microbiological analysis, where I contributed in the discussion section. I prepared the manuscript with the collaboration of Dr. Raquel Lebrero and Dr. Irene Sánchez-Andrea and under the supervision of Dr. Raúl Muñoz and Dr. Pedro A. García-Encina.

Introduction

Chapter 1

1. Introduction

1.1 *Methane emissions: origin and environmental concerns*

The steady rise in the average temperature of the Earth within the last decades and its associated detrimental impacts on the environment have resulted in an increasing concern about global warming and greenhouse gas (GHG) emissions [1–3]. Methane, which is considered the second most important GHG, represents 18 % of the total EU-28 GHG emissions and possesses a global warming potential 25 times higher than that of CO_2 (in a 100 yr horizon) [4,5]. CH_4 atmospheric concentration has increased yearly at 0.2-1 % since 1950, which together with the increase in CO_2 concentration have caused a rise in the global average temperature of 0.85 ± 0.2 ºC in the past 70 years. This temperature increase is severely damaging the environment and compromising human health and industrial development[3,6,7] (Figure 1.1).

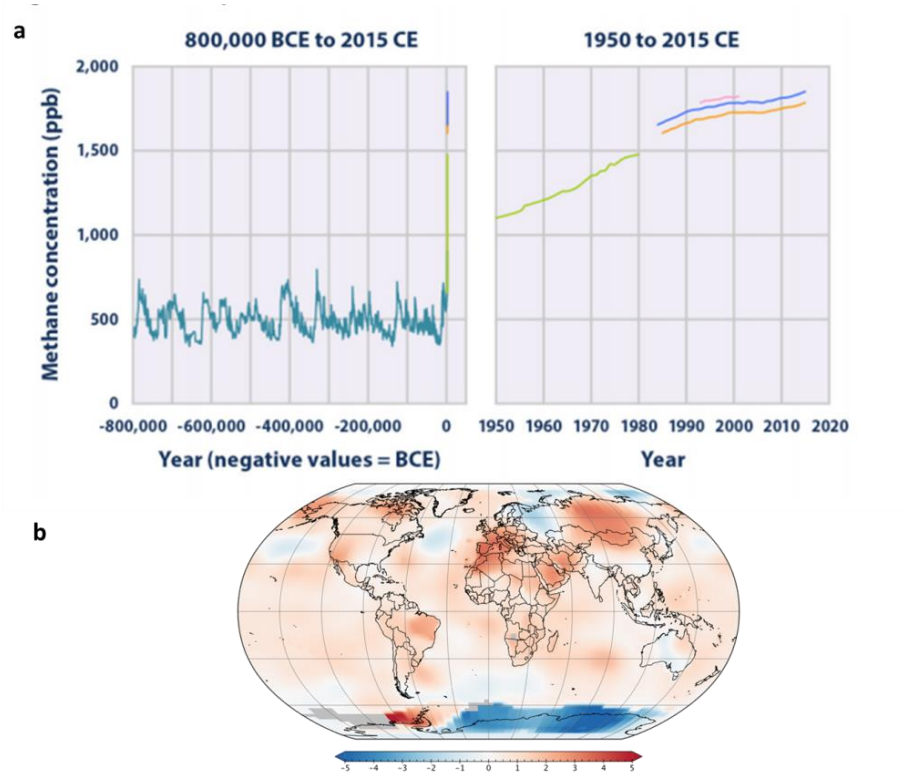

Figure 1.1 a) Time course of the concentration of methane in the atmosphere measured in parts per billion (ppb) [3]. b) A global map of the June 2017 LOTI (land-ocean temperature index) anomaly, relative to the 1951-1980 June average [8]**.**

1.1.1 *Methane sources and sinks: CH_4 budget*

Methane has been naturally generated during millions of years from a wide range of natural sources and it is considered the most abundant organic molecule in the atmosphere [4,5]. In this context, CH_4 natural emissions have been broadly grouped into three categories: biogenic, thermogenic and pyrogenic. Biogenic sources involve CH_4-generating organisms (methanogens), and comprise anaerobic environments such as natural wetlands and rice paddies (60.1 %), oxygen-depleted freshwater reservoirs (such as dams) (12.3 %) and digestive systems of ruminants and termites (8.0 %). Thermogenic CH_4, formed over millions of years through geological processes, is a fossil fuel that is vented from the subsurface into the atmosphere through natural mechanisms (such as terrestrial seeps, marine seeps and mud volcanoes) (18.7 %). On the other hand, pyrogenic CH_4 is produced from the incomplete combustion of biomass and soil carbon mainly during wildfires (0.9 %). Before the industrial revolution, the methane released from the above mentioned sources was destroyed or captured by different natural sinks such as the oxidation by hydroxyl radicals (OH) in the troposphere or by methanotrophic bacteria in aerated soils, the reaction with chlorine radicals and atomic oxygen radicals in the stratosphere and the reactions with chlorine radicals from sea salt in the marine boundary layer, thus balancing the methane cycle[6,9,10].

However, over the past 150 years, CH_4 concentration in the atmosphere has increased at a rate of 0.2-1% yr^{-1} due to human activities. Anthropogenic activities currently account for 50 % of all methane emissions at a global scale (Figure 1.2). The main anthropogenic sources are waste management (i.e manure, sewage and urban solid waste), livestock enteric fermentation (61.5 %), coal, oil and natural gas mining (28.8 %) and the incomplete combustion of biofuels and fossil fuels (9.7 %). This new scenario has created an imbalance between the emission (616 Tg CH_4 yr^{-1}) and removal (586 Tg CH_4 yr^{-1}) of CH_4 (Figure 1.2). Thus, although methane natural sinks are still able to remove higher amounts of methane than those emitted from natural sources, there is still a net emission of 30 Tg CH_4 yr^{-1} that accumulates in the atmosphere. It is therefore important to reduce the global CH_4 emissions below the natural CH_4 abatement capacity of the planet in order to decrease the methane concentrations in the atmosphere and its subsequent warming effect [9,10].

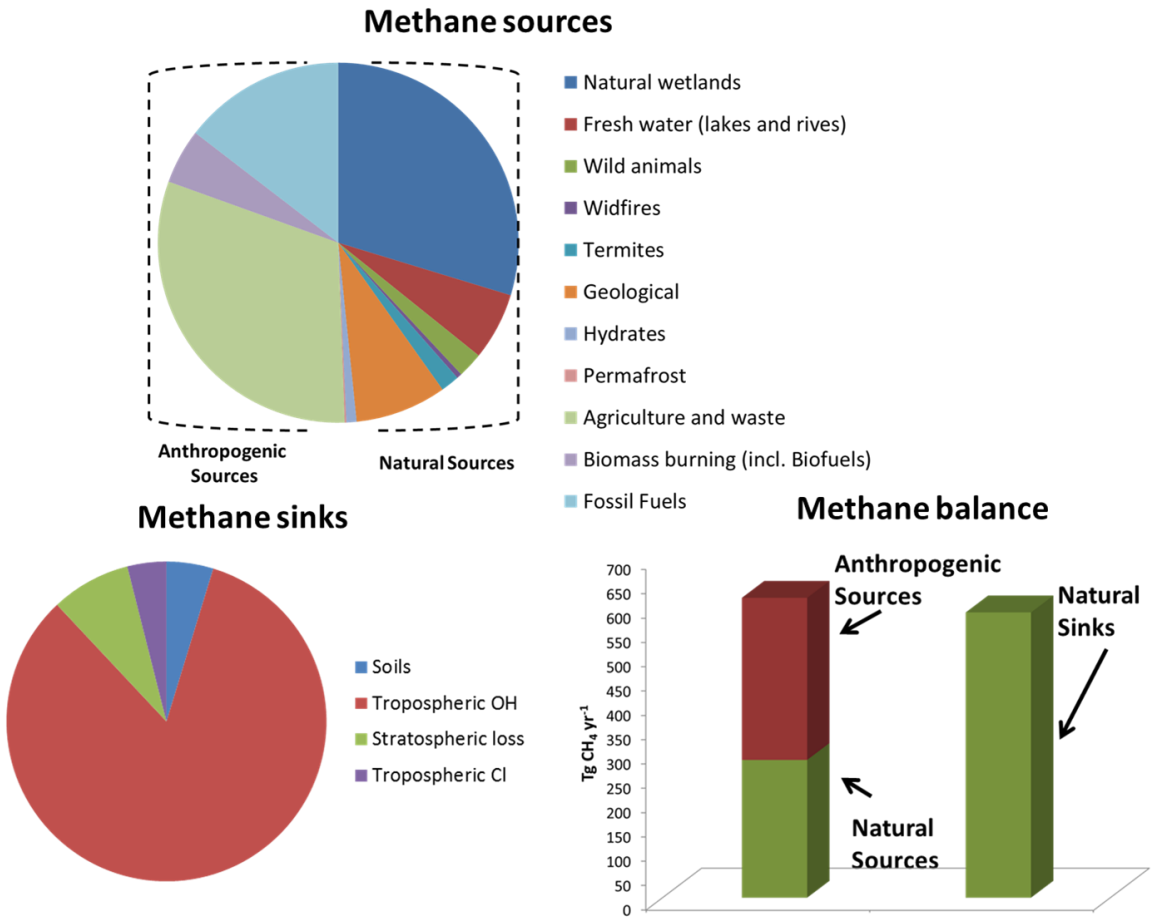

Figure 1.2 Methane sources, sinks and methane imbalance (sources-sinks). The numeric data were obtained from the methane budgets compilations carried out from 2000 to 2009 and 2012 [9,10].

In this context, an annual reduction in the global CH_4 emissions of 30 Mt would result in the stabilization of methane concentrations in the atmosphere. Such a reduction represents just 9.0 % of the total methane emissions and could be achieved via cost-effective end of the pipe abatement technologies [9].

1.1.2 *International initiatives for the reduction of methane emissions*

Climate change represents the greatest global environmental problem in this XXI century, which has forced governments to gradually implement policies in order to limit their GHG emissions. In this context, The United Nations Framework Convention on Climate Change (UNFCCC), which involves 173 countries (such as the member states of the European Union, North and South America, South Asia and North Africa), agreed during

the 2014 Conference of the Parties held in Paris to work on limiting the global temperature rise to well below 2 degrees Celsius above pre-industrial levels throughout boosting the ability to adapt to the adverse impacts of climate change, fostering climate resilience and low greenhouse gas emissions development policies and making finance flows consistent with a roadmap towards low greenhouse gas emissions and climate-resilient economy [7].

On the other hand, the Global Methane Initiative (GMI), which involves mainly the private and social sectors, represents also one of the most important initiatives for methane abatement. This initiative consists of a voluntary, international partnership that brings together national governments, private sector entities, development banks, non-governmental organizations and other interested stakeholders in a collaborative effort to reduce methane gas emissions and advance methane recovery. GMI Partner Countries account for approximately 70 % of the total anthropogenic methane emissions. Furthermore, the GMI established in 2016 new alliances with two key partners: the United Nations Economic Commission for Europe (UNECE) and the Climate and Clean Air Coalition (CCAC)[5].

In order to comply with the Paris agreement and GMI recommendations, an active abatement of the GHG emissions is mandatory and expected to become a general trend worldwide in the next decades.

1.2 *End of the pipe solutions for methane abatement*

Anthropogenic methane emissions from waste management have been typically controlled via traditional physical-chemical methods such as flaring or adsorption. However, these technologies are environmentally unfriendly (entail a large CO_2 footprint as a result of their intensive energy usage) and/or present prohibitive operating costs. In addition, the energy potential of methane is often wasted during flaring or incineration. Thus, the most cost-effective approach to control methane emissions from waste treatment relies on its conversion into an alternative income source. In this regard, methane derived from waste treatment can be collected and combusted for the generation of electricity and/or heat, thus replacing fossil fuels [5]. However, methane can only be used for energy recovery when its concentration in the emission is higher than 30 %. Unfortunately, more than 56 % of the

anthropogenic CH_4 emissions contain CH_4 concentrations below 4 %. For instance, dilute CH_4 emissions are typically found in old landfills (0–20%), ventilated coal mines (0.1–1%), covered liquid manure storage tanks (0–3%) or in confined cattle operations (<1%) [11,12]. Therefore, these methane sources result in emissions with low CH_4 concentrations, which significantly limits the implementation of energy recovery-based treatment technologies and require the application of cost-efficient abatement methods to mitigate the pernicious environmental effects of CH_4.

Biological technologies can become, if properly tailored, a low-cost and environmentally friendly alternative for the control of dilute CH_4 emissions. Off-gas treatment biotechnologies are based on the biocatalytic oxidation of pollutants following their transfer from the gas emission to an aqueous phase. The microbial oxidation of these gas pollutants occur at ambient temperature and pressure, and in the absence of an external supply of chemicals (only water and nutrients). The absence of extreme operating conditions and hazardous chemicals constitutes an additional advantage from an occupational safety viewpoint for on-site staff and operators. Biotechnologies have been successfully proven as robust and efficient abatement methods for the treatment of industrial volatile organic compounds (VOCs) and malodours, exhibiting a comparable abatement efficiency to their physical/chemical counterparts [13,14]. Additionally, dilute CH_4 emissions could be also used by microorganisms as raw materials to synthesize high added-value products such as biopolymers, exopolysaccharides or ectoine under specific environmental conditions. The valorization of residual CH_4 through its bioconversion into bioproducts with a high market price could turn their abatement into a sustainable process.

1.2.1 *Methane-oxidizing bacteria as the core of methane abatement biotechnologies*

Biotechnologies devoted to CH_4 treatment are based on the biocatalytic action of microorganisms, mainly aerobic methane-oxidizing bacteria (MOB, also called methanotrophs), that transform methane into carbon dioxide, biomass and water using oxygen as electron acceptor. Methane-oxidizing bacteria belong to the methylotrophic bacterial group, consisting of organisms that utilize reduced one-carbon substrates for their metabolism. Among methylotrophs, MOB were classified and considered the only group able to use CH_4 as their single energy and carbon source [15]. However, recent findings

have demonstrated that some methanotrophs are also able to utilize multicarbon compounds as their carbon and energy source in some environments [16].

Traditionally, MOB have been classified into three different groups according to their physiological and morphological characteristics: type I, type II and type X methanotrophs. Type I includes those MOBs that a) present intracytoplasmatic membranes as bundles of vesicular discs, b) use the ribulose monophosphate (RuMP) pathway for carbon assimilation and c) contain phospholipid fatty acids of 14 and 16 carbons length. Type II MOBs are characterized by a) an intracytoplasmatic membrane aligned along the periphery of the cell, b) the use of the serine pathway for carbon assimilation and c) phospholipid fatty acids of 18 carbons length. On the other hand, type X MOBs share characteristics of both type I and II, including the RuMP pathway to assimilate formaldehyde and the synthesis of the enzyme ribulose biphosphate carboxylase from the serine pathway to fix CO_2 [15,17]. A brief summary of the biochemical methane assimilation pathways of MOBs is shown in figure 1.3. The current classification of known aerobic methanotrophic genera based on 16S rRNA comprises a wide phylogenetic distribution within the three general groups: *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobia* [18,19]. The alphaproteobacterial methanotrophs can be further divided into the *Beijerinckiaceae* and *Methylocystaceae* families, while the *Methylococcaceae* and the *Methylacidiphilacecae* families belong to the gammaproteobacterial and verrucomicrobial methanotrophs, respectively. Furthermore, the phylogenetic relationships among methanotrophs are also commonly examined considering the enzyme methane monooxygenase (MMO), which is used in the conversion of methane to methanol. More specifically, the particulate methane monooxygenase (pMMO) is found in most known MOBs and is located in the cytoplasmatic membrane, while the soluble methane monooxygenase (sMMO) is present in the cytoplasm and can be expressed either as the sole form of MMO or together with pMMO [20]. Despite the enzyme sMMO has been traditionally associated with type II methanotrophs, it has been recently found that some genera of type I methanotrophs are also capable of synthesizing sMMO [21].

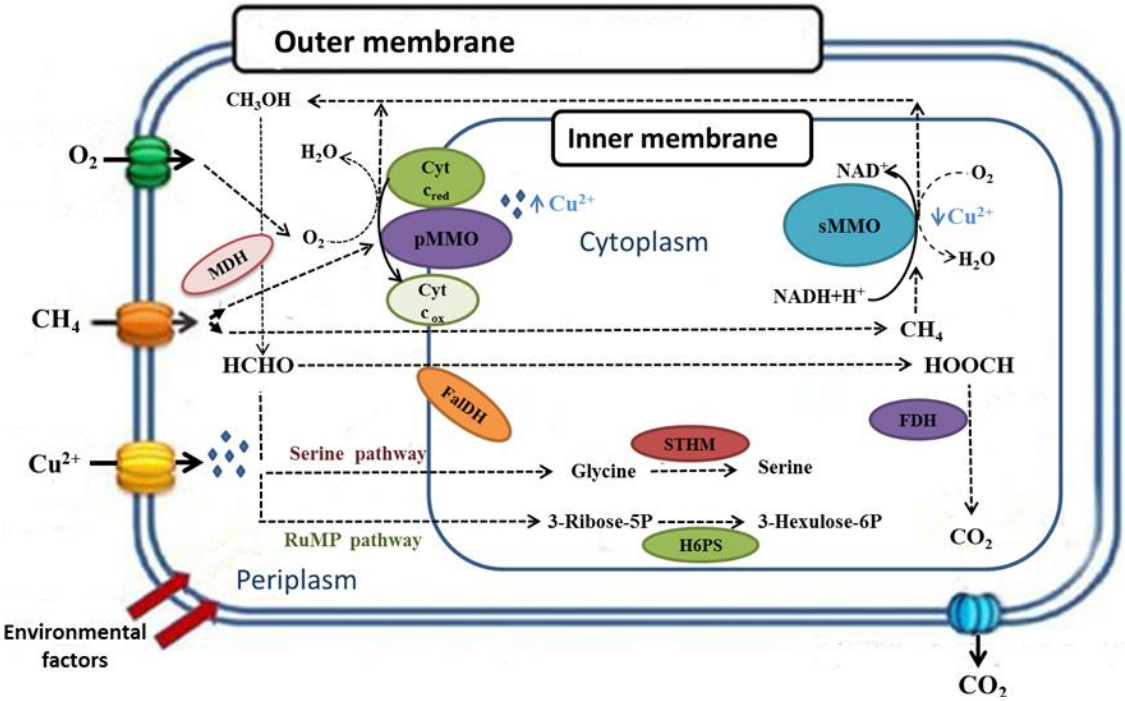

Figure 1.3 Methane oxidation pathways in methanotrophic bacteria, where: sMMO: soluble methane monooxygenase; pMMO: particulate methane monooxygenase; MDH: methanol dehydrogenase; FDH: formate dehydrogenase; FaIDH: formaldehyde dehydrogenase; Cyt red/ox: cytochrome reduced/oxidized; STHM: serine hydroxymethyltransferase; H6PS: hexulose-6-phosphate synthase. Adapted from Cantera et al. (2017)[25].

Aerobic methanotrophs are widely distributed in the environment and they can be found in the different ecosystems, such as wetlands, bogs, forests, rice paddies, groundwater, landfill cover soils, etc. [19]. The optimum temperature for methane oxidation has been established at 25 °C, with an optimum pH range of 7.0 to 7.6, even though these values can change depending on the species [23–25] (Table 1). Methane concentrations ranging from 4 to 23% do not significantly influence the microbial growth or CH_4 oxidation rates [26–28]. However, an increase in the concentration of methane over 20% leads to higher methanotrophic activity according to Hanson and Hanson (1996) [15]. The influence of the O_2 concentration on the methanotorphic growth and CH_4 biodegradation cultivation efficiency is quite ambiguous and depends on the source of the inoculum. For instance, Bussman et al. (2004) reported O_2 concentrations of 17% as the optimum values for methane oxidation in lakes, while O_2 concentrations of 0.1–11% resulted in a decrease in methanotrophic growth [26]. However, methanotrophs from rice soils grew better under O_2

concentrations lower than 1% [29,30]. Similarly, the ratio CH_4/O_2 constitutes an important parameter due to its influence on the type of MMO expressed. At low CH_4:O_2 ratios, methanotrophic communities preferentially express pMMO (low Ks, which entails a higher affinity for the substrate), while sMMO is preferentially expressed at high CH_4:O_2 ratios (high Ks, lower affinity for CH_4) [19,31,32]. High ammonium concentrations in the cultivation broth have a negative influence on the cultivation of methanotrophs. Indeed, NH_4^+ is a competitive inhibitor of methane, binding on the active site of pMMO [33]. Likewise, magnesium and sulfate can inhibit microbial growth at high concentrations (>1 mM) [26], the optimal reported magnesium concentration being 50 µM [34]. However, the most important nutrient governing methane degradation is copper, due to its key role on the relative expression of the enzymes sMMO and pMMO. Cu^{2+} increases the expression of pMMO at levels higher than 0.86 µmol Cu^{2+} g $biomass^{-1}$, thus enhancing CH_4 biodegradation [19,28].

Even if aerobic oxidation represents the main process implemented in biotechnologies treating methane, recent findings have demonstrated that some anaerobic archaea and bacteria are responsible for 7-25% of the total methane oxidation worldwide. The anaerobic oxidation of methane (AOM) is carried out by bacteria belonging to the NC10 phylum (*Candidatus* '*Methylomirabilis oxyfera*'), which couples methane oxidation to denitrification, and three groups of archaea: ANME-1 (distantly related to the *Methanosarcinales* and *Methanomicrobiales* spp.), ANME-2 (within the *Methanosarcinales* sp.), and ANME-3 (closely related to the *Methanococcoides* sp.) [35,36]. ANME-1 and ANME-2a, 2b and 2c oxidize methane using sulfate as electron acceptor (forming consortia with sulfate reducing bacteria), while some ANME-2 (ANME-2d) belong to a cluster that couples AOM to nitrate reduction in archaea of the order *Methanosarcinales* (related to '*Ca. Methanoperedens nitroreducens*'). Moreover, some ANME-2 are also able to oxidize methane using metals such as iron or humic acids as electron acceptors without a syntrophic partner. In the particular case of ANME-3, little is known about its metabolism. Additionally, some fungal genera such as *Graphium* have been reported as methane oxidizers [37,38].

Table 1. Main characteristics of methanotrophic bacteria				
Phylum	*Gammaproteobacteria*	*Alphaproteobacteria*	*Alphaproteobacteria*	*Verrucomicrobia*
Family	*Methylococcaceae,*	*Methylocystaceae*	*Beijerinckaceae*	*Methylacidiphilaceae*
Genera	*Methylococcus, Methylobacter, Methylocaldum, Methylohalobius, Methylomicrobium, Methylomonas, Methylosarcina, Methylosphaera, Methylothermus, Crenothrix, Clonothrix*	*Methylosinus, Methylocystis*	*Methylocapsa, Methylocella*	*Methylacidiphilum*
RuMP pathway	+	-	-	-
Serine pathway	-	+	+	+
Nitrogen fixation	SD	SD	+	SD
pH growth range	4-9	6-7.5	4.2-7.5	0.8-6
Temperature growth range (°C)	0-72 (SD)	23-31	6-32 (SD)	37-65 (SD)
Salt concentration range (%NaCl)	0.1-12 (SD)	0.5	NR	0.8-6.0
sMMO	SD	SD	SD	-
pMMO	+	+	SD	+

SD:specie-dependent

1.2.2 *Biological technologies for CH_4 abatement*

Biotechnologies for the treatment of methane have been widely studied over the past 30 years. Several bioreactor configurations have been applied at laboratory and/or full scale, which are classified into two different groups: (i) packed bed bioreactors and (ii) suspended growth bioreactors.

Among these conventional biotechnologies, biofiltration and biotrickling filtration are by far the most commonly implemented packed bed technologies for methane abatement, while the most representative suspended growth reactors are stirred tanks and airlift bioreactors [32] (Figure 1.4).

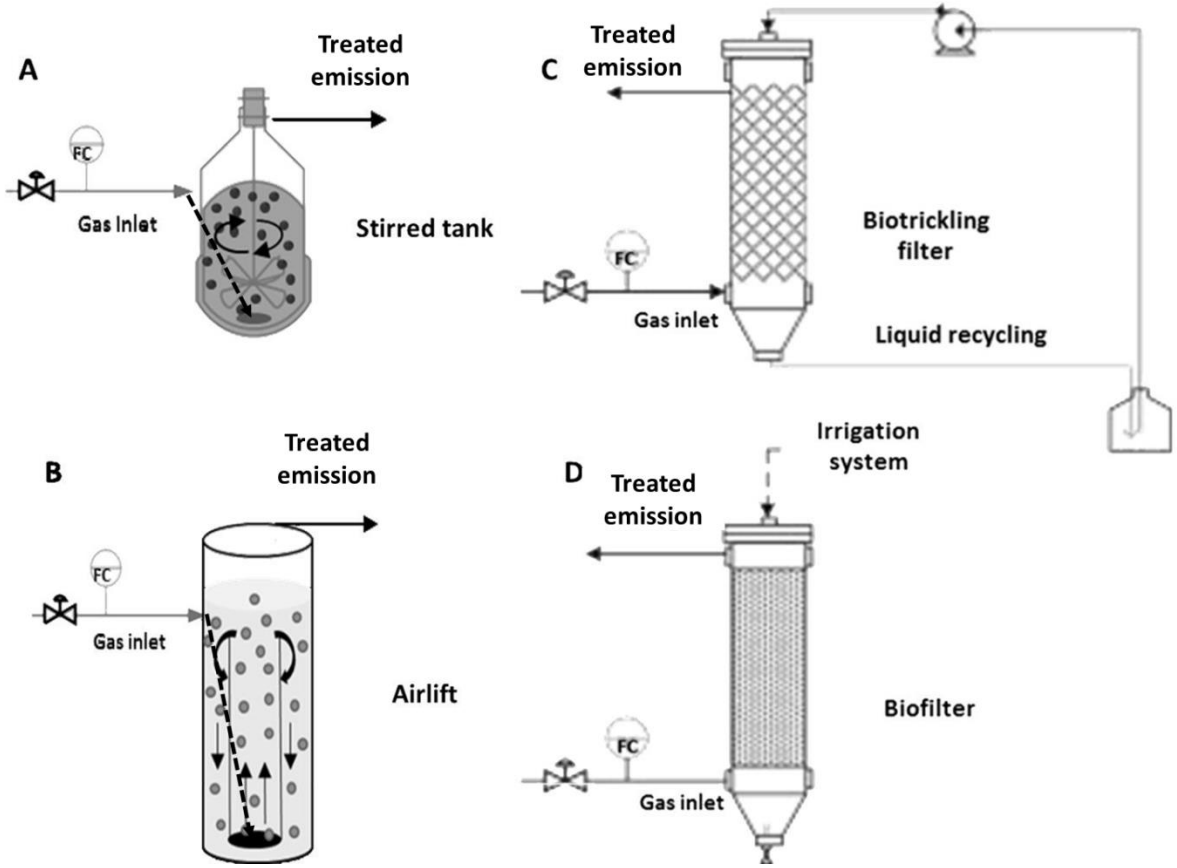

Figure 1.4 Main bioreactor configurations applied for CH_4 treatment. (A) Stirred Tank, (B) Airlift Bioreactor, (C) Biotrickling filter, (D) Biofilter.

Even though these bioreactor configurations can be the most cost-competitive technology to treat emissions with high concentrations of methane, and have been successfully

implemented under indoors and outdoors conditions to reduce CH_4 emissions [11,39], the CH_4 removal efficiencies (REs) reported during the treatment of dilute CH_4 emissions at gas residence times similar to those used during VOC/odour treatment (30-120 s) have been low. Hence, REs of 60-90% at EBRTs of 1-14 h were reported in passively vented biofilters, while actively vented bioreactors required EBRTs of 8-70 min to achieve an effective CH_4 abatement performance [39,40]). This results in bioreactor volumes 16-1600 higher than those typically used in VOC/odour applications [41].

1.3 Current limitations in biological CH_4 treatment

Despite the effectiveness and environmentally friendliness of biotechnologies devoted to the abatement of diluted emissions of methane, this platform technology is still restricted by three main limitations:

a) The poor mass transport of CH_4 from the gas phase to the microbial community due to its low aqueous solubility.

b) The lack of cost-competitive abatement methods for the treatment of diluted CH_4 emissions.

c) A limited knowledge of the microbial communities and biodegradation kinetics of methane at the trace level concentrations typically encountered during the treatment of diluted CH_4 emissions.

1.3.1 Methane mass transfer in bioreactors

The most important limitation of biological methane abatement is caused by the low aqueous solubility of this GHG (Henry's law constants (H) = 30). Thus, the high H of CH_4 results in low concentration gradients (low driving forces) for mass transport from the gas to the aqueous phase surrounding the biofilm and therefore, in a reduced GHG biodegradation performance[42]. Hence, this low mass transport entails process operation at high empty bed gas residence times (EBRT), which significantly increases both the investment and operating costs of methane treatment biotechnologies. In this regard, the implementation of a new concept of biological gas treatment based on the direct *gas-cell* transport of CH_4 could enhance the mass transport of this hydrophobic GHG[41,43]. Multiple innovative and high-performance technologies for biological gas treatment have

emerged in the last 10 years: two-phase partitioning bioreactors, membrane bioreactors, fungal-based biofilters and Taylor bioreactors (Figure 1.5). Unfortunately, the number of studies assessing the performance of these bioreactors during the treatment of CH_4 is still scarce.

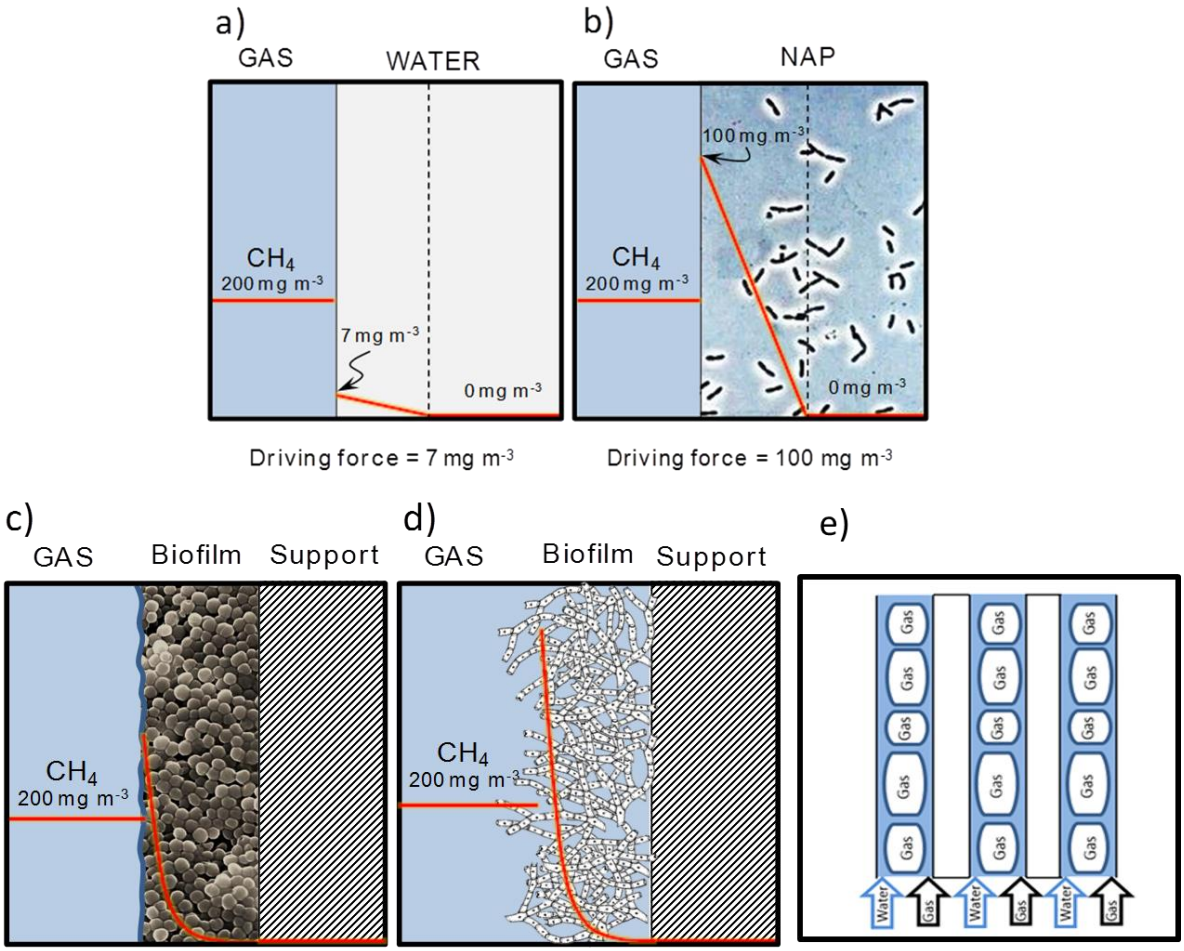

Figure 1.5 Typical concentration profiles and driving forces of CH_4 in a conventional mass-transfer limited bioreactor (a), in a two-phase partitioning bioreactor (b), in a bacterial biofilm (c) in a fungal biofilm (d) and in a segmented flow regime (Taylor flow) bioreactor (e).

Membrane bioreactors enhance the mass transfer of the target gas pollutant to the microbial community by promoting a direct gas-biofilm contact in the absence of a water layer surrounding the biofilm. This bioreactor configuration has been successfully tested for the treatment of volatile organic compounds [44], but only implemented for aerobic methane oxidation in liquid medium coupled to denitrification [45,46].

Fungal biofilters support an increase in methane mass transfer as a result of the large surface area of the fungal aerial mycelia and their high hydrophobicity (one order of magnitude larger than that of bacteria and higher at low moisture contents). In addition, fungi are often more resistant to drying out, acidification and nutrient limitations than bacteria. However, there is only one study in literature assessing the performance of a fungal-bacterial biofilter using the fungus *Graphium* sp., which was capable of co-metabolically biodegrading methane and methanol [47]. Although the results of this pioneer study did not support higher ECs (ECs ~ 40 g m^{-3} h^{-1} at an EBRT of 20 min) than other innovative bioreactor configurations (table 2), this fungal-bacterial biofilter exhibited a more stable performance compared to conventional bacterial biofilters [47].

Taylor Flow bioreactors are capillary multi-channel units where the gas-liquid hydrodynamics consist of an alternating sequence of gas bubbles and liquid slugs [48]. This segmented flow regime supports a high gas-liquid interfacial area along with a reduced liquid thickness and a high turbulence at the liquid side. In this regard, Rocha-Rios et al. (2013)[49] operated a capillary Taylor flow bioreactor for methane treatment, where the capillarity supported an increase in both the mass transfer coefficient and the bioconversion rate of methane compared to conventional bioreactors (EC ranging from 36.7 to 77.3 g m^{-3} h^{-1}). However, this bioreactor configuration entails nowadays high investment costs [49,50].

TPPBs are based on the addition to a conventional bioreactor of a non-toxic, non-volatile and non-biodegradable, non-aqueous phase (NAP) with a high affinity for the target gas pollutant [42]. Hence, the NAP supports an increase in both CH_4 and O_2 mass transfer from the gas phase to the microorganisms, while buffering the process against inlet pollutant surges or high metabolite concentrations potentially toxic to the microbial community. Different NAPs have been tested to enhance CH_4 mass transfer, although silicone oil (with an affinity for CH_4 15 times higher than water) was the most popular transfer vector [14,48,51,52]. The performance of two-phase partitioning bioreactors (TPPB) has been repeatedly evaluated using diluted CH_4 emissions, although the results seem to be configuration specific [14,44,46,49,53]. Avalos-Ramirez et al. (2012) reported ECs similar to those obtained with conventional biotrickling filters (ECs = 3.9 to 34 g m^{-3} h^{-1}) in a

biofilter packed with stones and operated with a non-ionic surfactant at an EBRT of 4.25 min [54], while an enhanced EC of 51 g m^{-3} h^{-1} was recorded by Rocha-Ríos et al. (2009)[55] in a two-liquid phase biotrickling filter (BTF) (10% v/v silicone oil) packed with polyurethane foam (PUF) and operated at an EBRT of 4.8 min. Similarly, the use of silicone oil (25 % v/v) by Lebrero et al. (2015) in a BTF packed with PUF supported CH_4 ECs of 45 g m^{-3} h^{-1} at EBRTs of 4 min, while a BTF operated under identical conditions without silicone oil reached a maximum EC of 35 g m^{-3} h^{-1} [52,54,55]. Thus, the addition of the NAP consistently supported a remarkable improvement in BTF performance compared to conventional aqueous phase systems. Rocha-Ríos et al. (2009) [55] also tested the potential of TPPB in stirred tank reactors, achieving a maximum EC of 106 g m^{-3} h^{-1} at an EBRT of 4.8 min under an IL of 187 g m^{-3} h^{-1}, 10% (v/v) of silicone oil and an agitation rate of 800 rpm [55]. However, Rocha-Rios et al. (2011) did not observe a significant improvement in the performance of a two-phase partitioning airlift bioreactor constructed with 10% (v/v) of silicone oil under a CH_4 IL of 170 g m^{-3} h^{-1} and an internal gas recirculation of 1-2 m_{air}^3 $m_{reactor}^{-3}$ min^{-1}, mainly due to the poor dispersion of silicone oil [56] (table 2).

Table 2: Conventional and innovative bioreactors for the treatment of methane

Ref.	Reactor type	System Characteristics	Inoculum	EBRT (h)	CH_4 load (g m^{-3} h^{-1})	Maximum EC (g m^{-3} h^{-1})
Nikiema and Heitz (2009)[57]	Biofilter	Biofilter packed with inorganic gravel and stones	Microorganisms from a biofilter treating CH_4 for 7 months	0.05-0.3	13-130	65
Park et al. (2009)[58]	Biofilter	Biofilter packed with landfill cover soil mixed with earthworm (60:40, w/w)	Microorganisms from the packing material	0.07-1.2	31-560	280
Girard et al. (2011)[59]	Biofilter	Biofilter with packing gravel (void space 40%)	Not specified	0.07	5-28	14.5

Girard et al. (2011)[59]	Biofilter	Biofilter with packing gravel (void space 40%)	Not specified	0.07	5-28	14.5
Ganendra et al. (2015)[60]	Biofilter	Biofilter setup using autoclaved aerated concrete (AAC) as a highly porous carrier material	MOB enriched from circum-neutral agricultural soil	0.013	39.3	28.7
Brandt et al. (2016)[61]	Biofilter	Mixtures of organic composted leaves and expanded vermiculite	MOB enriched from activated sludge and composted leaves	0.12	148.8	18.8
Rocha-Ríos et al. (2009)[55]	Two- phase partitioning stirred tank reactor	Stirred tank reactor operated at 800 rpm with 10 % SO200	Enriched methanotrophic consortium from a wastewater treatment plant	0.08	187	106
Rocha-Ríos et al. (2009)[55]	Two- phase partitioning biotrickling filter	Biotrickling filter packed with polyurethane foam, recycling rate 4-8 m h^{-1}, operated with 10 % silicone oil	Enriched methanotrophic consortium from a wastewater treatment plant	0.08	131	51
Rocha-Ríos et al. (2011)[56]	Two- phase partitioning airlift reactor	Concentric tube airlift reactor with internal gas recycling (1-2 m_{air}^3 $m_{reactor}^{-3}$ min^{-1}) and operated with 10 % (v/v) silicone oil	Enriched methanotrophic consortium from a wastewater treatment plant	0.12	170	22
Avalos-Ramírez et al. (2012)[54]	Two-phase partitioning biotrickling filter	Biotrickling filter operated in the presence of a non-ionic surfactant and packed with stones (0.73 cm, specific surface 470 m^2 m^{-3}).	Microorganisms from the lixiviate of a biofilter treating CH_4 for 1 year	0.07	62	34
Rocha-Ríos et al. (2013)[49]	Two-phase partitioning capillary bioreactor-taylor flow	Capillary bioreactor operated under Taylor flow with silicone oil at 10% (v/v)	Enriched methanotrophic consortium from a wastewater treatment plant	Not specified	Not specified	34-73

Lebrero et al. (2015)[52]	Two-partitioning phase biotrickling filter	Biotrickling filter operated with 25 % SO200 impregnated in the packing material, polyurethane foam (PUF).	*M. sporium* culture	0.065	420-450	45
Kennelly et al. (2014)[50]	Horizontal flow biofilm reactor (HFBR)	Horizontal flow biofilm reactor constructed with terram inorganic polymer sheets	A nitrifying activated sludge from a wastewater treatment plant	0.8	8.6	8
Estrada et al. (2014)[11]	Internal gas recycling biotrickling filter	Biotrickling filter packed with polyurethane foam (PUF) using internal gas recycling ($0.50\ m^3_g\ m^{-3}_r\ min^{-1}$)	*M. sporium* culture	0.065	420-450	35
García-Pérez et al (2017)[62]	Internal gas recycling bubble column reactor	PVC bubble column reactor with internal gas-recycling ($0.50\ m^3_g\ m^{-3}_r\ min^{-1}$)	*M. hirsuta* culture	0.5	48	35.2
Lebrero et al. (2016)[47]	Fungal bacterial biofilter	Biofilter packed with compost	Pure strain of the fungus *Graphium*	0.3	40	37

Although, TPPBs have achieved promising results for the treatment of hydrophobic volatile organic compounds, controversial results in terms of CH_4 biodegradation enhancement have been repeatedly reported [48,52,54,55]. In this context, the enrichment of hydrophobic methanotrophs capable of growing immerse inside the NAP, where much higher concentrations of methane are available for the growth of methanotrophs, could support a direct pollutant uptake and improve both, the overall CH_4 mass transfer capacity and the robustness of the bioreactor [41,53] (Figure 1.6).

The viability of microbial growth in the NAP-water interphase and even inside the NAP has been reported in previous studies for other volatile organic compounds, where bacteria were able to accumulate at the aqueous-NAP interphase or inside the NAP forming aggregates during the treat of hydrocarbons [43,63–65]. Unfortunately, the viability of

methanotrophic bacteria to grow inside the NAP, as well as the impact of the NAP on their biocatalytic activity, remains unknown [52,55]. In this context, it is of major importance to elucidate the operational conditions and selective enrichment strategies that could enable methanotrophs to be viable inside NAPs.

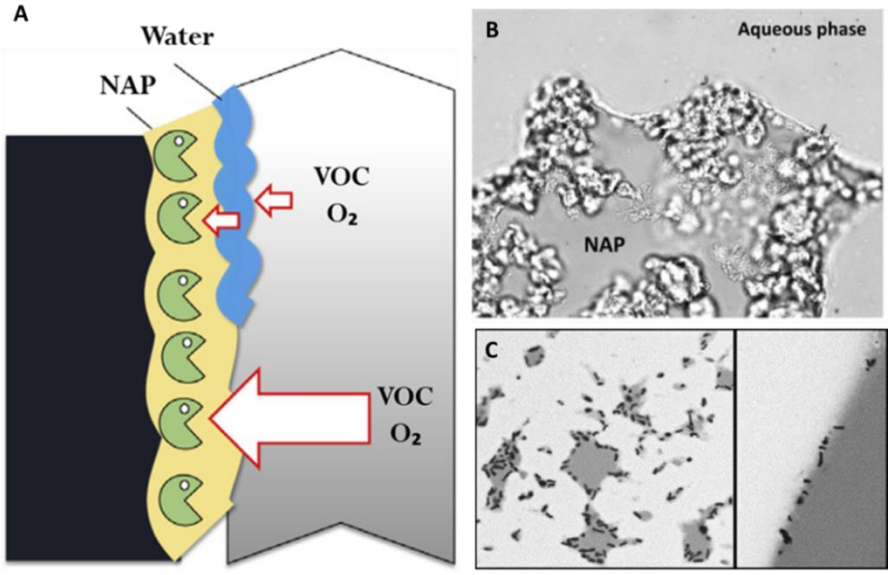

Figure 1.6 A) Gas pollutant biodegradation in two-phase partitioning biofilters operated with hydrophobic biomass [53], B) Photograph of silicone oil droplets used as the NAP for the treatment of Hexane [65], C) Photograph of *Mycobacterium* PYR-1 cells (black) grown in the presence of a sebacate(gray) and aqueous medium (white) in a reactor treating polycyclic aromatic hydrocarbons [43].

1.3.2 CH_4 biodegradation based on a waste-to-value approach

The lack of cost-competitive methods for the abatement of diluted CH_4 emissions could be overcome by the implementation of a new concept of biological gas treatment (using a methane bio-refinery approach) based on the bioconversion of GHG-laden emissions into multiple bioproducts with a high market value [66–70]. Although optimization from a micro and macroscopic perspective is still required to enhance microbial CH_4 bioconversion, this innovative CH_4 biorefinery can turn GHG abatement into a sustainable, profitable and competitive process (Figure 1.7).

In this context, during the past 20 years, a wide range of high added value compounds produced by methanotrophic bacteria has been described, the most promising ones based on

their market relevance and price being ectoine, bioplastics (polyhydroxyalkanoates or PHAs) and extracellular polysaccharides (EPS).

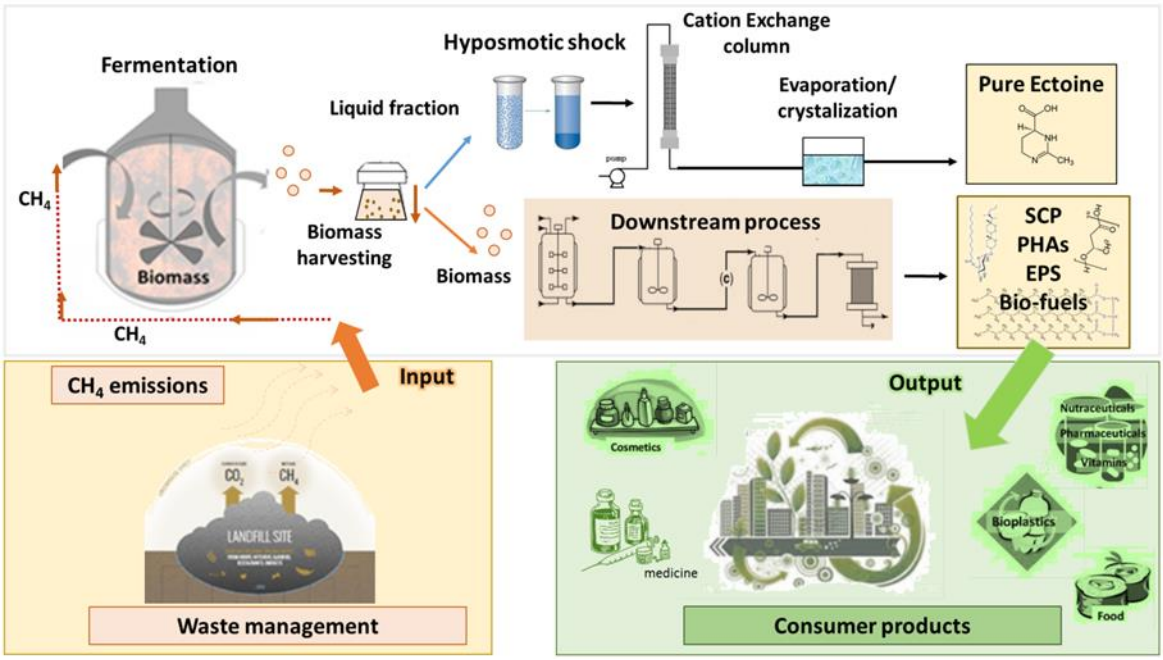

Figure 1.7 Process diagram for a methane bio-refinery producing ectoine, single cell protein, Polyhydroxyalkanoate, exopolysaccharides and biofuels from a landfill emission.

Ectoine

Ectoine is a cyclic imino acid that provides osmotic balance to a wide number of halotolerant bacteria [69,71,72]. Due to its high effectiveness as stabilizer of enzymes, DNA-protein complexes and nucleic acids, ectoine is used in medicine, cosmetology, dermatology and nutrition [73]. This osmolyte is probably one of the most valuable products synthesized by microorganisms, retailing in the pharmaceutical industry at approximately US$1000 kg^{-1} and representing a global consumption of 15000 tones yr^{-1} [69]. Despite its potential, ectoine is only currently produced biotechnologically by *Halomonas elongate* through a long fed-batch fermentation process called *biomilking* (total duration ~120 h), which consists of sequential hypo and hyper osmotic shocks [74]. For instance, the company BITOP (Witten, Germany) synthesizes this product on a scale of tons. However, this upstream processing is still inefficient due to the high amount of nutrients, oxygen and time required, besides entailing a complex and expensive downstream processing [71,72,75].

Since 1997, new species of halotolerant CH_4 MOB capable of synthesizing ectoine have been identified. Most of them belong to the genera *Methylomicrobium* (*i.e. M. alcaliphilum*, *M. buryatense*, *M. kenyense* or *M. japanense*), although other methanotrophic bacteria such as *Methylobacter marinus* and *Methylohalobius cremeensis* are also ectoine producers [76–78]. In the majority of these methanotrophs, ectoine synthesis relies on the sequential reactions of three enzymes (encoded by the conserved gene cluster, *ectABC*) that catalyze the conversion of the precursor aspartate into ectoine. However, an *ectABC–ask* cluster containing an additional gene of aspartokinase (*ask*) has been discovered in *M. alcaliphilum* (Figure 1.8). The Ask isoenzyme provides independent ectoine synthesis that results in the presence of a basal activity of ectoine biosynthesis and a relatively high salt tolerance. This versatile metabolism makes *M. alcaliphilum* a valuable platform for CH_4 mitigation combined with ectoine production [70,79]. In this regard, *M. alcaliphilum* has been proven to reach intracellular ectoine concentrations of up to 230 ± 20 mg ectoine g biomass^{-1} using CH_4 as the sole carbon and energy source, which largely exceeded the amounts synthesized by *M. marinus* and *M. kenyense* (60 and 70 mg g biomass^{-1}) [70].

These studies, conducted at high CH_4 concentrations, represented the first proof of the ability of MOB to produce ectoine. However, little is known about the influence of environmental conditions on the bioproduction of this secondary metabolite when combined with the abatement of diluted CH_4 emissions. Furthermore, no studies addressing the continuous production of ectoine by *M. alcaliphilum 20Z* or evaluating the technical feasibility of bio-milking have been carried out to date using methane as the sole carbon source.

Bioplastics (PHAs)

PHAs such as poly-3-hydroxybutyrate (PHB) and the copolymer poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) are intracellular biopolyesters produced under nutrient-limiting and carbon-excess conditions by a wide range of microorganisms as carbon and energy storage resources [80]. Their outstanding mechanical properties, similar to those of polypropylene and polyethylene, along with their biodegradability and biocompatibility make PHAs an attractive and potential alternative to oil-based plastics [81,82].

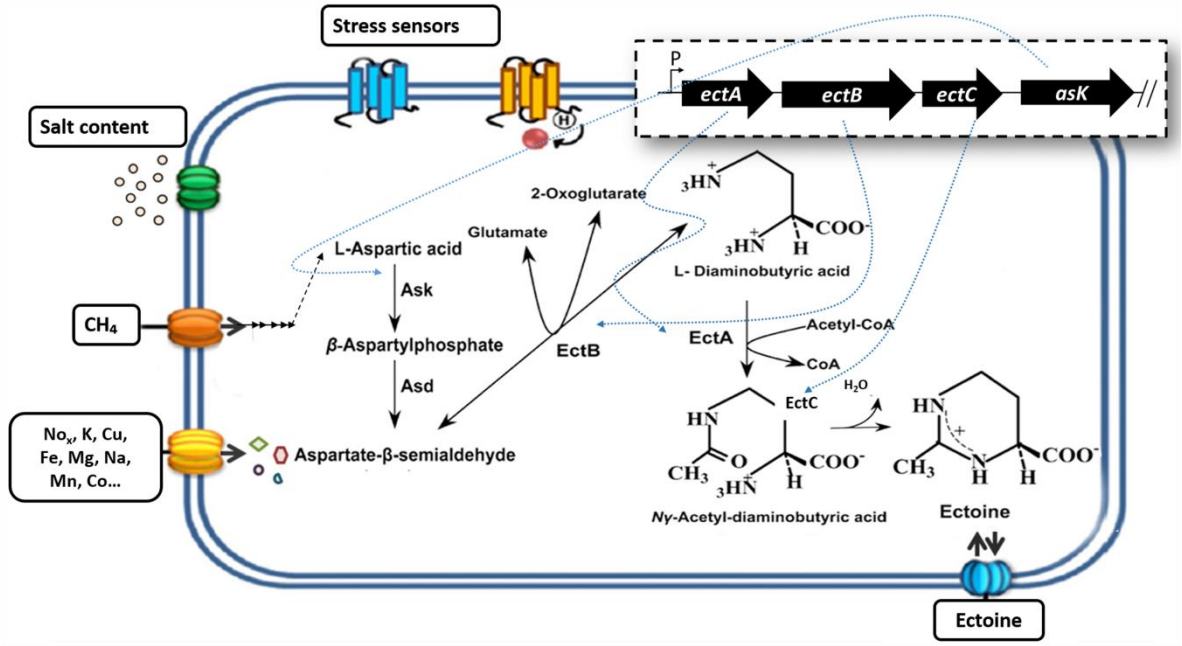

Figure 1.8 Accumulation of ectoine in *M. alcaliphilum 20Z*, pathway for the synthesis of ectoine and transcriptional control. Adapted from Pastor et al. (2010)[72].

PHAs are manufactured nowadays by nearly 30 companies, with Meredian Inc. (annual production of 300 kt) and Bio-On (annual production ~10 kt) as the leading manufacturers in U.S. and Europe, respectively[81]. *Ralstonia eutropha*, *Bacillus megaterium* and *Alcaligenes latus* are the main industrial PHA-producing heterotrophic organisms, while glucose and fructose represent the most common feedstocks. Nevertheless, the high cost of these carbon sources, which accounts for 30-40% of the total production costs, still hinders PHAs commercialization due to their uncompetitive market price (4-20 € kg_{PHA}^{-1})[83,84]. In this context, the methane contained in industrial diluted emissions (≤ 5 % *v/v*) has recently emerged as a potential feedstock for PHA production. The use of residual methane as a carbon source will significantly decrease PHAs production costs, while reducing the environmental impacts of GHG emissions [82,85]. Under nutrient-limiting conditions (i.e N-, P- or Mg-limitation), *Methylocystis*, *Methylosinus and Methylocella* are considered the main methanotrophic PHA producer genera (Figure 1.9). These methanotrophs can achieve PHBVs contents ranging from 20 to 51 % (wt) under batch [86,87] and continuous operation in suspended growth reactors [62,88–90]. In this regard, Mango Materials and Newlight Technologies (U.S.) are the pioneering companies in the development of methanotrophic-based technologies devoted to PHB production using CH_4 emissions [22].

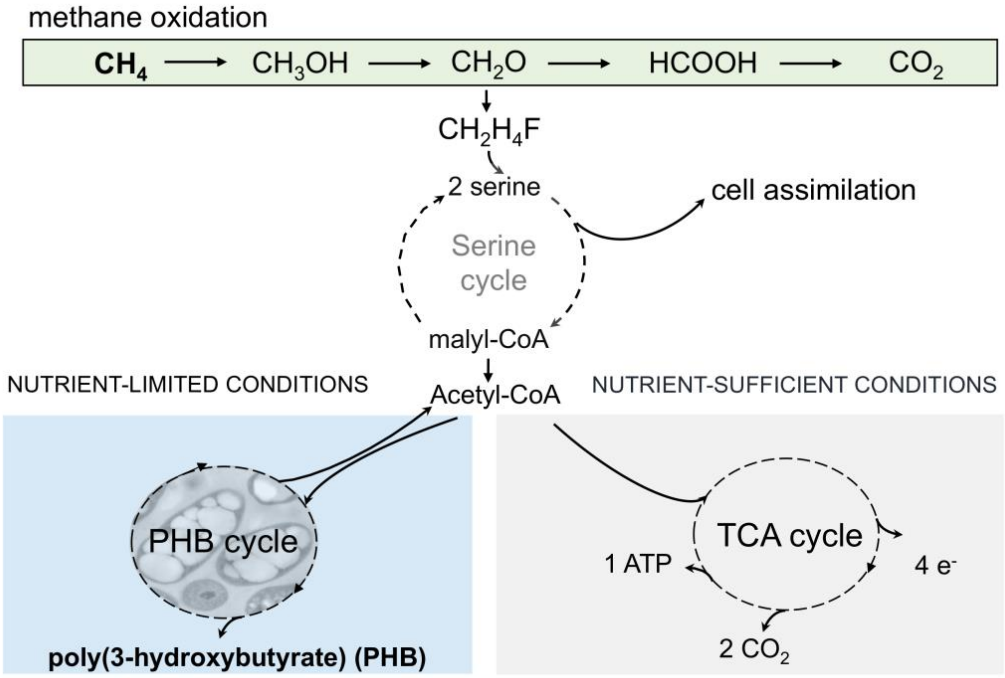

Figure 1.9. PHA production pathway in *α-proteobacter* methanotrophs, linked with the serine cycle. Adapted from Cantera et al. (2017).

Extracellular polysaccharides (EPS)

EPS consists of a wide variety of proteins, glycoproteins, glycolipids and polysaccharides [91]. In biofilms, EPSs provide microorganisms an effective protection against high or low temperature, salinity and predators [2, 37]. These bioproducts are of interest due to their colloidal and adhesive properties, and their effects on liquid rheology in the food, pharmaceutical, textile and oil industries [92]. CPKelco, Merck, Pfizer and Prasinotech Ltd. are companies currently focused on producing polysaccharides such as xanthan (4-13 € kg^{-1}) using *Xanthomonas campestris*, and dextran (30-50 € kg^{-1}) using *Leuconostoc mesenteroides* and *Streptococcus mutans*. However, the productivities and costs derived from the supplementation of the carbon source and EPS downstream still hamper their industrial production [93]. In this sense, CH_4 (capable of supporting EPS productivities of 300-430 mg g $biomass^{-1}$ in type I methanotrophs such as *Methylobacter*, *Methylomonas*) represents an alternative feedstock to reduce the production costs of EPS [94]. Overall, the

use of extreme conditions of salinity is one of the most effective strategies to promote EPS synthesis in methanotrophs [95].

Although the perspective of a near future methane based bio-refinery is promising, the implementation of these CH_4 bioconversion processes is still scarce due to the above mentioned limited mass transport of CH_4, which entails low CH_4 conversion rates and low biomass concentrations in the bioreactors. Suspended-growth bioreactors are the most suitable configurations for CH_4 fermentations, fed-batch cultivation in mechanically stirred fermenters with controlled nutrient feeding being the most popular approach [74,96]. However, this approach typically entails high operating costs during CH_4 bioconversion as a result of the intensive stirring required. Therefore, novel bioreactor configurations supporting high CH_4 mass transfer rates at lower energy inputs or the simultaneous production of multiple commercial metabolites from methane might support a cost-competitive CH_4 bio-refinery.

1.3.3 The need for a better understanding of methane-oxidizing bacteria

Besides the development of innovative high-performance methane mass transfer strategies, there is also a critical need to better understand the microbiology of CH_4 biodegradation in order to cope with the enhance CH_4 flow rates to cultivation broth and support a versatile CH_4 biconversion.

In this regard, the slow kinetics of methane uptake have been traditionally pointed out as one of the main limitations of methanotrophic bacteria. The enzyme MMO oxidizes methane to methanol in the first step of methane assimilation, but requires a high-energy electron donor (i.e NADPH) as an energy input to functionalize the otherwise inert methane molecule [97]. This phenomenon results on low biomass concentrations, which decreases the efficiency of the process and the cost-effectiveness of any potential CH_4 bioconversion. For instance, while sugar-based cultivation broths can achieved biomass concentrations up to 30 g L^{-1}, pure methanotrophic strains typically achieved average biomass concentrations of 1.0 g L^{-1}. This represents bioproduct productivities 10 to 100 times lower in CH_4 based fermentation than in conventional industrial processes based on sugars [62,72,98–101].

On the other hand, it is widely accepted that environmental conditions during culture enrichment control the structure and characteristics of methanotrophic microbial

communities, which ultimately determine their maximum specific degradation rate (q_{max}) and the affinity for CH_4 (K_s) [15,19,102]. In this regard, there is still a need to understand the effects of environmental factors during culture enrichment on the performance of methane treatment bioreactors. Of them, the CH_4 concentration in the gas phase, the gas-liquid CH_4 mass transfer rate and the Cu^{2+} concentration in the aqueous media have been identified as key parameters determining CH_4 abatement due to their key role on the nature and level of expression of MMO, and therefore on the population structure of methane-oxidizing bacteria [19,103–105].

Moreover, the strain or consortia used play a key role on the final performance of the bioreactor. Thus, the purer a culture is, the higher are the restrictions to grow under environmental stress factors, while higher population richness and diversity promotes higher resilience and therefore, better reactor performance and bio-product recovery. In this context, the impact of the microbiology on process performance is greater in the synthesis of specific bio-products from CH_4. There, the composition of the cultivation medium and the operational parameters in the bioreactor are crucial for an optimized bioconversion. For instance, few strains have been identified as PHA, EPS or ectoine producers, and sometimes the best producer has low growth rates or is too sensitive to the typical operational parameters in the bioreactor. For example, *M. alcaliphilum 20 Z* is sensitive to high agitation rates, which hinders methane transfer from the gas phase and results in low biomass concentrations [22]. Finally, it should be stressed that the recent advances in '-omics' techniques and the potential of systems biology must be oriented to the discovery of novel high-performance strains and of the untapped potential of classical strains in order to boost the development of CH_4-based bio-refineries.

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Aims and Scope of the Thesis

Chapter 2

2.1 Justification of the thesis

Methane (CH_4) is the second most important greenhouse gas worldwide, currently representing 18 % of the total GHG emissions, and its concentrations are expected to increase at a rate of 1.0-1.5 % in this XXI century. This current scenario has increased the concern about global warming, has encouraged the development of political initiatives for prevent GHGs emissions and has promoted an intensive research on novel biotechnological strategies for CH_4 treatment. Although methane emitted by anthropogenic activities can be used for energy recovery when its concentration is higher than 20 % (v/v), more than 56 % of anthropogenic CH_4 emissions contain concentrations below 3 %, which are neither technical nor economically suitable for energy recovery. These diluted emissions represent a threat to the environment and their cost-efficient and environmentally friendly abatement is still unresolved due to three main limitations: 1) The low aqueous solubility of CH_4, which hampers the transport of this GHG to the microbial community and increases the cost of CH_4 treatment biotechnologies. 2) Poor understanding of the bioconversion mechanisms of this GHG into products with high market value, which could turn GHG abatement into a sustainable and profitable process. 3) A limited knowledge of the microbial communities structure and biodegradation kinetics of methane at the trace level concentrations typically present in diluted emissions. In this context, the development of CH_4 treatment biotechnologies capable of enhancing the mass transfer of methane or bioconverting CH_4-laden emissions into high added value products, represent the most feasible and cost-effective solution for the mitigation of this GHG. Additionally, the availability of methanotrophic communities with high affinity for CH_4 is still required to enhance microbial CH_4 bioconversion.

2.2 Main objectives

The overall objective of the present thesis was to develop innovative microbiological, design and operational strategies in order to overcome the main limitations of the state-of-the-art biotechnologies devoted to the treatment of diluted CH_4 emissions. To this end, a multidisciplinary approach was adopted throughout the course of this thesis by combining three specific objectives**: objective 1** involved the design and operation of advanced off-gas

bioreactors with a high mass transfer capacity; **objective 2** consisted of the optimization of novel CH_4 bioconversion processes into valuable market products; and finally **objective 3** was based on the optimization of process performance from a microbiological viewpoint More specifically:

Objective 1- this research explored the potential and optimized the operational conditions of novel configurations such as hydrophobic TPPBs and column reactors with ultra-fine bubble diffusers to overcome mass transfer limitations during the treatment of methane.

Objective 2- this study evaluated the technical feasibility of ectoine production combined with CH_4 abatement by the methanotrophic bacterium *M. alcaliphilum* 20Z as well as the co-production of several products with high market value (ectoine, biopolymers and exopolysaccharides) from methane.

Objective 3- the role of copper and CH_4 concentration, and CH_4 mass transfer rate, on the microbial community structure and CH_4 biodegradation kinetics was investigated. Moreover, the isolation and characterization of novel halophilic and alkaliphilic methanotrophic strains was carried out in order to have alternative ectoine producers.

2.3 Development of the thesis

In the present thesis, innovative biotechnologies for the treatment of CH_4 emissions were proposed and evaluated. First, the elucidation of the differential microbial growth and specific strategies that could enable methanotrophs to grow inside a hydrophobic phase was carried out in **chapter 3** of the present thesis, along with a comparative study of the performance of TPPB and single phase bioreactors. Furthermore, an innovative biotechnological approach based on the bioconversion of CH_4 into the a product with a high market value, ectoine, was implemented for the first time during the development of this thesis using the methanotrophic strain *M. alcaliphilum 20Z*. In this regard, a preliminary optimization of the culture conditions for the transformation of CH_4 into ectoine followed by the continuous methanotrophic production of this osmoprotectant in stirred tank reactors was carried out in **chapter 4** and **5**. Furthermore, the potential of a bio-milking process similar to the one currently used in the industry for sugar-based ectoine production was

evaluated in **chapter 6** using CH_4 as the sole carbon and energy source. Despite the promising results obtained in **chapter 6**, a low ectoine productivity was achieved due to the low biomass concentrations prevailing in the reactor as result of the low CH_4 solubility and the sensitivity of *M. alcaliphilum 20Z* to high agitation rates. In this context, a bubble column reactor using ultra-fine bubble diffusers more suitable to the cultivation of shear sensitive microorganisms was used to assess the production of multiple high added market value metabolites during CH_4 mitigation in **chapter 7**.

Additionally, **chapter 8** focused on the enrichment and isolation of novel halotolerant methanotrophic strains able to support higher productivities of ectoine during CH_4 abatement. Finally, the effect of Cu^{2+} and CH_4 concentrations and CH_4 mass transfer rate during culture enrichment on the community structure and the CH_4 biodegradation kinetics was studied in **chapter 9** in order to standardize the enrichment conditions for highly efficient methanotrophs.

Comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors for methane : Mass Transfer and Biological Considerations

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Chapter 3

Comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors for methane abatement: Mass Transfer and Biological Considerations

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Abstract

This study demonstrated for the first time the capability of methanotrophs to grow inside silicone oil (SO200) and identified the optimum cultivation conditions for enrichment of hydrophobic methanotrophs (high dilution rates (D) and low CH_4 transfer rates). The potential of the hydrophobic methanotrophs enriched was assessed in a single-phase stirred tank reactor (1P-STR) and in a two-phase stirred tank reactor (2P-STR). Different operational conditions were systematically evaluated in both reactors (SO200 fractions of 30 and 60 %, stirring rates of 250 and 500 rpm, and D of 0.1-0.35 day^{-1} with and without biomass retention). The results showed that the TPPB only supported a superior CH_4 abatement performance compared to the 1P-STR (40% enhancement at 250 rpm and 25% enhancement at 500 rpm) at a D of 0.3 day^{-1} due to the retention of the biocatalytic activity inside the SO200, while the 1P-STR achieved higher elimination capacities (EC up to ≈3 times) than the TPPB under the rest of conditions tested (EC_{max} = 91.1 g m^{-3} h^{-1}). Furthermore, the microscopic examination and DGGE-sequencing of the communities showed that the presence of SO200 influenced the microbial population structure, impacting on bacterial biodiversity and favoring the growth of methanotrophs such as *Methylosarcina.*

Keywords:

Two-phase partitioning bioreactors, CH_4 abatement, hydrophobic bacteria, methanotrophs, mass transfer limitation.

1. Introduction

Methane (CH_4), with a global warming potential 34 times greater than that of CO_2 (over a 100-y window), is the second most important greenhouse gas (GHG) (IPPC 2013). The concentration of CH_4 increases at a yearly rate of 0.3-1 %, mainly due to anthropogenic activities (natural gas and petroleum systems, enteric fermentation, landfills, coal mining, waste management and energy production) (European Environmental Agency, 2015; United States Environmental Protection Agency, 2015). More than 56 % of anthropogenic CH_4 emissions contain this GHG at concentrations below 3 %, where current CH_4 treatment methods are neither efficient nor cost-effective (Avalos Ramirez et al., 2012). Dilute CH_4 emissions (< 3%) are typically found in old landfills (0–20%), in ventilated coal mines (0.1–1%), in covered liquid manure storage tanks (0–3%) and confined cattle operations (<1%) (Estrada et al., 2014). This situation has encouraged both political initiatives and an intensive research on novel strategies for CH_4 abatement (European Environmental Agency, 2015).

Conventional biological off-gas treatment technologies such as biofilters, biotrickling filters, stirred tank bioreactors and airlift bioreactors are based on the biocatalytic activity of specialized bacteria or fungi, and if properly tailored, they can be a cost efficient and environmentally friendly solution for the treatment of diluted CH_4 emissions from abandoned landfills, ventilated coal mines, manure storage tanks and confined cattle operations (López et al., 2013). However, these technologies are limited by the low water solubility of CH_4 when applied to CH_4 abatement, which hampers its gas-liquid transport to the methane oxidizing bacteria (MOB) and limits the overall CH_4 treatment performance of conventional biotechnologies (López et al., 2014). Two-phase partitioning bioreactors (TPPBs) have been proposed as an alternative to improve the removal of scarcely soluble atmospheric pollutants. This technology is based on the addition to the bioreactor of a non-toxic, non-volatile and non-biodegradable non-aqueous phase (NAP) with a high affinity for the target pollutant and a low vapor pressure (Daugulis, 2001). Strategy that has been successfully applied to the

treatment of hydrophobic volatile organic compounds (VOCs) such as toluene (Daugulis and Boudreau, 2003) and hexane (Hernández et al., 2010; Muñoz et al., 2013).

In the particular case of CH_4 biodegradation, the use of liquid NAPs has been tested in different bioreactor configurations with promising results (Avalos Ramirez et al., 2012; Kraakman et al., 2011; Lebrero et al., 2015; Rocha-Rios et al., 2009). However, there is still limited information about the mechanisms underlying the beneficial effects of NAPs at different operational conditions and the results were sometimes contradictory. In this context, the use of hydrophobic methanotrophs capable of growing inside the NAP could support a direct pollutant uptake and improve the overall CH_4 abatement capacity of the bioreactor. For instance, recent studies addressing the biodegradation of VOCs in TPPBs operated with the biomass growing inside the NAP reported significant increases in the elimination capacity (EC) under steady state operation (Hernandez et al., 2012).

Unfortunately, the role and mechanisms of the confinement of methanotrophic bacterial activity inside the NAP on bioreactor performance and cell viability when MOB grow inside the NAP remains still unclear (Lebrero et al., 2015; Rocha-Rios et al., 2009).

The present work aimed at exploring the potential and optimizing the operational conditions of TPPBs operated with hydrophobic bacterial communities for CH_4 abatement. First, the culture conditions resulting in the enrichment of hydrophobic methanotrophs were established, thus confirming the activity and viability of MOB in the NAP. Secondly, a systematic comparison of the performance of a conventional aqueous-phase stirred tank bioreactor (1P-STR) and a two-liquid phase stirred tank bioreactor (2P-STR) using silicone oil 200 cSt (SO200) as the model NAP was carried out. Both reactors were inoculated with the enriched MOB and operated with a special focus on the study of process microbiology and on the identification of the limiting step under each operational condition tested.

2. Materials and methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used during the enrichment of hydrophobic methanotrophs, as well as during

bioreactor operation, was a modified Brunner medium containing 25 μM Cu^{2+} and prepared according to López et al. (2014). All chemicals and reagents were purchased from Panreac (Barcelona, Spain) with a purity higher than 99.0%. CH_4 was obtained from Abelló-Linde, S.A. (Barcelona, Spain) with a purity higher than 99.5 %. Silicone oil 200 cSt (SO200) was procured from Sigma-Aldrich (Madrid, Spain). The CH_4 partition coefficient in SO200 accounts for 2 ± 0.1 compared to 30 in water at 25 ºC (Rocha-Ríos et al., 2011) and the Hildebrand Solubility Parameters of CH_4 and SO200 are also very similar (11.0 MPa1/2 and 14.9 MPa1/2, respectively), which confirmed the miscibility of both compounds, ergo the high solubility of methane in SO200 (Rocha Rios et al., 2011; Bacon et al., 2014). Due to these characteristics, SO200 was here used for enhancing the mass transport of CH_4 from the gas to the aqueous phase (whereas SO200 is also suitable for detoxification, this application was not the aim of the present study). Moreover, SO200 has been proven to support microbial growth inside, which enhances the biodegradation performance of the two-phase partitioning bioreactor due to the higher VOC and O_2 concentration in the NAP, besides being biocompatible, non-biodegradable, non-hazardous and odorless. Despite silicone oil has certain drawbacks as a transfer vector, since it is relatively expensive and its recovery may increase process cost, it constitutes, to the best of our knowledge, one of the few solvents evaluated that gathers all the above mentioned properties (Muñoz et al., 2014).

2.2. Microorganisms and enrichment procedure

Fresh aerobic settled activated sludge (≈ 6 g L^{-1}) from a denitrification-nitrification wastewater treatment plant (Valladolid, Spain) and fresh cow manure from a dairy farm (Cantabria, Spain) were used as inocula for the enrichment of hydrophobic methanotrophs. Briefly, a 10× dilution of the cow manure sample in MSM was mixed with activated sludge (50%/50% v/v). The enrichment was carried out for 45 days in six glass bottles (1.2 L) containing 100 mL of MSM and 50 mL of SO200, and provided with 10 mL of the inoculum above described. The bottles were closed with gas-tight butyl septa and plastic screw caps. O_2 was supplied via air flushing of the bottle

headspace prior to pure CH_4 injection to obtain a headspace concentration of 55 g CH_4 m^{-3}. This experimental protocol was daily repeated to restore O_2 and CH_4 headspace concentrations since CH_4 was totally depleted in 24 h. Cultures were grown at three different aqueous phase dilution rates (D = 0.12, 0.55 and 0.70 day^{-1}), under two different magnetic agitation conditions (300 and 650 rpm) (Table 1) and daily monitored for CO_2 and CH_4 concentrations in the headspace. The two MOB cultures exhibiting the best growth inside SO200 (test series 2 and 3, Table I) were chosen as inocula for 1P-STR and 2P-STR operation. During the 45 days of culture enrichment, random liquid samples from all 6 bottles were drawn to microscopically image the SO200, aqueous phase and SO200-aqueous interphase In addition, vital and dead bacteria inside the SO200 were identified.

2.3 Experimental set-up and operation

Two 1-L jacketed stirred tank reactors (Afora S.A., Spain) were used for continuous CH_4 abatement. The MOB enriched in TS2 and TS3 were mixed (50/50% v/v) and used as the inoculum for both reactors. The control 1P-STR was operated in the absence of SO200, filled with 700 mL of MSM and 300 mL of the aqueous phase of the hydrophobic

Table I: Cultivation conditions during hydrophobic microbial community enrichments

Test series (TS)	Dilution rate (day^{-1})	Stirring rate (rpm)	Presence of hydrophobic bacteria (day of appearance)
TS1	0.12	300	Yes (day 38)
TS2	0.55	300	Yes (day 21)
TS3	0.70	300	Yes (day 15)
TS4	0.12	650	No
TS5	0.55	650	No
TS6	0.70	650	No

bacterial inoculum. The 2P-STR was initially filled with 500 mL of MSM, 200 mL of new SO200, 100 mL of the organic phase of the inoculum and 200 mL of its respective aqueous phase. A 0.1 L min^{-1} CH_4 laden air emission containing 25.9 ± 2.1 g CH_4 m^{-3} (≈ 4%; concentration within the range of diluted CH_4 emissions typically found in abandoned landfills, coal mines, manure storage tanks and cattle farms) corresponding to a methane load of 150 g m^{-3} h^{-1} (loads similar to those typically applied in conventional biofilters and biotrickling filters devoted to the treatment of CH_4; Nikiema et al., 2007) was fed into the 1P- and 2P-STRs via 10 μm porous stainless steel diffusers located at the bottom of the reactors. The stream was obtained by mixing a pure CH_4 stream (controlled by means of a mass flow controller, Aalborg, USA) with a pre-humidified air flow, resulting in a gas empty bed residence time (EBRT) of 10 min. Six different sets of operational conditions were tested in both reactors. In the first and second stages (S1C and S2C in 1P-STR; S1 and S2 in 2P-STR) the stirring rate was fixed at 500 rpm, the dilution rate was 0.35 day^{-1} and the biomass drawn with the effluent was returned to the reactor following a total cell recycling strategy (the aqueous cultivation broth drawn was centrifuged at 10000 rpm for 5 min, the biomass pellet re-suspended in fresh MSM and returned to the bioreactor). This operation mode provided enough nutrients for microbial growth and avoided the accumulation of toxic inhibitory metabolites while efficiently retaining biomass in the system at high concentrations. In 2P-STR, 300 mL of MSM were replaced by fresh SO200 in S2 in order to increase the SO200 fraction from 30 to 60% v/v. The agitation rate was decreased to 250 rpm in stages S3 and S3C. In 2P-STR, 300 mL of MSM were replaced by fresh SO200 in S2 in order to increase the SO200 fraction from 30 to 60% v/v. The agitation rate was decreased to 250 rpm in stages S3 and S3C, while in stages S4 and S4C the dilution rate was decreased to 0.10 day^{-1} by daily replacement of 100 mL of aqueous cultivation broth with fresh MSM without biomass return. A slight increase in the dilution rate to 0.30 day^{-1} was implemented in stages S5 and S5C, while in the final stages S6 and S6C the stirring rate was increased back to 500 rpm (Table II).

Table II: Operational stages in the aqueous-phase stirred tank reactor (1P-STR) and in the two-phase stirred tank reactor (2P-STR).

	Stage (operating days)	SO200	Dilution rate (day^{-1})	Biomass return	Stirring rate (rpm)
1P-STR	**S1C/S2C** (14-29)		0.35	yes	500
	S3C (33-47)		0.35	yes	250
	S4C (68-83)		0.10	no	250
	S5C (105-120)		0.30	no	250
	S6C (130-149)		0.30	no	500
2P-STR	**S1** (3-19)	30%	0.35	yes	500
	S2 (20-27)	60%	0.35	yes	500
	S3 (30-45)	60%	0.35	yes	250
	S4 (50-65)	60%	0.10	no	250
	S5 (80-97)	60%	0.30	no	250
	S6 (104-124)	60%	0.30	no	500

Both systems were operated at 25 ºC and a pH of 7.5 ± 0.3 was maintained via daily replacement of the MSM. Distilled water (50 mL) was added weekly to compensate water losses by evaporation. The NAP was daily recovered from the withdrawn liquid and returned to the reactor in order to maintain the NAP/ MSM ratio corresponding to each operational stage. The elimination capacity (g m^{-3} h^{-1}), removal efficiency (RE, %) and volumetric CO_2 production rate ($TPCO_2$, g m^{-3} h^{-1}) were evaluated in both reactors. An operational steady state was achieved when neither the EC nor RE deviated >10% from the mean (only average values of stages S1 and S2 with deviations ~15% are shown due to the highly fluctuating performance of the TPPB). Gas samples for CH_4 and CO_2

determination were periodically taken from the sampling ports located at the inlet and outlet of the bioreactors using gas-tight syringes (HAMILTON, Australia). Aqueous samples of 20 mL were also periodically drawn to measure the biomass concentration (as total suspended solids, TSS). Biomass samples from the enriched hydrophobic inoculum and from the aqueous cultivation broth at the end of each steady state were drawn to determine the microbial population structure by denaturing gradient gel electrophoresis (DGGE)-sequencing.

2.4 Identification of mass transfer limitations

A mass transfer test was carried out at the end of each steady state in order to elucidate the limiting step during CH_4 biodegradation under the experimental conditions evaluated. In this regard, if the concentration of methane in the inlet emission increased by a factor of 2, the mass flow of methane potentially available for the microbial community also increased by a factor of 2. Under a microbial activity limiting scenario, not enough bacteria would be available to degrade this extra methane load and therefore the EC and $TPCO_2$ would remain constant (similar to the EC and $TPCO_2$ before the increase in the IL). However, if the process was limited by the mass transfer of the substrate to the community, the EC and the $TPCO_2$ would eventually increase by a factor of 2 as a result of the higher CH_4 concentration gradient available for CH_4 mass transfer and the availability of a microbial community active enough to cope with this additional CH_4 load. For this purpose the inlet CH_4 concentration was increased from 27.9 ± 0.9 g m^{-3} to 52.1 ± 1.6 g m^{-3} for 6 h and the concomitant change in EC and CO_2 production was periodically recorded (Lebrero et al., 2015).

In addition, the dissolved CH_4 concentration in the aqueous phase was determined after the mass transfer test in S4C and S5C to confirm the biological limitation. For this purpose, 5 mL of cultivation broth were injected in a 15 mL vial closed with a butyl septum, sealed with an aluminum cap and supplemented with 0.1 mL of H_2SO_4 to stop biological activity. The vial was immediately shaken vigorously and allowed to equilibrate for 2 h at 25 °C prior to the determination of the CH_4 headspace concentration (Frutos et al., 2014). The aqueous CH_4 concentration was then calculated using

the individual gas and liquid volumes and a Henry's law constant for CH_4 of 30 at 25 ºC (Sander, 1999).

2.5. Analytical procedures

CH_4 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD according to Lopez et al. (2014). TSS were measured according to Standard Methods (American Water Works Association, 2012). Vital and dead bacteria inside the SO200 were identified using the LIVE/DEAD BacLight™ Bacterial Viability Kit (L13152). The SO200, aqueous phase and SO200-aqueous interphase were imaged in a Leica DM4000B microscope equipped with a Leica DFC300FX.

2.6. Structure of the enriched communities

The total DNA was extracted using the Fast® DNA Spin Kit for Soil handbook (MP Biomedicals, LLC). Polymerase chain reaction (PCR) products of the bacterial 16S rDNA fragments from the samples were separated by DGGE according to Roest et al. (2005). Gels were stained with GelRed Nucleic Acid Gel Stain (biotium) for 1 h. Specific PCR-DGGE bands were manually excised from the gel, resuspended in 50 mL of sterile water and incubated at 60 ºC for 1 hour. The last PCR cycle was performed without the GC-clamp attached to the forward primer 968-F. The resulting PCR products were sent to Secugen S.L. (Madrid, Spain) for nucleotide sequencing. The taxonomic position of the sequenced DGGE bands was obtained using the RDP classifier tool (70% confidence level) (Wang et al., 2007). The closest matches to each band were obtained using the BLAST search tool from the National Centre for Biotechnology Information (NCBI) (McGinnis and Madden, 2004). The absence of chimeras in the sequences was assessed using the DECIPHER search tool (Wright et al., 2012) before deposit in the GenBank Data Library. The DGGE patterns obtained were compared using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium) to determine the Pearson product-moment correlation coefficient and the Shannon-Wiener diversity index (Hs) according to Lopez et al. (2014). The DNA sequences obtained were used to construct phylogenetic trees by using the ClustalX2.1 and MEGA 6.06 program package. The phylogenetic trees were constructed using the neighbor-joining

method (1000-fold bootstrap analysis) (Hall, 2013).

2.7. Statistical analysis

The statistical data analysis was performed using SPSS 20.0 (IBM). The occurrence of significant differences in the steady states achieved in both bioreactors operated under the same operating conditions were analyzed by ANOVA and post-hoc analysis. A Levene test was used to study homoscedasticity. Differences were considered significant at $p \leq 0.05$.

3. Results and Discussion

3.1 Enrichment of hydrophobic bacteria

The enrichment strategy was based on the exposure of microorganisms to different effective CH_4 mass transfer rates (determined by the stirring rate) under similar CH_4 headspace concentrations, and to different dilution rates (D) in the aqueous phase, which selectively washed-out MOB growing in the aqueous-phase (Table I). The lowest stirring rate (300 rpm), which supported both a limited transfer of CH_4 to the microbial community and a reduced SO200 dispersion, favored microbial growth inside SO200 (microscopic observations, Figure 1). However, the MOB enriched at 650 rpm did not grow inside the SO200 since the higher agitation rates likely resulted in a higher CH_4 mass transfer into the aqueous phase (where nutrients were easily available), and therefore the environmental pressure for bacteria to migrate inside the organic phase disappeared. A higher D also promoted microbial growth in the organic phase. Thus, the first hydrophobic microorganisms in cultures at 300 rpm were observed by day 15 at 0.70 day^{-1} (TS3), by day 21 at 0.55 day^{-1} (TS2), and by day 38 at 0.12 day^{-1} (TS1). The MOB found inside the NAP grew in the form of aggregates (Figure 1). A similar growth pattern has been reported in previous studies for different NAPs, where bacteria were able to accumulate in the aqueous-NAP interphase and grow inside NAPs forming aggregates (Fu et al., 2015; Han et al., 2009b; Yamashita et al., 2007). Moreover, McLeod and Daugulis (2005) observed that certain hydrophobic bacteria formed aggregates in the aqueous phase instead of inside the NAP. Overall, cell aggregates offer an advantage to colonize a new habitat and to enhance the environmental quality of hostile habitats (Langer et al., 2014; Lazarova and Manem, 1995). In our particular case, the

main factor likely promoting the formation of aggregates inside SO200 was the limitation in nutrients or water activity inside the NAP, since cell aggregation may have supported the preservation of the aqueous niches required for the growth of *Methylosarcina*. In fact, the entrapment of MSM droplets inside NAPs has been empirically proven (Cordiki et al., 2012). However, the mechanisms that allow some bacteria to grow inside the NAP still remain unclear, and more research in this field is needed.

Previous studies reported cells growing inside other NAPs using fluorescence staining (McLeod and Daugulis (2005). However, to the best of our knowledge, this is the first study in literature demonstrating that the bacteria observed inside SO200 were viable cells. Hence, fluorescence in-situ hybridization of the cells present in the SO200, in the aqueous phase and in the interphase showed that those bacteria were alive (fluorescent green staining) (Figure 1b), thus confirming that SO200 was non-toxic for the MOB.

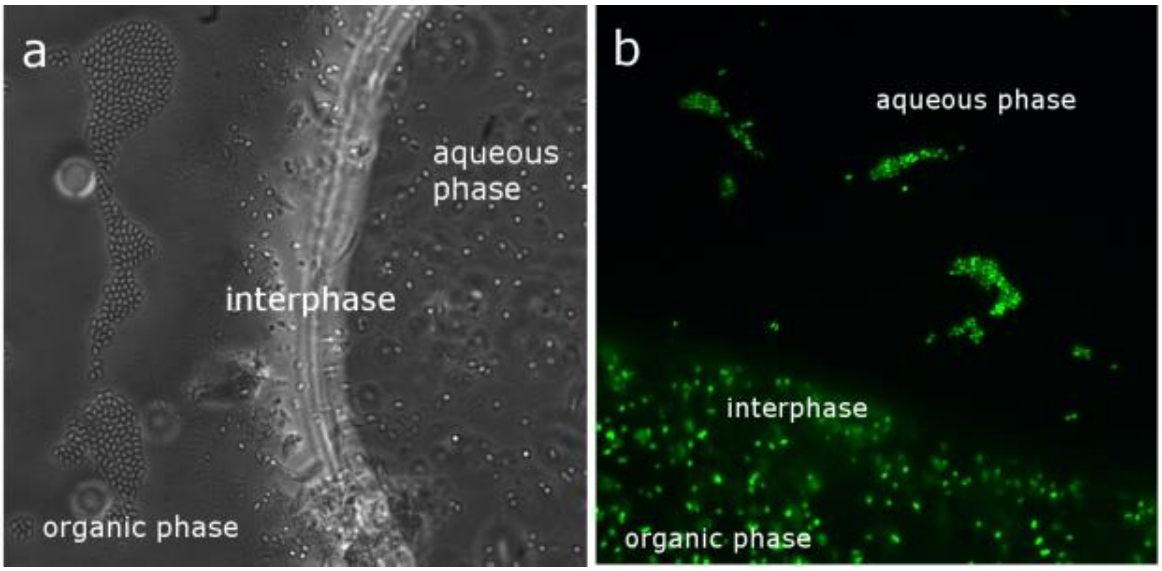

Figure 1. Microscopic images of the enriched bacteria in TS2 (300 rpm, D= 0.55 day^{-1}) using a) a phase contrast microscope Leica DM40000b AT 40× magnification (focused on the organic phase to identify microbial growth inside the NAP) and b) a Leica DM40000b AT fluorescence microscope 20× magnification and specific filters sets for alive bacteria (SYTO®9 green-fluorescent nucleic acid stain) and dead bacteria (propidium iodide red-fluorescent nucleic acid stain).

3.2 Comparative evaluation of a 1P-STR and a 2P-STR for CH_4 abatement

Six operational stages were evaluated in both reactors to compare their performance and to determine the optimal operational parameters for continuous CH_4 abatement.

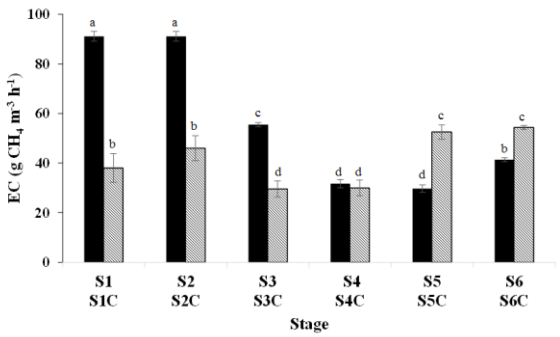

Figure 2. Average EC in the 1P-STR (black) and 2P-STR (scratched). Vertical bars represent standard deviations from replicates during steady state in each operational stage. Columns with different letters were significantly different at $p<0.05$.

On day 14, the first steady state was reached in 1P-STR (S1C/S2C, Table 2), with average ECs and REs of 91.1 ± 1.9 g m^{-3} h^{-1} and 57.0 ± 1.0 %, respectively. On the contrary, the ECs obtained in the 2P-STR during the first 27 days were highly variable, exhibiting average values of 38.0 ± 5.8 and 46.0 ± 3.6 g m^{-3} h^{-1} and REs of 27.1 ± 3.5 and 34.9 ± 4.5 % in S1 and S2, respectively (Figure 2). Surprisingly, the increase in SO200 fraction from 30 to 60 % did not significantly affect the performance of the 2P-STR. The results of the mass transfer limitation tests in these stages (S1C/S2C, S1 and S2) revealed that a ×1.9 ± 0.03 increase in the inlet load resulted in both a ×1.6 ± 0.02 increase in the EC and a concomitant increase of ×1.3 ± 0.03 in the $TPCO_2$ (Figure 3a). Therefore, the higher concentration gradient at increasing CH_4 concentration in the gas phase supported higher ECs, thus confirming the mass transfer limitations regardless of the presence and fraction of the NAP. During stages S1C/S2C, S1 and S2, an average TSS concentration of 5.6 ± 1.3 g L^{-1} was recorded in the 1P-STR while in the 2P-STR, the aqueous TSS concentrations oscillated within a similar range (7.3 ± 2.4 g L^{-1}). Microscopic observations revealed that biomass grew mainly in the aqueous phase of both reactors, supporting a high biodiversity during these stages (diverse protozoans, including *Vorticella*, *Opercularia* or *Euplotes*, and bacteria were identified).

The agitation rate was decreased to 250 rpm on day 33, which mediated in the 1P-STR (stage S3C) a decrease in the steady state EC to 55.5 ± 1.6 g m^{-3} h^{-1} and in the RE to 34.9 ± 1.1 %. The EC and RE in the 2P-STR operated at 250 rpm (stage

S3) were ×1.8 times lower than those reached in the 1P-STR. Unfortunately, a significant deterioration in the performance of the 2P-STR at this lower agitation rate could not be confirmed based on the high variations in EC and RE recorded in the two first operational stages. As expected, the mass transfer tests confirmed that both reactors remained mass transfer-limited during stages S3C and S3. Indeed, a ×2.0 ± 0.05 increase in CH_4 inlet load resulted in increases of ×1.9 ± 0.3 and ×1.6 ± 0.4 in the EC and the $TPCO_2$, respectively (average test results of both reactors). The maximum TSS concentration in the aqueous phase was reached in this third stage due to biomass retention (10.6 ± 0.9 g L^{-1} and 10.3 ± 1.6 g L^{-1} in 1P-STR and 2P-STR, respectively). The increasing biomass concentrations observed in stages 1, 2 and 3 suggested the use of methane for cell maintenance purposes as well as cell growth. Similarly to stages S1 and S2, the microscopic images showed that most microorganisms grew preferentially in the aqueous phase. These results supported those obtained in the enrichment tests, where low dilution rates did not favor the abundance of bacteria able to grow inside the SO200.

Stages S4C and S4 were characterized by process operation with no biomass return at a D of 0.1 day^{-1}. No statistical differences existed between the ECs and REs recorded in both reactors during this stage (according to the one-way ANOVA test combined with a Games-Howell post hoc analysis, $p<0.05$). The EC obtained decreased to 31.2 ± 1.6 g m^{-3} h^{-1} and the RE to 19.1 ± 1.6 % in the 1P-STR. However, the average EC and RE recorded in the 2P-STR did not show statistical differences with those recorded in S3 (30.0 ± 3.1 g m^{-3} h^{-1} and 20.6 ± 2.1 %) (Figure 2). A biological limitation was identified in the 1P-STR at a TSS concentration of 2.7 ± 0.1 g L^{-1}, since a 2.1-fold increase in the CH_4 inlet load resulted in negligible variations in EC and $TPCO_2$ (Figure 3b). This biological limitation was further confirmed by the relatively high CH_4 concentration measured in the aqueous phase (0.43 g m^{-3}), which clearly showed that CH_4 was transferred to the aqueous phase at higher rates than its microbial consumption. However, and despite the decrease in aqueous TSS concentration to 4.5 ± 0.6 g. L^{-1} in the 2P-STR due to daily biomass removal, the mass transfer test confirmed that the TPPB was still mass transfer

limited, and therefore the concentrations of CH_4 in both liquid phase were theoretically 0 mg CH_4 L^{-1} (otherwise the system would be limited by microbial activity). This discrepancy between both reactors can be explained by the fact that SO200 prevented the washout of the biomass entrapped inside the NAP in the 2P-STR. In this context, the microscopic observations (Figure 4a, d) revealed that a new population of sarcine-shape bacteria established inside the SO200 during S4. It is important to notice that the possibility of all methane being sequestered inside the organic phase was unlikely in the particular case of SO200 and CH_4 based on the moderate CH_4 partition coefficient of CH_4 in silicone oil (Déziel et al., 1999).

In this context, the dilution rate was increased to 0.3 day^{-1} during S5 and S5C in order to further promote the growth of hydrophobic bacteria inside the NAP. The steady ECs reported were 29.7 ± 1.5 g m^{-3} h^{-1} and 52.5 ± 2.8 g m^{-3} h^{-1} for the 1P-STR and 2P-STR, corresponding to REs of 20.3 ± 1.6 % in the 1P-STR and 33.8 ± 1.4 % in the 2P-STR. No statistical differences were observed in the performance of the 1P-STR when compared with the previous stage S4C.

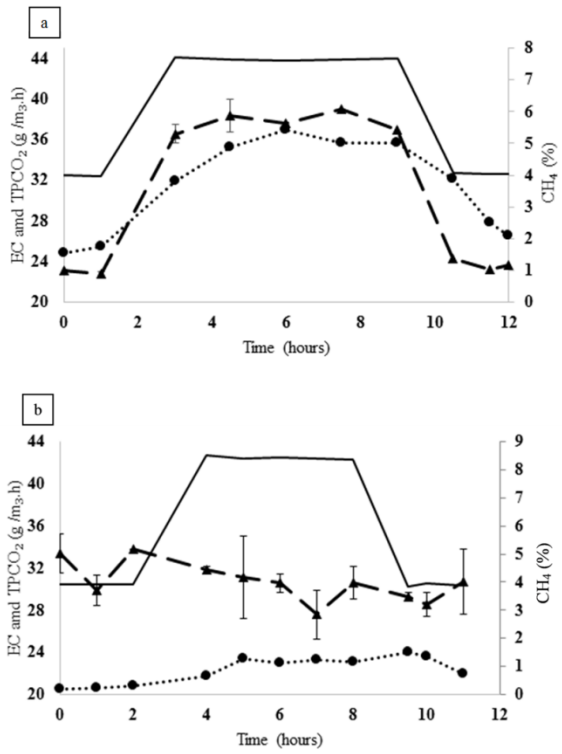

Figure 3. Time course of CH_4 concentration (black continuous line), the EC (▲, dashed line) and the CO_2 production rate (●, dotted line) during the mass transfer limitation test in a) S1C/S2C (mass transfer limitation scenario), b) S4C (biological limitation scenario).

However during S5, the 2P-STR achieved ECs and REs 1.6 times higher than in S4. The lower biomass concentration (0.9 ± 0.1 g L^{-1}) imposed by the higher dilution rate supported the occurrence of biological limitation in the 1P-STR. Thus, a ×1.9 increase in the CH_4 inlet load did not result in a significant increase in the EC or the $TPCO_2$ in the control reactor. On the contrary, the 2P-STR continued to

be mass transfer limited despite the aqueous concentration of biomass decreased to 1.9 ± 0.7 g L^{-1}. In this regard, microscopic observations confirmed the active bacterial growth inside the SO200 (Figure 4b).

Finally, the agitation rate was increased from 250 to 500 rpm at a D of 0.3 day^{-1} during S6 and S6C. Both the EC (41.3 ± 0.8 g m^{-3} h^{-1}) and RE (24.2 ± 0.8 %) increased compared to the previous stage in the 1P-STR. However, the EC and RE (54.4 ± 0.4 g m^{-3} h^{-1}, 32.2 %) recorded in the 2P-STR did not show statistical differences with S5.

The mass transfer experiments showed that both reactors were mass transfer limited during stage 6. The increase in the stirring rate resulted in an increase in the TSS concentration up to 1.8 ± 0.1 g L^{-1} in the 1P-STR likely as a result of the higher CH_4 mass transport to the microbial community. This increase in biomass concentration shifted process limitation from microbial activity to mass transport. The average aqueous TSS concentration in the 2P-STR was not significantly different from that in S5 (1.7 ± 0.6 g L^{-1}).

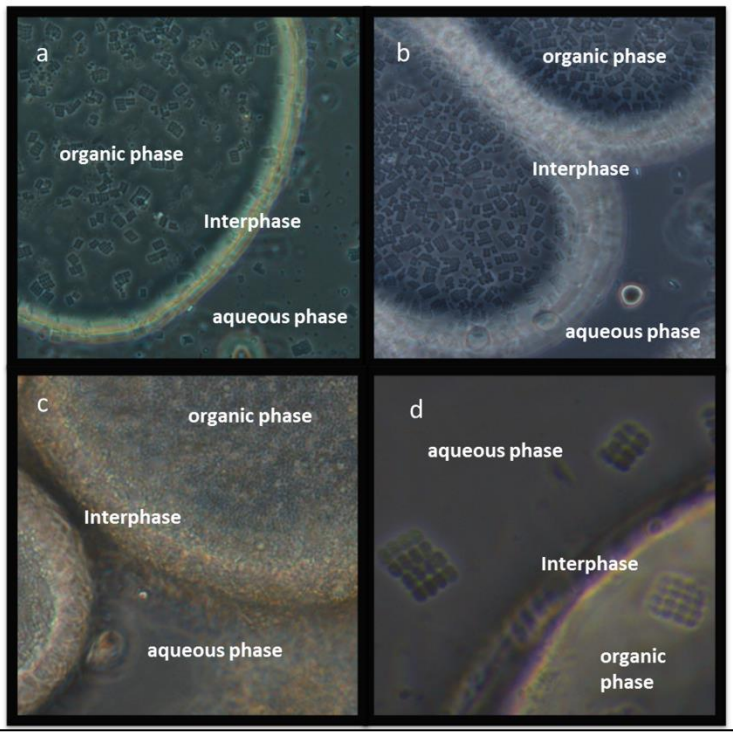

Figure 4. Microscopic images using a phase contrast microscope Leica DM40000b AT of the hydrophobic MOB established in a) S4 (20× magnification), b) S5 (20× magnification) c) S6 (40× magnification) and d) S4 (100× magnification).

The steady biomass concentrations reached in stages 4, 5 and 6 were the result of the equilibrium between cell growth and cell evacuation under steady state conditions. The system never operated under completely cell maintenance as previously observed by Daugulis and co-workers in TPPBs (Boudreau and Daugulis, 2006). According to microscopic observations, bacteria actively grew inside the SO200 and NAP droplets were surrounded by bacterial flocs (Figure 4c). The surprising absence of performance enhancement in the 2P-STR despite the expected increase in CH_4 mass transfer at higher agitation rates might be due to the fact that CH_4 diffusion inside the SO200 rather than gas-liquid mass transport was limiting CH_4 abatement during stage 6.

3.3 *Microbial population diversity and community profile*

The enriched hydrophobic inoculum showed the lowest number and evenness of species among the analyzed samples according to its low Shannon Wiener diversity index (*H*=1.4) (Fig. 5). This fact was mainly due to the selective cultivation conditions of the inoculum carried out during the enrichment stage.

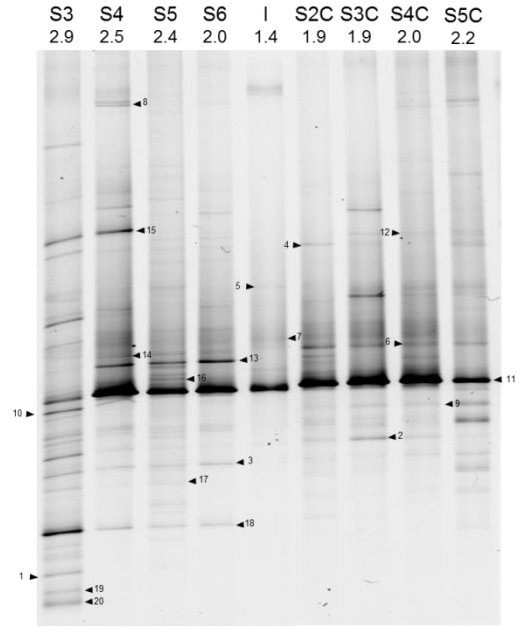

Figure 5: DGGE profile of the main bacterial communities present in: the 2P-STR (S3, S4, S5, S6), Initial Inoculum (I) and in the 1P-STR (S3C, S4C, S5C, S6C). The Shannon-Wiener diversity indexes are indicated in the upper part of the gel. The sequenced bands are indicated by "▶'' and the corresponding number of each band.

CH_4 was the only carbon source and the high dilution rate applied washout the majority of bacteria not associated with SO200. The biodiversity increased over time in the 1P-STR, with *H* values ranging from 1.9 to 2.2. In the 2P-STR, there was an initial biodiversity raise, with the maximum *H* found in S3 when biomass was returned to the reactor and the aqueous TSS concentration was 10.3 ± 1.6 g L^{-1}. Despite this initial increase, the biodiversity decreased throughout the

following stages probably due to bacterial specialization and biomass washout, with *H* values of 2.5, 2.4 and 2 in stages S4, S5 and S6, respectively. At this point it is important to remark that mixed samples (including both NAP and aqueous phase) were analyzed. The diversity values obtained in this study were within the range previously reported for long-term cultures exposed to diluted CH_4 emissions (Estrada et al., 2014; Lebrero et al., 2015). Relatively low *H* values, compared to samples from other environments exposed to varied energy and rich carbon sources, were expected since CH_4 was the only carbon and energy source provided.

Despite the high similarity found between the inoculum and the communities established in the final stage of the 2P-STR (85.5 %), the similarity obtained between stage S3 and the inoculum was considerably lower (40.7 %). This demonstrated that the enrichment with SO200 determined a common specialization strategy that favored only a few hydrophobic MOB populations, while supporting a lower bacterial biodiversity. In the particular case of the 1P-STR, a significant shift in microbial population was observed, with a gradual decrease in the similarity with the inoculum from stage S3C onward (90.0, 76.7, 68.9 and 55.3 %). A low similarity was also observed between S6 and S6C (52.2 %), which confirmed that SO200 had a clear influence on the bacterial population structure.

The DGGE-sequencing results (Figure 6) showed that all bands obtained belonged to two phyla according to the RDP classifier tool: the phylum *Proteobacteria* (bands 1 to 18) and the phylum *Verrucomicrobia* (bands 19 and 20) (Table I, supporting information). Most bands (5-18) were classified into the *Gammaproteobacteria* class. All methanotrophs identified belonged to the *Gammaproteobacteria* class were classified as type I and X MOB. The MOB genera found were *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina* and *Methylococcus* ((Hanson and Hanson, 1996; Kao et al., 2004; Wise et al., 1999), which have been previously identified in bioreactors devoted to CH_4 treatment (Estrada et al., 2014; López et al., 2014). The genus *Methylomonas* (bands 5, 7 and 11) was the only methanotroph detected by DGGE in the inoculum, and dominated the population of both bioreactors along the

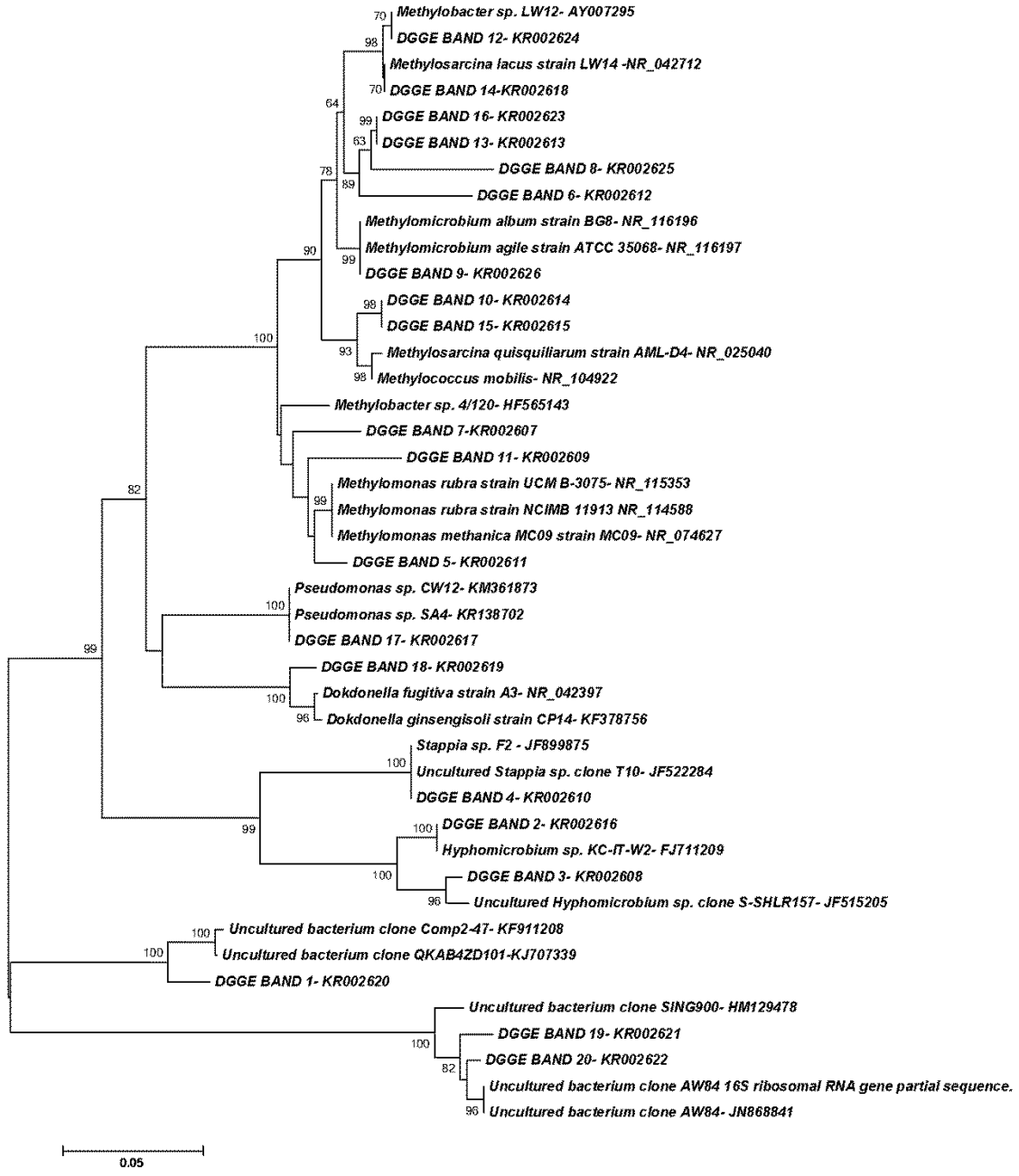

Figure 6. Bacterial phylogenetic tree based on neighbor-joining analysis of the 16S rRNA sequences from the enriched populations of both reactors by PCR-DGGE and their closest relatives (similarity > 96%) in GenBank obtained by the Blast search tool. Accession numbers are indicated. Numbers on the nodes indicate bootstrap values (1000 replicates). The scale bar indicates 10% sequence difference.

entire experimental period (likely due to the high growth rates of this MOB).

Interestingly, the genus *Methylosarcina* (bands 13, 14, 15 and 16) was only detected in the 2P-STR from stage S4 to S6, which agreed with the microscopic

observations of the NAP conducted during these operational stages (Figure 4). The genus *Methylosarcina* has been previously detected in STR bioreactors treating diluted emissions of CH_4 in the absence of SO200 (López et al., 2014). In addition to MOB, bacteria from the genus *Hypomicrobium* (band 2-3; a common methylotroph that utilizes the methanol from CH_4 oxidation (Hanson and Hanson, 1996)) were present both in the inoculum and in the cultivation broth of the 1P,2P-STR regardless of the operational conditions evaluated (Calhoun and King, 1998; Han et al., 2009a; López et al., 2014).

Other non-methanotrophic heterotrophic genera such as *Dokdonella* (band 18), *Pseudomonas* (band 17) and *Spartobacteria* (bands 19 and 20), which have been retrieved in two-phase partitioning biotrickling filters treating CH_4 (Lebrero et al., 2015), were detected in the 2P-STR during the stage with the highest diversity (S3). Nevertheless, these microorganisms gradually disappeared due to MSM exchange, while those that occupied the SO200 protected niche created a reservoir for stable colonization. On the other hand, the genus *Stappia* (band 4), a non- methanotrophic versatile organism which occupy several niches and participate in CO oxidation and denitrification (Weber and King, 2007) was identified in every stage in the 2P-STR and in S3C, S4C and S6C in the 1P-STR (Veillette et al., 2011).

4. Conclusion

This study confirmed, for the first time, the ability of hydrophobic methanotrophs to grow and maintain their viability inside a NAP. High dilution rates together with low CH_4 transfer rates were identified as the best enrichment conditions for hydrophobic methanotrophs. Under continuous bioreactor operation, this study demonstrated that the addition of a non-aqueous phase (60% v/v SO200) at high biomass concentrations (mediated by biomass retention) did not result in an enhancement in methane abatement compared to a conventional 1P-STR. However, process operation at high dilution rates promoted the washout of biomass in the aqueous phase and the accumulation of hydrophobic methanotrophs in the NAP, which resulted in a superior performance of the 2P-STR. The 2P-STR was always mass transfer limited, while in the absence of biomass retention at low stirring rates (conditions typically encountered under

full-scale operation) the 1P-STR was limited by biological activity. Both the microscopic observations and the DGGE analysis showed that the presence of SO200 severely influenced the community structure and bacterial biodiversity. Finally, the investigation of new NAPs with higher affinities for CH_4, and the elucidation of the differential microbial growth and specific strategies that enable some MOB to be viable inside the NAP will be of major interest for the optimization of TPPBs with the biocatalytic activity confined in the NAP.

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Chapter 4

Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement

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Abstract

This study assessed an innovative strategy for the valorization of dilute methane emissions based on the bio-conversion of CH_4 (the second most important greenhouse gas (GHG)) into ectoine by the methanotrophic ectoine-producing strain *Methylomicrobium alcaliphilum 20 Z*. The influence of CH_4 (2-20 %), Cu^{2+} (0.05-50µM) and NaCl (0-9 %) concentration as well as temperature (25-35 ºC) on ectoine synthesis and specific CH_4 biodegradation rate was evaluated for the first time. Concentrations of 20 % CH_4 (at 3 % NaCl, 0.05 µM Cu^{2+}, 25 ºC) and 6 % NaCl (at 4 % CH_4, 0.05 µM Cu^{2+}, 25 ºC) supported the maximum intra-cellular ectoine production yield (31.0 ± 1.7 and 66.9 ± 4.2 mg g biomass^{-1}, respectively). On the other hand, extra-cellular ectoine concentrations of up to 4.7 ± 0.1 mg L^{-1} were detected at high Cu^{2+}concentrations (50 µM), despite this methanotroph has not been previously classified as an ectoine-excreting strain. This research demonstrated the feasibility of the bio-conversion of dilute emissions of methane into high-added value products in an attempt to develop a sustainable GHG bioeconomy.

Keywords: Ectoine, Greenhouse Gas, Methane treatment, Methane biorefinery

1. Introduction

Methane (CH_4) is the second most important greenhouse gas (GHG) emitted nowadays as a result of its high global warming potential (25 times higher than that of CO_2) and emission rates (United States Environmental Protection Agency, 2015). Despite CH_4 can be used as an energy vector for electricity and heat generation when concentrations are higher than 20 %, more than 56 % of anthropogenic CH_4 emissions worldwide contain concentrations lower than 5 %. When applied to these dilute emissions (such as exhaust gases from landfills or coal mines), current CH_4 abatement technologies are neither environmentally friendly nor cost-effective (Avalos Ramirez et al., 2012).

Nowadays, the lack of a suitable approach to prevent the adverse environmental effects of CH_4 has encouraged both political initiatives to control these GHG emissions and an intensive research on novel strategies for CH_4 abatement (European Environmental Agency, 2015). In this regard, the biological abatement of dilute CH_4 emissions combined with the production of high-added value products represents, if properly tailored, a cost-effective alternative to mitigate CH_4 emissions. This CH_4 bio-refinery approach would avoid the negative environmental effects of methane emissions while turning its treatment into a profitable process.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most valuable microbial protective compounds against osmotic dehydration, as well as an efficient stabilizer for enzymes and nucleic acids (Pastor et al., 2010). This compound has attracted recent attention based on the high retail value that purified ectoine reaches in the cosmetic industry (approximately \$1300 kg^{-1}) (Strong et al., 2015). In 1999, Khmelenina et al. demonstrated that some moderate halophilic methanotrophs such as *Methylomicrobium alcaliphilum 20Z* were able to produce and accumulate ectoine inside the cell (But et al., 2013; Kaluzhnaya et al., 2001; Kalyuzhnaya et al., 2008; N. V. Khmelenina et al., 2000; Khmelenina et al., 1999). These studies, conducted at high CH_4 concentrations, represented the first proof of the ability of CH_4-oxidizing bacteria to produce ectoine. However, little is known about

the influence of environmental conditions on the bioproduction of this secondary metabolite when combined with the abatement of dilute CH_4 emissions. Furthermore, no studies on the production of extra-cellular ectoine (naturally excreted to the medium by specific excreting strains) by methanotrophs have been carried out to date.

The present study aimed at systematically elucidating the influence of CH_4, copper (Cu^{2+}) and NaCl concentrations, as well as temperature, on the extra and intra-cellular ectoine production using the strain *Methylomicrobium alcaliphilum 20Z*.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used was a modified Brunner medium prepared according to Kalyuzhnaya et al. (2008) with a final pH of 9.1. NaCl and $CuCl_2 \cdot 2H_2O$ were supplemented to the MSM at the different concentrations tested All chemicals and reagents were purchased from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 was purchased from Abello-Linde, S.A. (Barcelona, Spain) with a purity of at least 99.5 %.

2.2. Microorganisms and inoculum preparation

The methanotrophic strain used in this study, *Methylomicrobium alcaliphilum 20Z* (Kalyuzhnaya et al., 2008), was purchased from DSMZ (Leibniz-Institut). *Methylomicrobium alcaliphilum 20Z* is an halophilic alkalitolerant methanotrophic strain able to produce ectoine in the presence of NaCl (V. N. Khmelenina et al., 2000). Briefly, a 10× dilution of the liquid *Methylomicrobium alcaliphilum 20Z* stock culture from DSMZ was grown at 25 ºC in 120 mL glass bottles containing 90 mL of MSM at 3 % of NaCl and 0.05 µM Cu^{2+}. The bottles were closed with gas-tight butyl septa and metallic caps and 50 % v/v of the air headspace was replaced by CH_4. The inoculum was ready to use in the batch cultivation tests when a bacterial biomass concentration of 0.1 ± 0.06 g L^{-1} was achieved.

2.3. Batch cultivation tests

Five series of 13-day tests (TS) were performed in duplicate to evaluate the influence of different environmental factors (CH_4, NaCl, Cu^{2+}, T) on the production of extra and intra-cellular ectoine by *Methylomicrobium*

alcaliphilum 20 Z (Table 1). Sterile batch gas-tight reactors (1.2 L) containing 190 mL of MSM and inoculated with 10 mL of the inoculum above described (to an initial concentration of 0.05 ± 0.001 g L^{-1}) were used in each tests series. The reactors were closed with gas-tight butyl septa and plastic screw caps. Unless otherwise specified, all tests were initially supplied with a CH_4 headspace concentration of 25 g CH_4 m^{-3} (4 %), 3 % of NaCl, 0.05 µM Cu^{2+} and incubated at 25 ºC under a continuous magnetic agitation of 600 rpm. All tests were periodically monitored until complete CH_4 consumption, with no replacement of the headspace along the test. The parameter evaluated in each specific test series was: TS1: methane concentration (2, 4 and 20 %), TS2: copper concentration (0.05, 25 and 50 µM), TS3: NaCl concentration (0, 3, 6 and 9 %), and TS4: temperature (25, 30, 35 ºC). A final test (TS5) combining the optimum conditions for ectoine production obtained in the previous test series (20 % CH_4, 50 µM Cu^{2+}, 6 % NaCl and 30 ºC) was also carried out.

Aliquots of pure CH_4 were supplied to the headspace of the reactors in TS1 using a 100 mL gas tight syringe. Copper and NaCl were supplied by addition of the corresponding amount of salt to the cultivation broths in TS2 and TS3, respectively, while the different temperatures used in TS4 (25, 30 or 35º C) were maintained using thermostatic baths (Digiterm-S-150 20).

The O_2, CO_2 and CH_4 headspace

Table 1. Cultivation conditions evaluated during *Methylomicrobium alcaliphilum 20Z* batch cultivation tests.

Test series (TS)	Operating conditions			
	CH_4 (%)	**Cu^{2+} (µM)**	**NaCl (%)**	**T (ºC)**
TS1	2, 4, 20	0.05	3	25
TS2	20	0.05, 25, 50	3	25
TS3	20	0.05	0, 3, 6, 9	25
TS4	20	0.05	3	25, 30, 35
TS5	20	50	6	30

concentrations were daily monitored. Aliquots of 10 mL from the cultivation broth were also daily drawn with a liquid syringe to determine biomass concentration and the intra and extra-cellular ectoine concentration. Biomass concentration was estimated via culture absorbance (OD) measurements at 650 nm, which were previously correlated to dry biomass concentrations (g L^{-1}) determined as total suspended solids (TSS) concentration (TSS = 0.30×OD). A cell viability assay was performed in the cultivation of the tests incubated with Cu^{2+} at 25 µM

2.4 Extraction and Analysis of Ectoine

The intra-cellular ectoine concentration was determined using 2 mL of cultivation broth centrifuged at 9000 g and 4 ºC for 15 min. Then, 2 mL of 80 % ethanol and 25 ± 5 mg of 0.1-mm-diameter zirconia/silica beads (BioSpec, Spain) were added to the Eppendorf tube containing the pellet. Microbial cells were then disrupted in a Mini-BeadBeater-16 (BioSpec, Spain) at 1048 g for 1 min and the suspension was kept overnight at room temperature (modified from Lang et al., 2011). The supernatant of these suspensions was used for ectoine analysis prior centrifugation at 9000 g and 4 ºC for 15 min and filtration through 0.22 µM filters (Filter-lab, Barcelona). The specific intra-cellular ectoine concentration (g ectoine g $biomass^{-1}$) was calculated using the TSS concentration (g L^{-1}) of the corresponding cultivation broth. An aliquot of 1 mL of cultivation broth was also drawn and filtered through 0.22 µM filters (Filter-lab, Barcelona) to measure the extra-cellular ectoine concentration. The concentration of ectoine was measured by HPLC-UV in a HPLC 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA detector) at 220 nm using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, EEUU) and a C18 AQ + pre-column (Waters, Bellefonte, EEUU). A phosphate buffer, consisting of 0.8 mM K_2HPO_4 and 6.0 mM Na_2HPO_4, was used as a mobile phase at 25 ºC and a flow rate of 1 mL min^{-1} (Becker et al., 2013). Ectoine quantification was carried out using external standards of commercially available ectoine ((S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %, Sigma Aldrich, Spain) (Tanimura et al., 2013). The detection and quantification limits (DL and QL) were

calculated via determination of the signal-to-noise ratio performed by comparing the measured signals from samples with known low concentrations of analyte with those of blank samples. This procedure allowed establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 and 2:1 is generally considered acceptable for estimating the detection limit while a signal-to-noise ratio of 10:1 is necessary to determine the quantification limit (ICH Expert working Group, 2005).

2.5 *Analytical procedures*

CH_4, O_2 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were maintained at 45 ºC, 150 ºC and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

Culture absorbance measurements at 650 nm were conducted using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). TSS concentration was measured according to standard methods (American Water Works Association, 2012). Vital and dead bacteria were identified using the LIVE/DEAD BacLight™ Bacterial Viability Kit (L13152) using a Leica DM4000B microscope equipped with a Leica DFC300FX.

2.6. *Data analysis*

The specific CH_4 degradation rate (SDR, g CH_4 $g^{-1}_{biomass}$ h^{-1}) was calculated from the slope of the time course plot of methane concentration within the linear range in the batch cultivation tests carried out. The statistical data analysis was performed using SPSS 20.0 (IBM, USA). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were analysed by ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

3. Results

3.1. Influence of cultivation conditions on intra-cellular ectoine production

Intra-cellular ectoine reached its maximum concentration between days 5 and 7 of cultivation regardless of the conditions tested. Subsequently, the

concentration of intra-cellular ectoine remained constant until the end of the assay (Figure 1a).

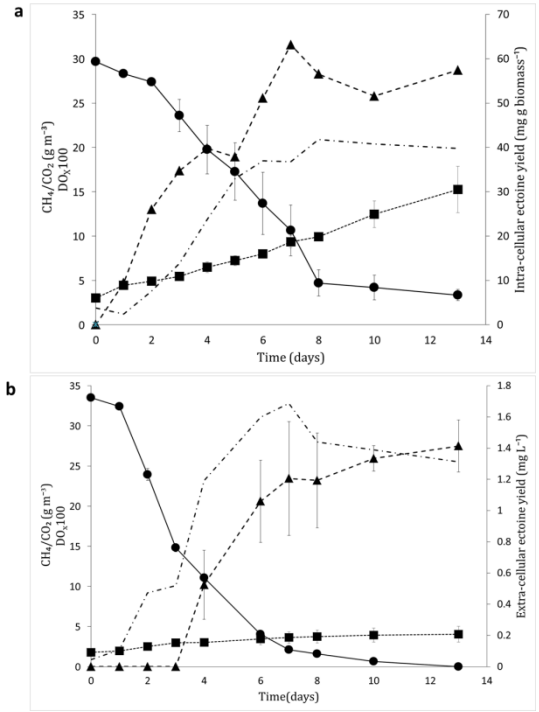

Figure 1. Time course of the concentration of CH_4 (●, continuous line), CO_2 (■, dotted line). DO×100 (dashed and dotted line) and intra-cellular (a) or extra-cellular (b) ectoine (▲, dashed line) at a) during *Methylomicrobium alcaliphilum 20Z* cultivation at 6 % NaCl, 0.05 µM Cu^{2+}, 25 ºC and 4 % CH_4, and b) at 3 % NaCl, 50 µM Cu^{2+}, 25 ºC and 4 % CH_4.

No significant difference was recorded in the intra-cellular ectoine concentration at 4 % CH_4, 3 % NaCl, 0.05 µM Cu^{2+} and 25 ºC in TS1-TS4, which confirmed the reproducibility and consistency of the results here obtained. Both CH_4 and NaCl concentrations had a significant influence on the production of intra-cellular ectoine (Figure 2). A CH_4 concentration of 20 % supported maximum specific yields of 31.0 ± 1.7 mg ectoine g biomass^{-1} by the end of the cultivation, while the maximum yields obtained at CH_4 concentrations of 2 and 4 % were 9.9 ± 0.6 and 13.6 ± 3.8 mg ectoine g biomass^{-1}, respectively. A NaCl concentration of 6 % was identified as the optimum value for the accumulation of intra-cellular ectoine, which reached a maximum concentration of 66.9 ± 4.2 mg ectoine g biomass^{-1}. Higher or lower salt concentrations supported lower ectoine yields (30.4 ± 7.5 mg ectoine g biomass^{-1} at 9 % NaCl; 12.5 ± 3.9 mg ectoine g biomass^{-1} at 3 % NaCl; 1.2 ± 0.5 mg ectoine g biomass^{-1} at 0 % NaCl).

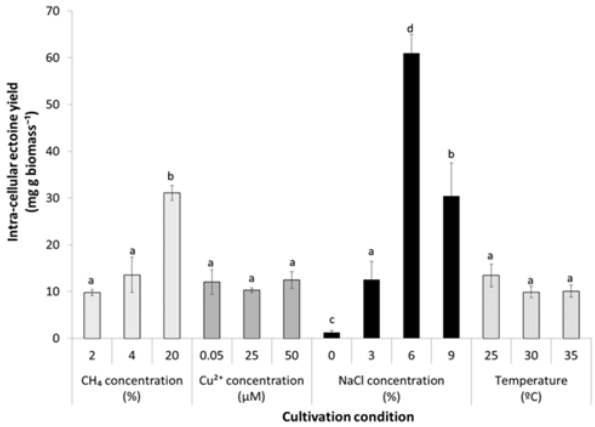

Figure 2. Maximum intra-cellular ectoine yield under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

On the contrary, no significant effect ($p<0.05$) of temperature or Cu^{2+} concentration was observed on the production of intra-cellular ectoine (Figure 2).

The maximum specific intra-cellular ectoine yields recorded at the different Cu^{2+} concentrations tested were 12.0 ± 2.6 mg ectoine g biomass^{-1} at 0.05 µM Cu^{2+}, 10.3 ± 0.5 mg ectoine g biomass^{-1} at 25 µM Cu^{2+} and 12.4 ± 0.7 mg ectoine g biomass^{-1} at 50 µM Cu^{2+}. Similarly, no significant effect of temperature on ectoine accumulation was observed within the tested T range, with an average yield of 11.70 ± 1.1 mg ectoine g biomass^{-1}.

3.2. Influence of cultivation conditions on ectoine excretion

While no extra-cellular ectoine was detected (DL= 0.4 mg/L and QL= 0.65 mg/L) during the 13 days of cultivation under the different concentrations of CH_4, NaCl and temperature tested, ectoine excretion was detected at high Cu^{2+} concentration (Figure 3).

Ectoine excretion was observed by day 4 in tests supplemented with high Cu^{2+} concentrations (Figure 1b). The maximum concentrations recorded were 0.7 ± 0.05 and 1.2 ± 0.01 mg extra-cellular ectoine L^{-1} (0.5 and 1.1 % (g g^{-1})) at 25 and 50 µM of Cu^{2+}, respectively.

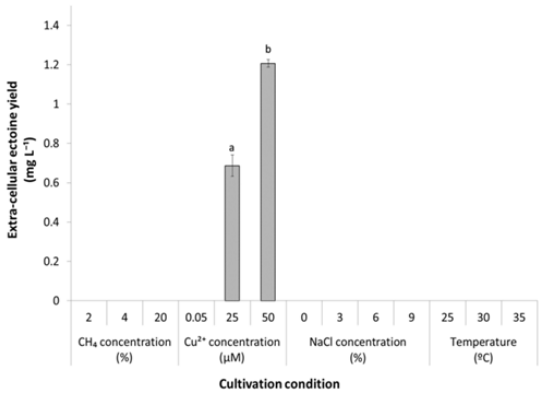

Figure 3. Extra-cellular ectoine excreted under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

3.3. Influence of cultivation conditions on the specific CH_4 degradation rate

In the TS1 (0.05 µM Cu^{2+}, 3 % NaCl and 25ºC), the results showed that a CH_4 headspace concentration of 20 % supported a significantly ($p<0.05$) higher SDR (1.50 ± 0.08 g CH_4 h^{-1} g biomass^{-1}) compared to the SDRs recorded at 4 and 2 % of CH_4 (0.33 ± 0.05 and 0.29 ± 0.03 g CH_4 h^{-1} g biomass^{-1}, respectively) (Figure 4). The results showed that a CH_4 headspace concentration of 20 % supported a significantly ($p<0.05$) higher

SDR (1.50 ± 0.08 g CH_4 h^{-1} g biomass^{-1}) compared to the SDRs recorded at 4 and 2 % of CH_4 (0.33 ± 0.05 and 0.29 ± 0.03 g CH_4 h^{-1} g biomass^{-1}, respectively) (Figure 4). On the contrary, the specific CH_4 oxidation rates decreased at higher NaCl concentrations, with CH_4 SDRs of 0.05 ± 0.005, 0.22 ± 0.02, 0.34 ± 0.02 and 0.38 ± 0.02 g CH_4 h^{-1} g biomass^{-1} at 9, 6, 3 and 0 % NaCl, respectively.

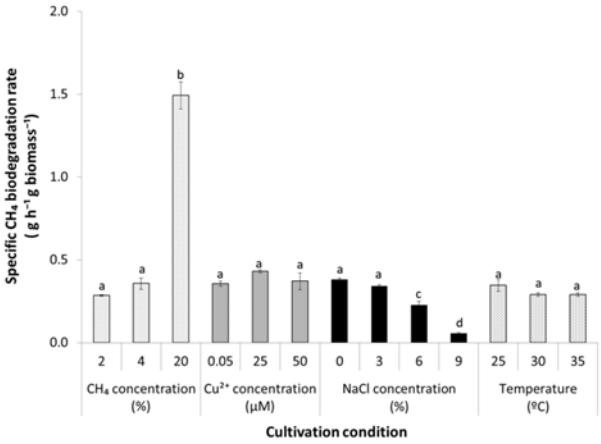

Figure 4. Specific CH_4 biodegradation rate under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Neither temperature (25, 30 and 35 ºC supported SDRs of 0.34 ± 0.04, 0.30 ± 0.01 and 0.30 ± 0.01 g CH_4 h^{-1} g biomass^{-1}, respectively) nor Cu^{2+} concentration (0.05, 25 and 50 µM of Cu^{2+} supported SDRs of 0.35 ± 0.01, 0.43 ± 0.01 and 0.37 ± 0.05 g CH_4 h^{-1} g biomass^{-1}, respectively) showed a significant effect on the specific CH_4 degradation rate.

3.4. Production of extra and intra-cellular ectoine under optimum cultivation conditions

A final study was carried out combining the optimum parameters from previous tests TS1-TS4 in order to determine the production of extra and intra-cellular ectoine (20 % CH_4, 6 % NaCl, 30 ºC, 50 µM Cu^{2+}) (Table 2). These cultivation conditions promoted the excretion of ectoine to the extra-cellular medium (4.7 mg L^{-1}, which would correspond to 33.3 mg ectoine g biomass^{-1}) and resulted in a high production of intra-cellular ectoine (40.7 ± 0.02 mg ectoine g biomass^{-1}).

4. Discussion

This research investigated for the first time the effect of 4 environmental parameters (i.e. CH_4 concentration, Cu^{2+} concentration, temperature and NaCl concentration) on the production and excretion of ectoine and on the specific CH_4 degradation by *Methylomicrobium alcaliphilum 20 Z* in order to elucidate the optimum operational conditions to maximize ectoine production during the abatement of dilute CH_4 emissions.

Table 2. Maximum values of ectoine concentration and specific CH_4 degradation rates for each test

	Maximum extra-cellular ectoine		Maximum intra-cellular ectoine		Maximum specific CH_4 degradation rate
	[Ectoine] (mg L^{-1})	**% Ectoine** ((g Ect. g biomass^{-1}) ×100)	**[Ectoine]** (mg g biomass^{-1})	**% Ectoine** ((g Ect. g biomass^{-1}) ×100)	(g CH_4 h^{-1} g biomass^{-1})
TS1					
20% CH_4, 25ºC, 0.05µM Cu^{2+}, 3% NaCl	N/D	N/D	31.0 ± 1.7	3.1 %	1.50 ± 0.08
TS2					
4% CH_4, 25ºC, 0.05µM Cu^{2+}, 6% NaCl	N/D	N/D	66.9 ± 4.2	6.7 %	0.22 ± 0.02
TS3					
4% CH_4, 25ºC, 50µM Cu^{2+}, 3% NaCl	1.2 ± 0.01	1.1 %	12.4 ± 0.7	1.2 %	0.30 ± 0.01
TS4					
4% CH_4, 30ºC, 0.05µM Cu^{2+}, 3% NaCl	N/D	N/D	10.6 ± 0.15	1.1 %	0.37 ± 0.05
TS5					
20 % CH4, 30ºC, 50µM Cu^{2+}, 6% NaCl	4.7 ± 0.05	3.2 %	40.7 ± 0.02	4.1 %	2.03 ± 0.11

A high concentration of CH_4 in the GHG emission significantly enhanced the production of intra-cellular ectoine likely due to an increase in substrate availability for the bacterial community, which induced high growth rates and therefore a high metabolite production (Estrada et al., 2014). In this sense, an increase in CH_4 concentration from 4 to 20 % enhanced the production of intra-cellular ectoine by a factor of 2.7 (from 9.9 ± 0.6 to 30.4 ± 7.5 mg ectoine g biomass^{-1}). However, no significant effect was observed on the accumulation of ectoine within the lower range of CH_4 concentrations tested (2 and 4 %). The key role of CH_4 concentration in the intra-cellular ectoine accumulation is in agreement with the results observed by Khemelenina et al. (2000), who recorded a maximum ectoine concentration of 200 mg ectoine g biomass^{-1} in *Methylomicrobium alcaliphilum 20 Z* under a CH_4 concentration of 50 % (v/v) in a MSM with 6 % of NaCl, 0.05 µM Cu^{2+} at 29 ºC (V. N. Khmelenina et al., 2000; Trotsenko et al., 2005). However, only dilute CH_4 emissions (<20 %) not suitable for energy recovery can be considered as a substrate of this novel CH_4 bio-refinery.

The salinity of the cultivation medium exhibited a positive effect on the intra-cellular ectoine yield up to a concentration of 6 % NaCl (66.9 ± 4.2 mg ectoine g biomass^{-1}).). The same effect has been observed by other authors using *Methylomicrobium alcaliphilum 20Z*. For instance, But et al. (2013) observed a 4

times increase in the concentration of intra-cellular ectoine in *M. alcaliphilum 20Z* when salt concentration was raised from 3 to 7 % (from 10 to 40 μg mg biomass^{-1}). However, in our particular study, higher salt concentrations resulted in lower ectoine yields (30.4 ± 7.5 mg ectoine g biomass^{-1} at 9 % of NaCl).

Some authors have also observed a decrease in ectoine accumulation at increasing external salinity due to the regulation of the ectoine biosynthesis at the enzyme activity level (Reshetnikov et al., 2005). It is noteworthy that the concentration of ectoine herein obtained at 6 % NaCl does not differ much from the values commonly encountered during the industrial production of ectoine using the glucose fermentative microorganism *Halomonas elongate* (yielding ectoine at an average value of 155.5 mg ectoine g biomass^{-1} when reused 9 times) (Strong et al., 2015). Nowadays, the process implemented at industrial scale (bacterial milking) involves the cyclic increase and decrease of the salt concentration in the cultivation broth up to 12 % NaCl. This process involving salt shocks increases reactor corrosion and hinders the downstream processing of ectoine due to the discontinuous nature of the production procedure and the high concentrations of salt. Moreover, this process requires an expensive carbon source such as glucose. Alternatively, *Methylomicrobium alcaliphilum 20Z* can continuously synthesize a comparable high yield of ectoine in a less harsh cultivation medium coupled with CH_4 abatement from dilute emissions, which lowers production costs and mitigates climate change. Ectoine production was also observed in the absence of NaCl, although the concentrations detected were 55 times lower than the maximum ectoine yield recorded at 6 % of NaCl. The presence of a basal activity of the specific enzymes responsible for ectoine biosynthesis was likely related to the constitutive transcription of the ectoine gene cluster by this strain as previously observed by Khmelenina et al. (2000) and Reshetnikov et al. (2006). Moreover, the high concentration of sodium carbonates present in the cultivation media could have mediated the expression of the *ectabc* operon.

Likewise, Reshetnikov et al. (2006) confirmed that the optimum temperature for the enzymes catalyzing the key reactions of ectoine biosynthesis in *Methylomicrobium alcaliphilum 20Z* was

20 ºC, while temperatures higher than 30 ºC inhibited this reaction. However, no pernicious effect of temperature on the production of intra-cellular ectoine was observed in the present study. Moreover, no significant differences occurred among the ectoine yields recorded in the cultivation media containing different Cu^{2+} concentrations.

Ectoine excreting bacterial strains can accumulate ectoine and excrete it into the cultivation medium, thus enhancing the economics of the industrial production process of ectoine (Lang et al., 2011). Up to date, *Methylomicrobium alcaliphilum 20Z* has never been described as a strain able to excrete ectoine to the extra-cellular medium (Trotsenko et al., 2005). Whereas no extra-cellular ectoine was detected at the concentrations of NaCl and CH_4 and temperatures tested, cultivation at high Cu^{2+} concentrations promoted the excretion of a significant fraction of intra-cellular ectoine. This excretion could be have been caused by a release from dead or injured cells by Cu^{2+}. However, no significant differences were observed among the final biomass concentrations or SDR recorded under the three Cu^{2+} concentrations tested (Figure 1. Supplementary Materials). Moreover, a cell viability assay of *Methylomicrobium alcaliphilum 20Z* grown at high copper concentrations demonstrated that this element was not toxic (Figure 2, Supplementary Materials). In this regard, copper could be associated with the activation of unspecific channels or specific transporters able to excrete ectoine as a result of passive diffusion or an active transport of the cation Cu^{2+}. A research carried out by Schubert et al. (2007) observed that a transgenic *E.coli* genetically modified with the genes for ectoine biosynthesis, *ectABC*, from the halophilic bacterium *Chromohalobacter salexigens*, was able to excrete ectoine via expression of a specific transporter (Schubert et al., 2007). In this context, the presence of high Cu^{2+} concentrations in the cultivation medium could affect the activation of some specific transporters. Gram negative bacteria can express the tubular trans-membrane proteins porins, which allow the diffusion of small solutes and constitute a likely route for the simultaneous uptake of unchelated Cu^{2+} and excretion of intra-cellular ectoine in methanotrophs (Balasubramanian et al., 2011).

Along with the optimization of ectoine production, the maintenance of an

efficient CH_4 abatement from dilute emissions of this GHG is also of key importance in the context of climate change mitigation. Hence, the highest SDRs were obtained at a CH_4 concentration of 20 % as a result of the enhanced gas-liquid concentration gradients, which likely induced higher aqueous CH_4 concentrations and therefore higher specific growth rates (Figure 3. Supplementary Materials) (Cantera et al., 2016). However, no SDR enhancement was observed in the low range of CH_4 concentrations (2 and 4 %), as previously reported by Cantera et al. (2016). The complete oxidation of 1 mol of CH_4 requires a maximum of 2 mol of O_2, this ratio being lower in practice as a result of biomass growth. On the one hand, the K_La of O_2 and CH_4 are relatively similar, which means that the transfer of oxygen and methane from the gas phase to the microbial community is a function of their concentration in the gas phase (Yu et al., 2006). On the other hand, our particular study focused on the treatment of dilute CH_4 emissions, where the concentration of oxygen is typically larger than the concentration of CH_4 (> 20%) and O_2 limitation is unlikely to occur. On the other hand, high salt concentrations negatively affected methane biodegradation in *Methylomicrobium alcaliphilum 20Z*, the SDR obtained at 9 % NaCl being 7.7 times lower than that obtained at 3 % NaCl. This strain is an halotolerant alkaliphilic methanotroph (Khmelenina et al., 1997) that can tolerate higher salt concentrations than other methanotrophs, but highly saline environments are not its optimum habitat. Thus, despite higher NaCl concentrations mediated the highest ectoine yields (6 and 9 %), a reduced CH_4 abatement performance was recorded. On the other hand, temperature and Cu^{2+} did not affect the SDR.

Finally, the optimum conditions (30ºC, 50 µM Cu^{2+}, 20 % of CH_4 and 6 % of NaCl) were combined in a test in order to maximize both extra and intra-cellular ectoine production. In this particular assay, the production of extra-cellular ectoine was 4 times higher than that obtained in TS2 (25ºC, 50 µM Cu^{2+}, 4 % of CH_4 and 3 % of NaCl), while the intra-cellular ectoine yield was lower than that recorded in TS3 (6 % of NaCl, 0.05 µM Cu^{2+} and 4 % of CH_4). Thus, the combination of these parameters favored the excretion of 44.4 % of the total intra-cellular ectoine produced (4.7 % (g g^{-1})).

The total (intra-cellular + extra-cellular) ectoine produced would account for 73 mg ectoine g biomass^{-1} if not excreted, which was similar to the intracellular ectoine concentration (66.9 ± 4.2 mg gbiomass^{-1}) in TS3 at 6 % NaCl. These concentrations were lower than those reported by other ectoine producing strains grown on costly high-quality carbon sources (e.g. glucose). For example, *Halomonas elongate* has been reported to produce 4.1 g L^{-1} of extra-cellular ectoine (90% of the total ectoine) in industrial fed-batch fermentation (Sauer and Galinski, 1998), while *Brevibacterium epidermis* excretes 8 g L^{-1} (50% of the total ectoine produced) (Onraedt et al., 2005). However, these systems were operated with a high cell density of 48 and 49 g L^{-1}, respectively, while the biomass concentration maintained in our batch study was ~ 100 mg L^{-1}. Therefore, the specific amount of extra-cellular ectoine here obtained could be comparable with previous industrial studies when expressed as g ectoine per g biomass.

5. Conclusion

A proper selection of the environmental parameters (temperature, Cu^{2+}, NaCl and CH_4 concentration) during *Methylomicrobium alcaliphilum 20Z* cultivation is crucial to simultaneously, maximize both the intra-cellular production and excretion of ectoine and CH_4 abatement. The study here presented constitutes a first attempt to evaluate the feasibility of producing ectoine combined with methane abatement. The promising results obtained in this preliminary study support further research in order to implement this CH_4 biorefinery in a continuous system capable of creating value out of GHG mitigation using extremophilic methanotrophs.

6. Acknowledgements

This research was supported by MINECO/FEDER/EU (CTM2015-70442-R project and Red NOVEDAR), the European Union through the FEDER Funding Program and the Regional Government of Castilla y León (PhD Grant contract Nº E-47-2014-0140696 and UIC71).

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Supplementary material

Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement

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Figure 1

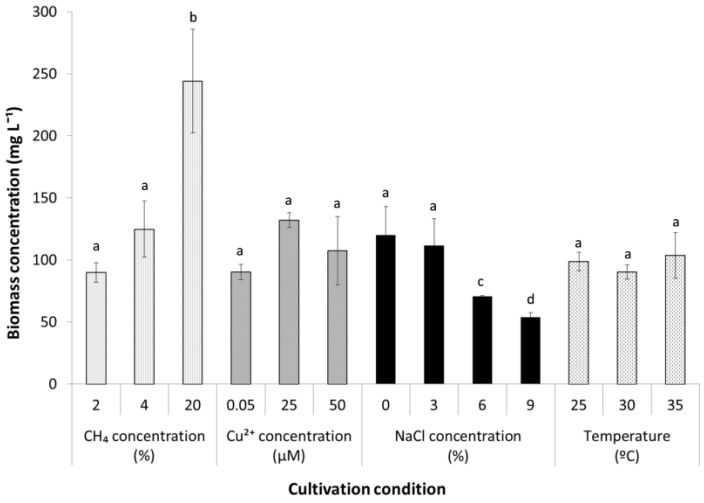

Figure 1: Final biomass concentration under the different cultivation conditions evaluated. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Figure 2

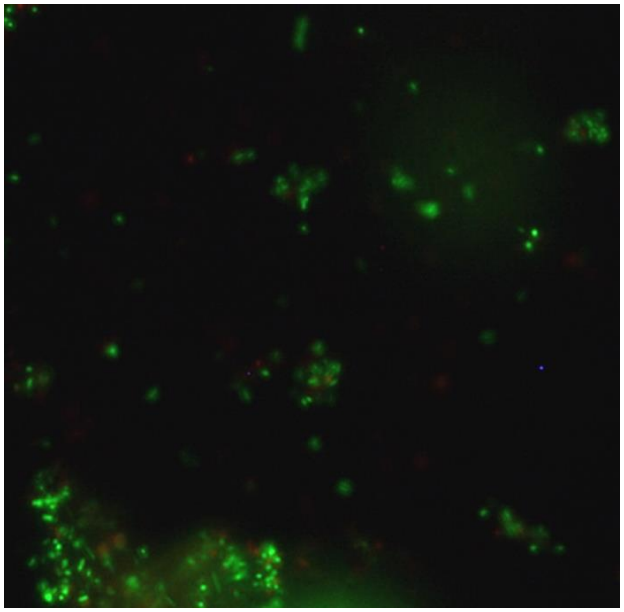

Figure 2. Microscopic image of the enriched bacteria in TS2 (25 µM Cu^{2+}) using a Leica DM40000b AT fluorescence microscope 40× magnification and specific filters sets for alive bacteria (SYTO®9 green-fluorescent nucleic acid stain) and dead bacteria (propidium iodide red-fluorescent nucleic acid stain).

Figure 3

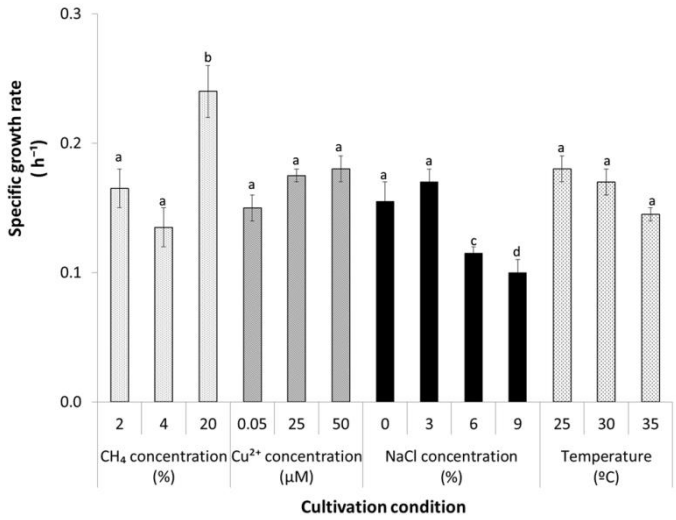

Figure 3: Specific growth rate under the different cultivation conditions evaluated. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Continuous abatement of methane coupled with ectoine production by Methylomicrobium alcaliphilum 20Z in stirred tank reactors: a step further towards greenhouse gas biorefineries.

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Chapter 5

Continuous abatement of methane coupled with ectoine production by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors: a step further towards greenhouse gas biorefineries

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Abstract

This study demonstrates for the first time the feasibility of producing ectoine (a high added value osmoprotectant intensively used in the cosmetic industry) during the continuous abatement of diluted emissions of methane by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors under non-sterile conditions. An increase in NaCl concentration in the cultivation broth from 3 to 6 % increased the intra-cellular ectoine yield by a factor of 2 (from 16.5 to 37.4 mg ectoine (g biomass)$^{-1}$), while high stirring rates (600 rpm) entailed a detrimental cellular stress and 3 times lower ectoine yields (5.6 mg ectoine (g biomass)$^{-1}$) compared to process operation at 300 rpm. An increase in Cu^{2+} concentration from 0.05 to 25 µM enhanced methane abatement by a factor of 2 (up to elimination capacities of 24.5 g m^{-3} h^{-1}), did not enhance intra-cellular ectoine production but promoted the excretion to the cultivation broth of 20 % of the total ectoine synthesized regardless of the NaCl concentration and stirring rate. The results obtained by culture hybridization with the specific probe Mγ1004 showed that *Methylomicrobium alcaliphilum 20Z* accounted for more than 80 % of the total bacterial population in most experimental runs. This work confirmed the technical feasibility of a new generation of biorefineries based on the abatement of diluted CH_4 emissions using extremophile methanotrophs.

Keywords: Ectoine, climate change, methane abatement, methane biorefinery, *Methylomicrobium*

1.Introduction

Methane (CH_4), with a global warming potential 25 times higher than that of CO_2 in a 100 y horizon, is nowadays the second most relevant greenhouse gas (GHG) emitted to the atmosphere (European Environmental Agency, 2015). CH_4 can be used as an energy vector for the production of electricity and heat when its concentration is higher than 20 %, however, more than 56 % of the anthropogenic CH_4 emissions worldwide contain concentrations of this GHG lower than 5 % (Estrada et al., 2014; IPCC, 2014; EEA, 2015). CH_4 abatement using conventional physical/chemical technologies is either inefficient or too costly at such low concentrations, and often entails a large CO_2 footprint (Estrada et al., 2014; Nikiema et al., 2007). In this context, biological treatment technologies can become a low cost and environmentally friendly alternative to their physical/chemical counterparts for the treatment of diluted CH_4 emissions (López et al., 2013).

However, the widespread implementation of methane treatment biotechnologies is still restricted by i) the low mass transfer of CH_4 from the emission to the bacterial community due to its high hydrophobicity, which entails high investment and operating costs; and ii) the lack of knowledge about the potential industrial applications of methanotrophic bacteria for the bioconversion of CH_4 into high-added value products, which would significantly enhance the economic viability of the process (López et al., 2013; Strong et al., 2016). Indeed, the biological oxidation of diluted CH_4 emissions combined with the production of high added value products could be, if properly tailored, a cost-competitive approach to mitigate climate change.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most valuable bioproducts synthezised by microorganisms, retailing in the pharmaceutical industry at approximately US$1000 kg^{-1} (among the most important pharmaceutical companies that produce ectoine nowadays can be found Merk, Johnson & Johnson, Larens, Bioderma…). The global demand of this compound accounts for 15000 tones $year^{-1}$ (Strong et al., 2016). This inmino acid, which is synthesized by bacteria to survive in salt-rich environments, is an effective stabilizer for enzymes, DNA-protein complexes and nucleic acids (Pastor et al., 2010). Currently, ectoine is

commercially produced using the halophilic heterotroph *Halomonas elongata*. The most common industrial process, namely *bio-milking*, consists of a fed-batch two-stage at different salt concentrations (12 and 0%) to obtain first a high density culture (25 g L^{-1}) that is subsequently subjected to a hypoosmotic shock. The sudden decrease in medium salinity results in the excretion of ectoine from the cell to the culture broth, where the product is collected for its downstream purification. Despite the extensive design and operation experience in this industrial process, the high cost of the glucose required as a carbon source, and the need for an intensive O_2 supply, entail high operating costs (Pastor et al., 2010; Strong et al., 2015).

In 1999, Khmelenina et al. (1999) demonstrated that moderately halotolerant methanotrophs, such as *Methylomicrobium alcaliphilum 20Z*, could express the three specific enzymes involved in ectoine synthesis, and were thus able to accumulate ectoine inside the cell (Kaluzhnaya et al., 2001; Khmelenina et al., 1999; Khmelenina et al., 2000; Reshetnikov et al. 2006). These studies, conducted at high CH_4 concentrations, represented the first proof of the ability of CH_4-oxidizing bacteria to produce ectoine (Khemelenina et al., 2000; But et al., 2013, Strong et al., 2016). Further studies demonstrated that the bio-conversion of CH_4 into ectoine by the methanotrophic ectoine-producing strain *Methylomicrobium alcaliphilum 20 Z* could be carried out batch wise. Moreover, it was observed that environmental conditions involving CH_4 (2-20 %), Cu^{2+} (0.05-50µM) and NaCl (0-9 %) concentrations, as well as temperature (25-35 ºC), were crucial to boost ectoine synthesis and the specific CH_4 biodegradation rate (Cantera et al., 2016b). This finding supports the treatment of diluted CH_4 emissions coupled with the synthesis of ectoine in suspended growth bioreactors which would potentially reduce the costs associated to ectoine production while boosting climate change mitigation via active CH_4 abatement. In this context, the optimization of the cultivation conditions to promote ectoine accumulation and its continuous excretion from the cell to the culture broth, either following a process similar to the currently used with *Halomonas elongata*, namely "bio-milking", or by promoting the natural excretion of ectoine to the culture broth in

bioreactors, is crucial to ensure the technical and economic viability of the process (Cantera et al., 2016b).

The present study aimed at systematically elucidating the influence of copper (Cu^{2+}), NaCl concentrations and stirring rate on the continuous abatement of methane combined with the production of extra and intra-cellular ectoine in stirred tank bioreactors using the strain *M. alcaliphilum 20Z*.

2.Materials and Methods

2.1. Chemicals and mineral salt medium

A high-alkalinity mineral salt medium (MSM) with a final pH of 9.0 was used (Kalyuzhnaya et al. 2008). NaCl and $CuCl_2 \cdot 2H_2O$ were added to the MSM at the concentrations tested in each experimental run (Table 1). Unless otherwise specified, all chemicals and reagents were obtained from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (> 99.5 %) was purchased from Abello-Linde S.A (Barcelona, Spain).

2.2. Microorganisms and inoculum preparation

Methylomicrobium alcaliphilum 20Z, an halophilic alkalitolerant methanotrophic strain able to synthesize ectoine (Kalyuzhnaya et al., 2008), was acquired from DSMZ (Leibniz-Institut, Germany). An aliquot of 1 mL of *M. alcaliphilum 20Z* stock liquid culture was inoculated in 120 mL glass bottles containing 90 mL of 3 % NaCl/0.05 µM Cu^{2+} MSM. The bottles were closed with gas-tight butyl septa and aluminum caps, and CH_4 was then injected to the headspace in order to reach an initial concentration of 50 % v/v air/CH_4. The inocula were incubated at 25 ºC under orbital agitation at 220 rpm to a final biomass concentration of 0.1 ± 0.06 g L^{-1}.

2.3 Experimental set-up and operational conditions

1-L jacketed stirred tank reactors (STR) (Afora S.A., Spain) equipped with a Rushton turbine were used for the continuous abatement of CH_4 coupled with ectoine production. The STRs were filled with 950 mL of MSM and 50 mL of the inoculum previously described. A 0.033 L min^{-1} CH_4-laden air emission containing 26.8 ± 2.1 g CH_4 m^{-3} (≈ 4%), corresponding to a methane load of 53 g m^{-3} h^{-1}, was fed to the STRs via 10 µm porous stainless steel diffusers located at the bottom of the reactors. This polluted air emission was obtained by mixing a continuous pure CH_4 stream (from a CH_4

gas cylinder stored in a safety gas cylinder cabinet and regulated by a mass flow controller, Aalborg, USA) with a continuous pre-humidified air flow, resulting in a gas empty bed residence time (EBRT) of 30 min. 500mL of culture broth were replaced by fresh MSM every two days to prevent the accumulation of inhibitory metabolites and to maintain optimal nutrient concentrations. 400 mL of the total 500 mL drawn were centrifuged at 10000 rpm for 10 min and the biomass pellet was returned to the bioreactor prior resuspension in 500 mL of fresh MSM. The remaining 100 mL of aqueous cultivation broth were used for the determination of the biomass concentration (measured as culture absorbance and total suspended solids (TSS)), total nitrogen (TN), as well as intra and extra-cellular ectoine. Samples for the determination of TSS, TN and ectoine concentrations were drawn every two days. Six different operational conditions were tested (Table 1). In experimental runs 1 and 2, the stirring rate and Cu^{2+} concentration were fixed at 300 rpm and 0.05 µM, respectively, while two salt concentrations (3 and 6 % NaCl, respectively) were used in order to test the influence of medium salinity on CH_4 abatement and ectoine production. The influence of NaCl concentration (3 and 6 %) was also evaluated at a Cu^{2+} concentration of 0.05 µM and a stirring rate of 600 rpm in experimental runs 3 and 4. Finally, experimental runs 5 and 6 were carried out at a Cu^{2+} concentration

Table 1. Operational conditions evaluated during *Methylomicrobium alcaliphilum 20Z* cultivation in STRs for the optimization of CH_4 abatement and ectoine production

Experimental Run	Factors of study		
	NaCl (%)	Cu^{2+} (µM)	Agitation rate (rpm)
1	3	0.05	300
2	6	0.05	300
3	3	0.05	600
4	6	0.05	600
5	3	25	300
6	6	25	300

of 25 µM, 300 rpm and NaCl concentrations of 3% and 6 %, respectively, in order to assess the influence of Cu^{2+} on the simultaneous production of ectoine and CH_4 removal (by comparison with experimental runs 1 and 2). No experimental runs at a Cu^{2+} concentration of 25 µM and agitation rate of 600 rpm were performed due to the poor process performance recorded in test 3 and 4. All experimental runs were maintained for 50 days, which ensured process operation under steady state conditions. Prior to inoculation, an abiotic test with MSM was performed for 5 days at the above described operational conditions to assess any potential removal of CH_4 by adsorption or photodegradation in the experimental set-up.

Temperature was maintained at 25 ºC in all experiments. Distilled water was weekly added to compensate water evaporation losses. Steady state conditions were achieved when the elimination capacity (CH_4-EC) and CO_2 production rates ($TPCO_2$) deviated <10 % from the mean for at least 20 days. Gas samples for CH_4 and CO_2 analysis were periodically taken from the sampling ports located at the inlet and outlet of the bioreactors using 100 µl gas-tight syringes (HAMILTON, Australia). Biomass samples were taken at the end of each steady state for the identification and quantification of *Methylomicrobium alcaliphilum 20Z* by double labeling of oligonucleotide probes fluorescence *in situ* hybridization (DOPE-FISH).

2.4 Analytical procedures

The intra-cellular ectoine contained in 2 mL of cultivation broth was extracted to the extra-cellular medium according to Cantera et al. (2016b). The specific intra-cellular ectoine concentration (g ectoine g biomass^{-1}) was calculated using the corresponding TSS concentration (g L^{-1}). An aliquot of 1 mL of cultivation broth was also drawn and filtered through 0.22 µM filters (Filter-lab, Barcelona) to determine the extra-cellular ectoine concentration. The concentration of ectoine was measured by HPLC-UV in a 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA) at 210 nm and 40 ºC using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, USA) and a C18 AQ + pre-column (Waters, Bellefonte, USA). A phosphate buffer, consisting of 0.8 mM $K_2HPO_4.3H_2O$ and 6.0 mM $Na_2HPO_4.12H_2O$, was used as a mobile

phase at 25 ºC and a flow rate of 1 mL min^{-1} (Tanimura et al., 2013). Ectoine quantification was carried out using external standards of commercially available ectoine ((S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %, Sigma Aldrich, Spain). The ectoine retention time ranged from 2.32 to 2.35 min depending on the column pressure. The detection and quantification limits (DL and QL, respectively) were calculated via determination of the signal-to-noise ratio. In this sense, a signal-to-noise ratio of 3:1 - 2:1 is considered acceptable for estimating the detection limit, while a signal-to-noise ratio of 10:1 is necessary to determine the quantification limit (ICH Expert working Group, 2005). The DL and QL of ectoine in our specific MSM was 0.4 mg L^{-1} and 0.65 mg L^{-1}, respectively.

CH_4 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were maintained at 45, 150 and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

Culture absorbance measurements at 650 nm were conducted using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). TSS concentration was measured according to Standard Methods (American Water Works Association, 2012). pH was determined using a pH-meter Basic 20 (Crison, Spain), while the concentrations of TOC and TN were measured using a Shimadzu TOC-VCSH analyzer (Japan) equipped with a TNM-1 chemiluminescence module.

2.5. *Fluorescence in situ hybridization of the microbial cultures*

Methylomicrobium cells were identified and enumerated by Fluorescence *In Situ* Hybridization with double labeling of oligonucleotide probes (DOPE-FISH), which improves signal intensity and increases rRNA accessibility (Stoecker et al., 2010). A Cy3-double labeled oligonucleotide probe specific for *Methylomicrobium* genus (Mγ1004) (Eller et al., 2001; Stoecker et al., 2010) was used for quantification in comparison to DAPI-stained bacteria. The samples drawn at the end of each experimental run (250 µL) were fixed in 4 % (*w/v*) paraformaldehyde (750 µL) for 3 h and

then washed three times with phosphate-buffered saline (PBS) medium and preserved in 96 % (*v/v*) alcohol. Aliquots of 10 µL were placed on glass microscope slides and dehydrated with ethanol at 50, 80 and 96 % (*v/v*). Hybridization was carried out at 46 ºC for 12 h. The hybridization buffer contained 360 µL 5M NaCl, 40 µl 1MTris/HCl, 4µL 10 %SDS and Milli-Q water to a final volume of 2 mL. Hybridization stringency was set at 0 % formamide in the hybridization buffer as indicated in literature (Eller et al., 2001). After hybridization, the slides were submerged in 50 ml washing buffer for 20 minutes to remove unbound oligonucleotides. Total DNA was stained using DAPI (4P, 6-diamidino-2-phenylindole) (Sigma, Spain). After air-drying at room temperature, cover glasses were mounted with 2 µL Citifluor to reduce the fading of the fluorescent dyes used for labelling (Citifluor Ltd., UK). For quantitative FISH analysis, 15 images were randomly obtained from each condition using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC300FX camera. The relative bio-volumes (percentage) of *Methylomicrobium* from the total DAPI-stained bacterial population were calculated using the software DAIME (Daims et al., 2006). The averaged bio-volume fractions of *Methylomicrobium* and the standard error of the mean were calculated for each experimental run. Phase contrast images were also acquired to monitor the appearance of *Methylomicrobium* population at the end of the six experimental runs.

2.6. Data analysis

The statistical data analysis was performed using SPSS 20.0 (IBM, USA). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were determined by ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

3.Results and Discussion

3.1 Influence of operational conditions on intra-cellular ectoine production

The concentration of intra-cellular ectoine peaked at days 5-7 regardless of the operational conditions evaluated (Figure 1). The intra-cellular ectoine yield sharply decreased afterwards, increasing

again to achieve another relative maximum concentration between days 11 and 14 (Figure 1).

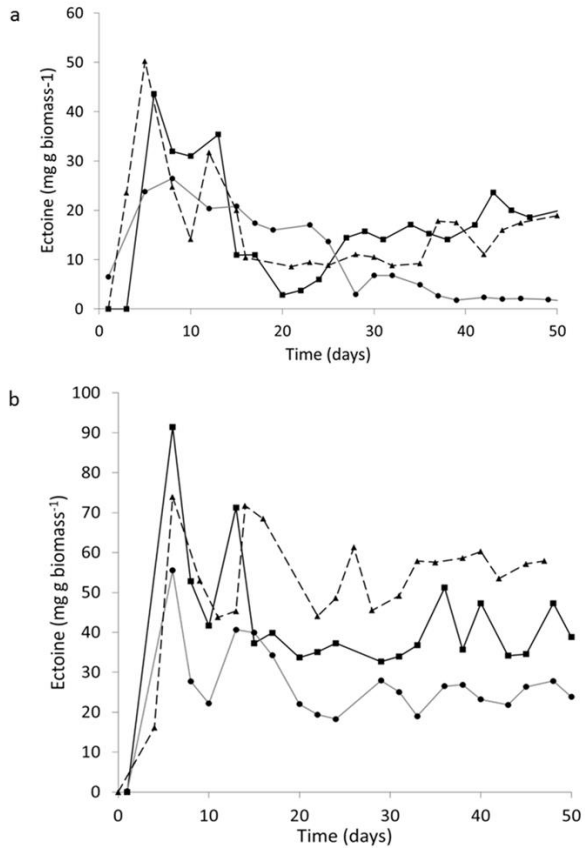

Figure 1: Time course of the intra-cellular ectoine concentration at a) 3 % NaCl-300 rpm-0.05µM Cu^{2+} (continuous black line), 3 % NaCl-600 rpm-0.05µM Cu^{2+} (continuous grey line) and 3 % NaCl-300 rpm-25µM Cu^{2+} (dotted line) and b) 6 % NaCl-300 rpm-0.05µM Cu^{2+} (continuous black line), 6 % NaCl-600 rpm-0.05µM Cu^{2+} (continuous grey line) and 6 % NaCl-300 rpm-25µM Cu^{2+} (dotted line). The symbols ■, ● and ▲ represent the sampling times.

Finally, the intra-cellular ectoine concentration decreased and stabilized from day 28 onward (Figure 1).

The correlation between an intensive production of ectoine and *M. alcaliphilum 20Z* exponential growth has been previously reported in batch wise cultivations. Khemelenina et al. (2000) observed that intra-cellular ectoine concentration peaked in the mid-exponential growth phase of *M. alcaliphilum 20Z*, and decreased afterwards during the growth-retardation phase, which suggested that ectoine can be used for the synthesis of other cell constituents. Cantera et al. (2016b) confirmed the over-production of intra-cellular ectoine during the exponential growth phase of *M. alcaliphilum 20Z*. This phenomenon could be associated to a hyperosmotic shock that initially resulted in an over-expression of the ect*abc* operon followed by a decrease in the concentration of ectoine, which was likely re-assimilated by cell metabolism.

The steady state intra-cellular ectoine yield was comparatively evaluated at the operational conditions tested (Figure 2). Cu^{2+} concentration did not show an effect on ectoine accumulation despite Cu^{2+} is a key micronutrient for type I methanotrophs (Semrau et al., 2010). Nevertheless, NaCl concentration showed a major influence on the accumulation of

intra-cellular ectoine in the long-term operation. A NaCl concentration of 6 % was identified in this study as the optimum salinity for the accumulation of intra-cellular ectoine, supporting average concentrations of 31.4 ± 1.5 and 37.4 ± 3.8 mg ectoine (g biomass)$^{-1}$ at 300 rpm under low and high Cu^{2+} concentrations (experimental runs 2 and 6), respectively. On the contrary, a NaCl concentration of 3 % supported lower ectoine yields (17.6 ± 0.9 and 16.5 ± 1.8 mg ectoine (g biomass)$^{-1}$ at 300 rpm and 0.05 and 25 µM (experimental runs 1 and 5), respectively). In spite of the improved ectoine accumulation detected at higher salt concentrations (~ 2.2 times higher), this increase was lower compared with previous studies carried out batch wise, which reported ectoine productions up to 4 times higher when increasing the cultivation broth salinity from 3 to 6-7 % during the exponential growth phase (But et al., 2013; Cantera et al., 2016b).

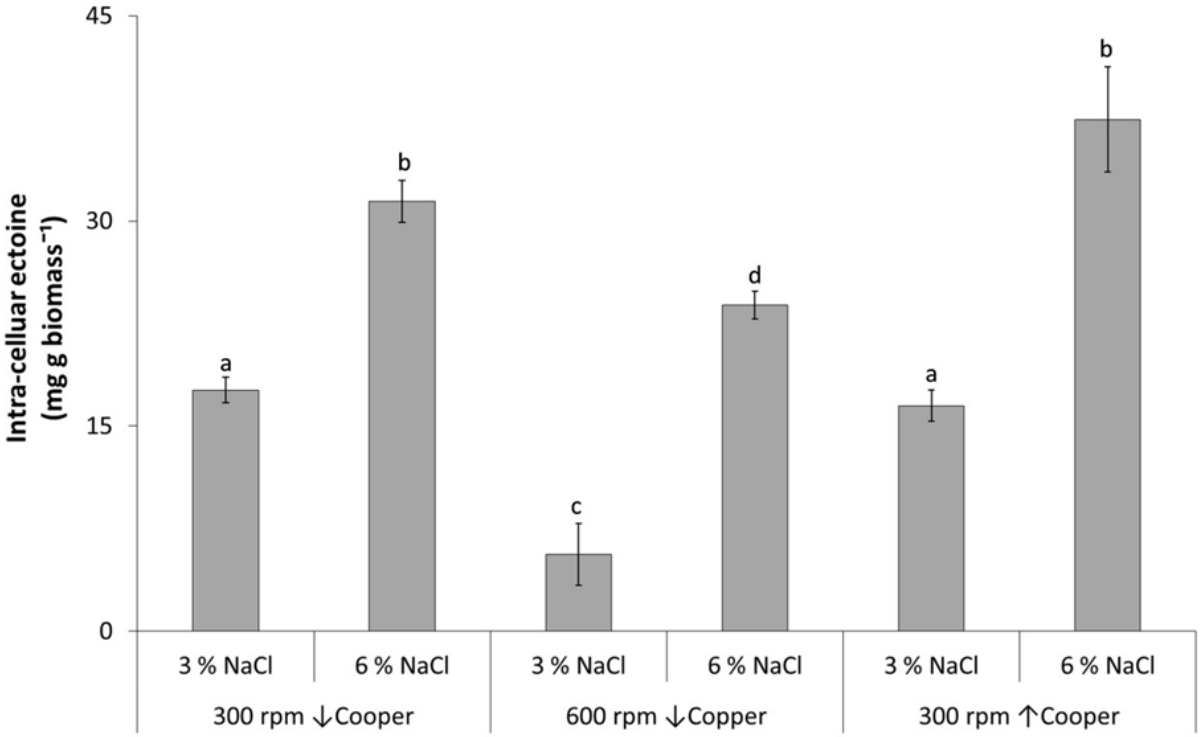

Figure 2: Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state intra-cellular ectoine yield. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

In contrast, the increase in agitation rate from 300 to 600 rpm negatively affected the accumulation of intra-cellular ectoine. In the experimental run 3 (3% NaCl and 600 rpm), the accumulation of intra-cellular ectoine decreased by a factor of 3 % (5.6 ± 2.9 mg ectoine (g biomass)$^{-1}$) compared to the experimental runs at 3% NaCl and 300 rpm. In the case of the experimental run 4 (6 % NaCl and 600 rpm) the accumulation of intra-cellular ectoine decreased by a factor of 1.5 (23.8 ± 1.1 mg ectoine (g biomass)$^{-1}$) compared to the experimental runs at 6% NaCl and 300 rpm. Although higher agitation rates can enhance the mass transfer of methane from the emission to the aqueous microbial community (Cantera et al., 2015), they can also induce a pernicious cellular stress, which resulted in a severe damage of the *Methylomicrobium alcaliphilum 20Z* culture.

3.2. Influence of operational conditions on ectoine excretion

Extra-cellular ectoine was always detected under all operational conditions but with a different accumulation pattern to that recorded for the intra-cellular ectoine. In this context, the maximum extracellular ectoine concentration at 300 rpm and 0.05 µM Cu^{2+} was recorded by day 27 at 3 % NaCl (1.9 mg L^{-1}, corresponding to 1.3 mg g^{-1}) and by day 22 at 6 % NaCl (2.2 mg L^{-1}, corresponding to 1.5 mg g^{-1}) (experimental runs 1 and 2, respectively). However, negligible extra-cellular ectoine concentrations were detected from day 29 onward, regardless of the culture salinity (Figure 3a). At 600 rpm and 0.05 µM Cu^{2+} the maximum ectoine excreted to the medium was 3.1 mg L^{-1} (corresponding to 3.4 mg g^{-1}) at 3 % NaCl on day 13 and 3.8 mg L^{-1} (corresponding to 3.8 mg g^{-1}) at 6 % NaCl on day 13 (experimental runs 3 and 4, respectively). Ectoine excretion remained stable until the end of the operation at steady values of 1.3 ±0.2 and 1.7 ±0.4 mg L^{-1}, respectively (Figure 3b). The presence of ectoine in the extra-cellular medium along the 50 days of bioreactor operation at agitation rates of 600 rpm was likely mediated by a mechanical cell disruption induced by shear stress.

M. alcaliphilum 20Z is not described as a strain able to naturally excrete ectoine to the culture broth (Khmelenina et al., 1999; Reshetnikov et al., 2006). Therefore, the cost-efficient industrial production of ectoine by this strain

requires a two-stage fed-batch process to first attain a high cell density culture with a high concentration of intra-cellular ectoine, and then excrete the accumulated ectoine by hypoosmotic shocks (Pastor et al., 2010; Strong et al., 2016). However, this study showed that at a Cu^{2+} concentration of 25 µM and 300 rpm the extra-cellular concentrations recorded were significantly higher than at 0.05 µM Cu^{2+}. Ectoine excretion remained low during the first week of operation, but increased up to 5.9 ± 0.6 and 12.9 ± 0.7 mg L^{-1} by the end of experimental runs 5 and 6, corresponding to 6.1 ± 0.9 and 8.3 ± 1.2 mg g^{-1}, respectively. Recent literature studies have reported that an increase in copper concentration decreased the expression-level of the proteins conformin the S-layer in M. alcaliphilum (Shchukin et al.,2011), which could support the higher secretion of ectoine (Figure 4).

In this sense, the total concentration of ectoine in non-ectoine excreting strains is equal to the intra-cellular concentration required to preserve the osmotic balance, while ectoine excreting strains maintain the required concentrations of intra-cellular ectoine despite releasing ectoine into the culture broth (Lang et al., 2011).

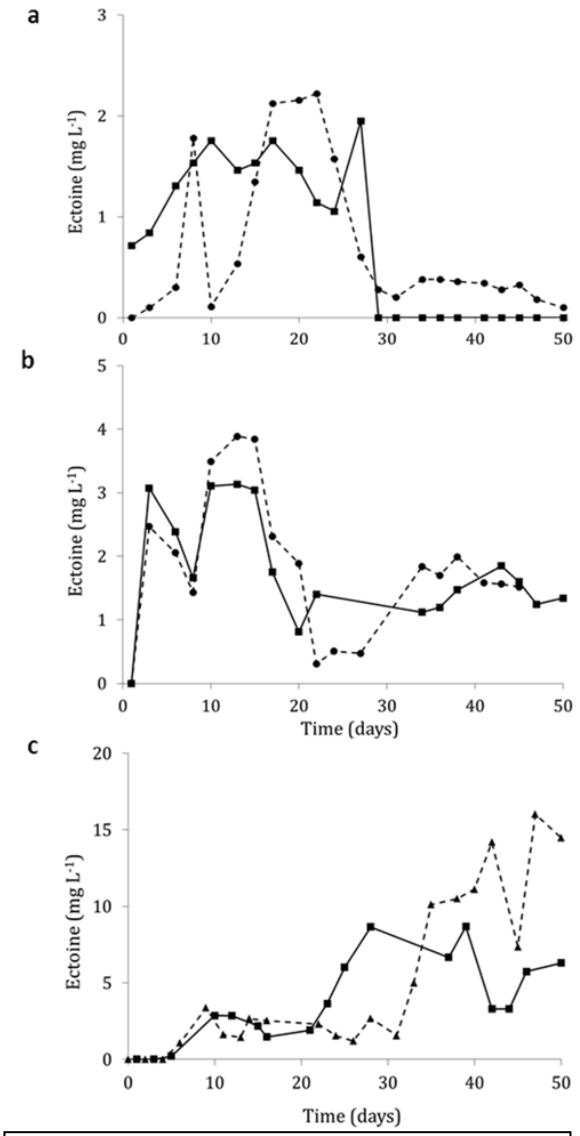

Figure 3: Time course of the extra-cellular ectoine concentration at **a)** 3 % NaCl-300 rpm-0.05µM Cu^{2+} (continuous black line) and 6 % NaCl-300 rpm-0.05µM Cu^{2+} (dotted line) **b)** 3 % NaCl-600 rpm-0.05µM Cu^{2+} (continuous black line) and 6 % NaCl-600 rpm-0.05µM Cu^{2+} (dotted line) **c)** 3 % NaCl-300 rpm-25µM Cu^{2+} (continuous black line) and 6 % NaCl-300 rpm-25µM Cu^{2+} (dotted line). The symbols ■ and▲ represent the sampling times.

Nevertheless, our study showed that modifications in the cultivation conditions can promote the excretion of ectoine by inducing changes in cell physiology. Therefore, those conditions that promote the excretion of ectoine in non-naturally excreting strains can enhance the cost-competitiveness of industrial ectoine production since no further mechanical extraction would be needed.

3.3 Influence of operational conditions on methane abatement

A constant pH of 8.6 ± 0.4 was recorded in all experimental runs regardless of the operating conditions, which favored the growth of the alkalophilic *M. alcaliphilum 20Z* while preventing opportunistic contamination. The concentration of nitrogen was also daily monitored as total nitrogen and maintained between 100 and 130 mg L^{-1} to avoid nitrogen limitation, since nitrogen has been previously identified as a key factor for ectoine synthesis (Khmelenina et al., 2000). No significant CH_4 degradation occurred along the abiotic removal test, as shown by the negligible difference (<1%) between inlet and outlet CH_4 gas concentrations in the STR.

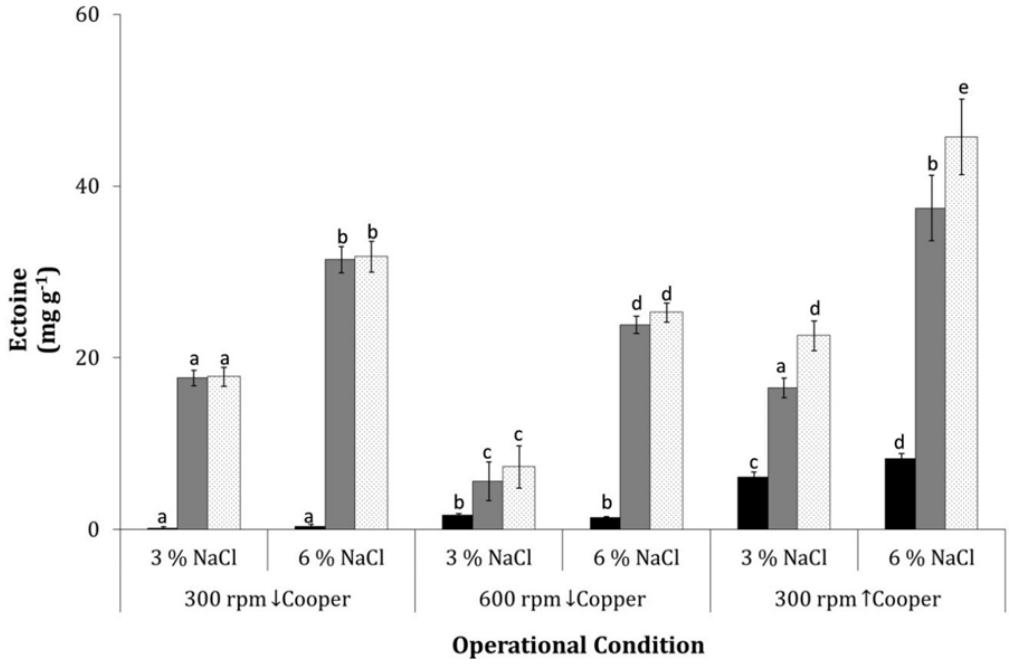

Figure 4: Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state concentrations of extra-cellular ectoine (black column), intra-cellular ectoine (grey column) and total ectoine (white column). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

Process operation at low Cu^{2+} concentrations (experimental runs 1-4) was characterized by a steady CH_4 abatement performance from day ~27 onward, while both CH_4-EC and $TPCO_2$ stabilized by day ~23 when operating at high Cu^{2+} concentrations. NaCl concentration did not influence significantly CH_4-ECs at 300 rpm and low Cu^{2+} concentrations, which remained constant at 16.5 ± 2.0 g CH_4 m^{-3} h^{-1} at 3 % NaCl and 14.8 ± 1.1 g CH_4 m^{-3} h^{-1} at 6 % NaCl (Figure 5).

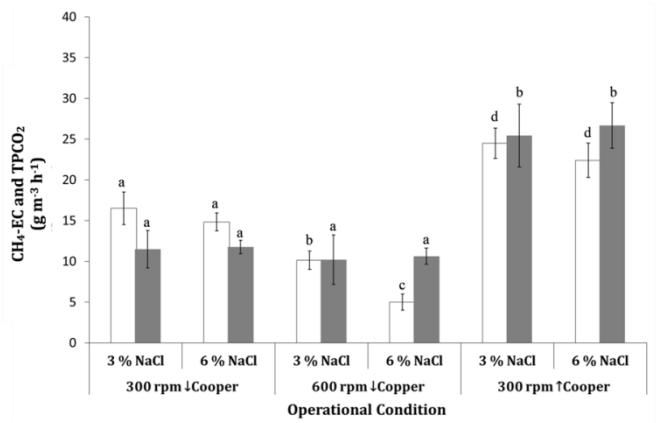

Figure 5: Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state CH_4 elimination capacity (CH_4-EC) (white column) and total CO_2 production ($TPCO_2$) (grey column). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

Methane has an inherently low solubility in water based on its hydrophobic nature. In this regard, the dimensionless CH_4 partition coefficient in water is 30 at 25 ºC (Rocha-Ríos et al., 2009), which often entails a low CH_4 availability to the microbial community. Moreover, there is a consistent evidence that CH_4 solubility in water gets reduced at higher cultivation broth salinities, thus limiting even more the mass transfer of CH_4 from the emission to the microbial community (Duan and Mao, 2006). However, in this study no significant effect of salinity to the methane mass transfer was recorded, which always limited the process under steady state (Figure S2).

NaCl concentration did affect the growth of *M. alcaliphilum 20Z* during the initial stages of experimental run 2 (300 rpm, 0.05 Cu^{2+} and 6% NaCl), which resulted in a longer lag phase (19 days compared to 10 days at 3% NaCl). However, no significant differences between the steady state biomass concentrations were observed at low and high salt concentrations (Figure 6).

On the contrary, CH_4 abatement was significantly affected by the agitation rate. Hence, the CH_4-ECs recorded at 600 rpm (CH_4-ECs of 10.1 ± 1.1 g CH_4 m^{-3} h^{-1} at 3 % NaCl and 5.0 ± 1.0 g CH_4 m^{-3} h^{-1} at 6 % NaCl) were significantly lower than those achieved at 300 rpm (Figure 5).

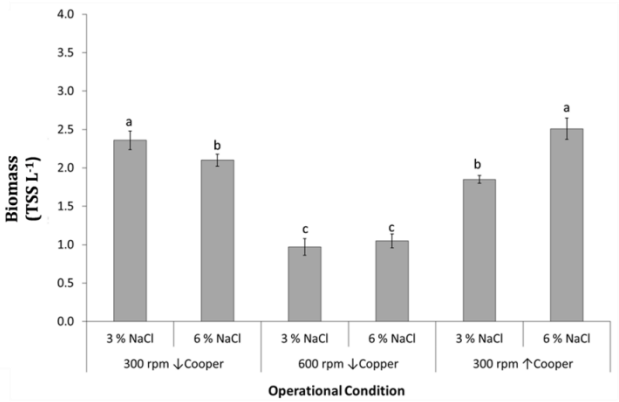

Figure 6: Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state biomass concentration. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Higher agitation rates often support a better mass transfer of methane from the gas to the microbial community, thus enhancing CH_4-EC (Estrada et al., 2014; Cantera et al. 2015). However, higher agitation rates in our study promoted an unexpected cell disruption as a result of a high shear stress on *M. alcaliphilum 20Z* (Figure S2). Indeed, the concentration of biomass (g L^{-1}) at 300 rpm was two times higher than that at 600 rpm (Figure 6), which shifted the mass transfer limitation typically encountered in methane-treating bioreactors to a microbial activity limitation. Surprisingly, despite lower CH_4-ECs were achieved at 600 rpm, the CO_2 productions recorded at 300 rpm were significantly similar to those achieved at 600 rpm. The $TPCO_2$ at 3 and 6 % were, respectively, 11.5 ± 2.3 and 11.7 ± 0.83 g CO_2 m^{-3} h^{-1} under 300 rpm, and 10.2 ± 3.0 and 10.6 ± 0.9 g CO_2 m^{-3} h^{-1} under 600 rpm. Thus, the mineralization ratios at 300 rpm were 47.0 ± 7.7 at 3% NaCl and 43.6 ± 7.7 at 6% NaCl, which entails that more than 50% of the C-CO_2 is used for biomass production. However, the average mineralization ratios recorded at 600 rpm were 79.6 ± 9.1 % at 3% NaCl and 79.9 ± 10.0 % at 6% NaCl. This is in agreement with literature studies reporting a higher share of the organic substrate being directed to energy-yielding reactions under cell stress scenarios (Chung et al., 2006).

Finally, the increase in Cu^{2+} concentration from 0.05 to 25 µM enhanced methane abatement regardless of the salinity in the cultivation broth, with CH_4-ECs of 24.5 ± 1.8 and 22.4 ± 2.1 g CH_4 m^{-3} h^{-1} at 3 and 6 % NaCl, respectively. This finding confirmed the key role of Cu^{2+} on the expression of the enzyme particulate methane monooxygenase in type I methanotrophs, and revealed that CH_4

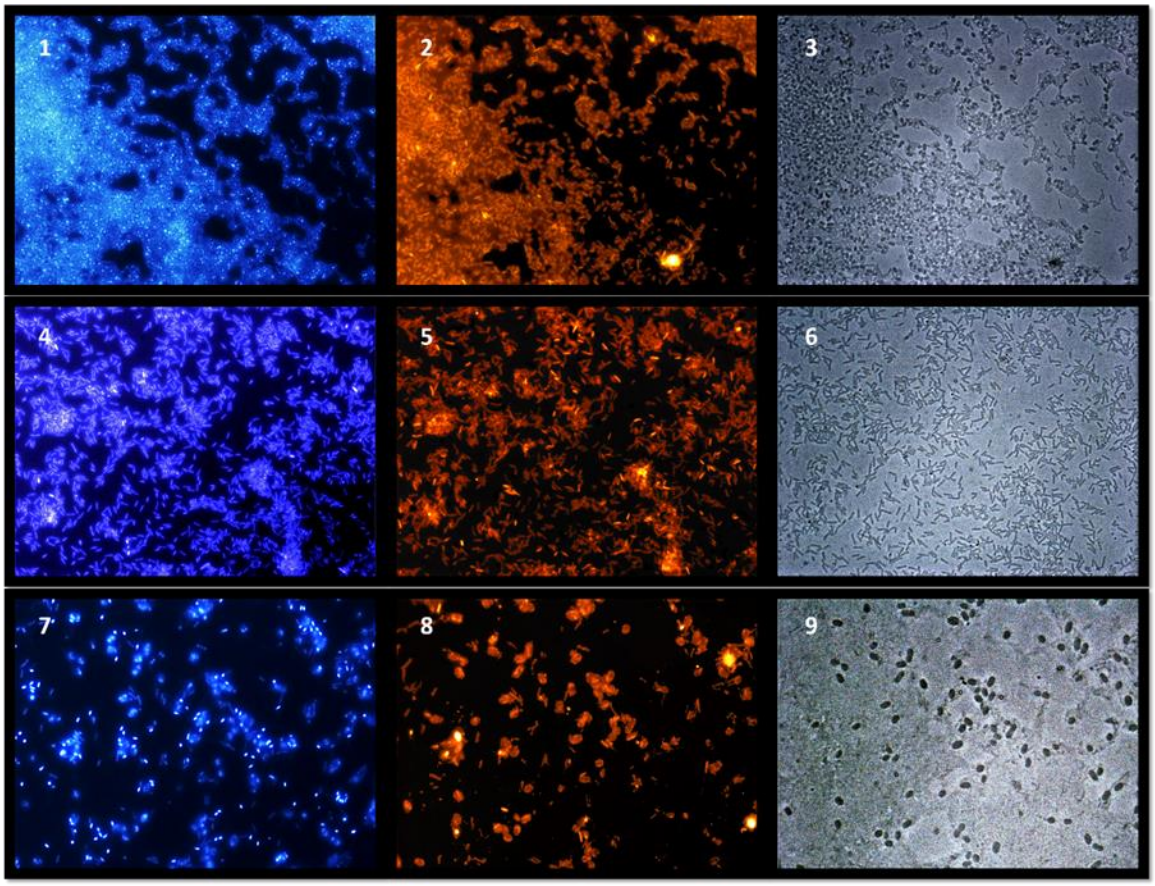

Figure 7: Epifluorescence images (100×) of *Methylomicrobium* culture hybridized with Mγ1004 (Cy3-labeled) (2, 5, 8) in contrast to DAPI staining (1, 4, 7). The corresponding phase contrast image of each field of view is shown (3, 6, 9). Images 1-3 correspond to experimental run 1 (3% NaCl-300 rpm-low Cu^{2+}), images 4-6 correspond to experimental run 3 (3% NaCl-600 rpm-low Cu^{2+}), images 7-9 correspond to experimental run 6 (6% NaCl-300 rpm,-high Cu^{2+}). Images from experimental runs 2, 4 and 5 are not shown due to their similar characteristics to experimental runs 1, 3 and 6, respectively.

abatement and ectoine production were limited by Cu^{2+} availability during experimental runs 1-4 (Semrau et al., 2010, Cantera et al., 2016a). Similarly to the results obtained at low Cu^{2+} concentrations, a higher salt content increased the culture lag-phase but did not influence the steady state CH_4-ECs achieved. The $TPCO_2$ of 25.4 ± 3.8 and 26.7 ± 2.8 g CO_2 m^{-3} h^{-1} recorded in experimental runs 4 and 5, respectively, yielded average mineralization ratios of 55.8 ± 6.0 % and 57.4 ± 5.0 %.

No significant difference between the steady state biomass concentrations was observed at low and high Cu^{2+} concentrations, despite the presence of Cu^{2+} enhanced CH_4-ECs (Figure 6).

3.4 Process microbiology

Phase contrast microscopy observations revealed two different morphologies of *Methylomicrobium*-like bacteria depending on the operational conditions tested. Agitation rates of 600 rpm favored rod-shaped bacteria under low Cu^{2+} concentrations regardless of salinity, while agitation rates of 300 rpm supported the predominance of sphered-shaped bacteria regardless of the Cu^{2+} and NaCl concentrations (Figure S1, supplementary materials).

Pleomorphology (the ability of bacteria to alter their shape or size in response to environmental conditions) has been previously observed in *Methyloccocus* and *Methylosarcina* cultures, which are the closest phylogenetical lineages to the genus *Methylomicrobium* (Wise, 2001). *Methylomicrobium* cells at high Cu^{2+} concentrations appeared embedded in a dense matrix likely composed of the excreted ectoine (Figure7).

Methylomicrobium-like bacteria population was quantified by FISH analysis with the specific probe Mγ1004 using the total DAPI-stained bacteria as a reference (Figure 7). The results showed that the genus *Methylomicrobium* accounted for more than 80% of the total bacterial population in most experimental runs. *Methylomicrobium* population in experimental runs 1, 3, 4 and 5 accounted for 89.2 % (*se* = 3.2), 102.9 % (*se* = 4.5), 104.9 % (*se* = 4.9) and 81.0 % (*se* = 3.4) of the total bacterial population, respectively. The biovolume fractions of *Methylomicrobium* in experimental runs 2 and 6 were slightly lower: 73.0 % (*se* = 3.5) and 69.1 % (*se* = 4.1), respectively. Biovolume shares higher than 100 % can be attributed to the fact that probe-hybridized cells can slightly increase their size/volume after hybridization. The high hybridization shares recorded suggested that the extreme conditions prevailing during the 6 experimental runs (alkaline pH and high salinity) prevented culture contamination with opportunistic microorganisms, which guarantees process robustness even under non-sterile conditions.

4.Conclusions

This study confirmed for the first time the feasibility of coupling ectoine production with the continuous abatement of dilute emissions of methane. NaCl concentration was the main factor influencing the accumulation of intra-cellular ectoine, with high salt concentrations inducing higher intra-cellular ectoine yields without compromising methane abatement. Apart from an unexpected increase in CH_4-ECs, the increase in Cu^{2+} concentration mediated the excretion of 20 % of the ectoine synthesized, thus enhancing its subsequent recovery. Process operation at high agitation rates damaged the bacterial population, with a subsequent decrease in both CH_4-ECs and ectoine yields. FISH analysis showed that *Methylomicrobium alcaliphilum 20Z* was the dominant microorganism regardless of the operational conditions as a result of the high pH and salinity prevailing in the culture broth. The promising results obtained in this study support the need for further research in order to implement CH_4 biorefineries for the production of ectoine (either in a process similar to the currently used by *Halomonas elongata*, namely "bio-milking", or by promoting the natural excretion of ectoine to the culture broth concentration at high Cu^{2+} concentrations) and open up a door to the development of a new generation of GHG biorefineries based on extremophile methanotrophs capable of creating value out of methane mitigation.

5.Acknowledgements

This research was supported by the Spanish Ministry of Economy and Competitiveness (CTM2015-70442-R project and Red NOVEDAR), the European Union through the FEDER Funding Program and the Regional Government of Castilla y León (PhD Grant contract Nº E-47-2014-0140696 and UIC71).

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Supplementary material

Continuous abatement of methane coupled with ectoine production by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors: a step further towards greenhouse gas biorefineries

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Figure S1

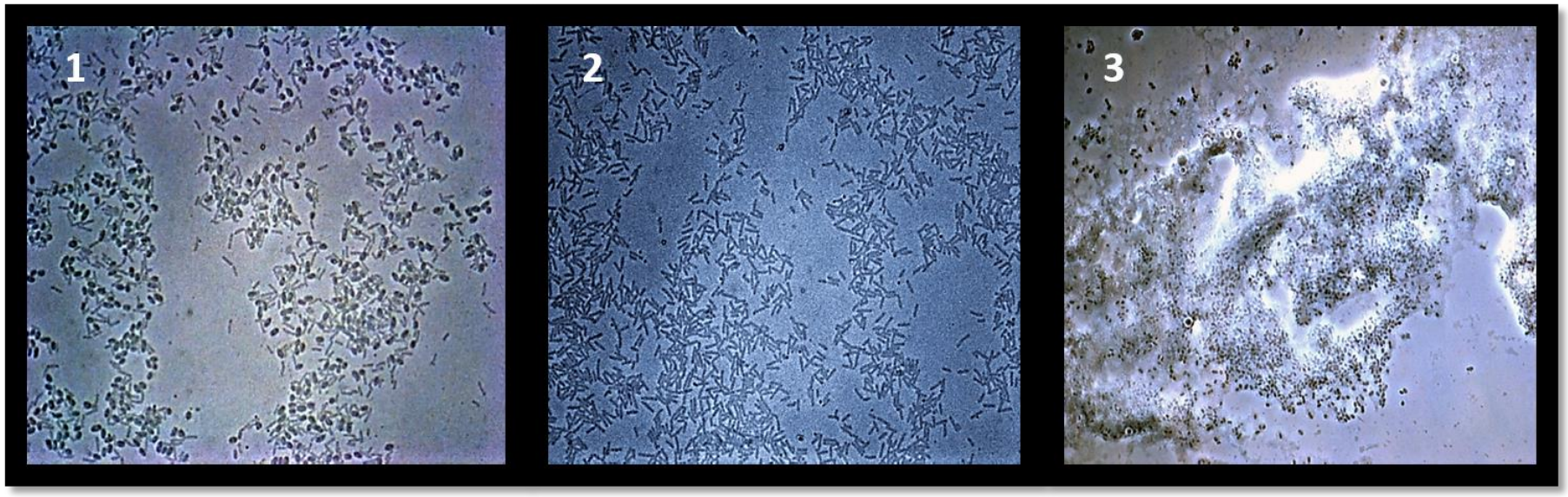

Figure S1. Phase contrast images showing cell morphology and *Methylomicrobium* population structure in 1- experimental run 1 (3% NaCl, 300 rpm, low Cu^{2+}) (sphered-shaped bacteria predominance), 2- experimental run 3 (3% NaCl, 600 rpm, low Cu^{2+}) (rod-shaped bacteria predominance) and 3- experimental run 6 (6% NaCl, 300 rpm, high Cu^{2+})(sphered-shaped bacteria predominance)

Figure S2

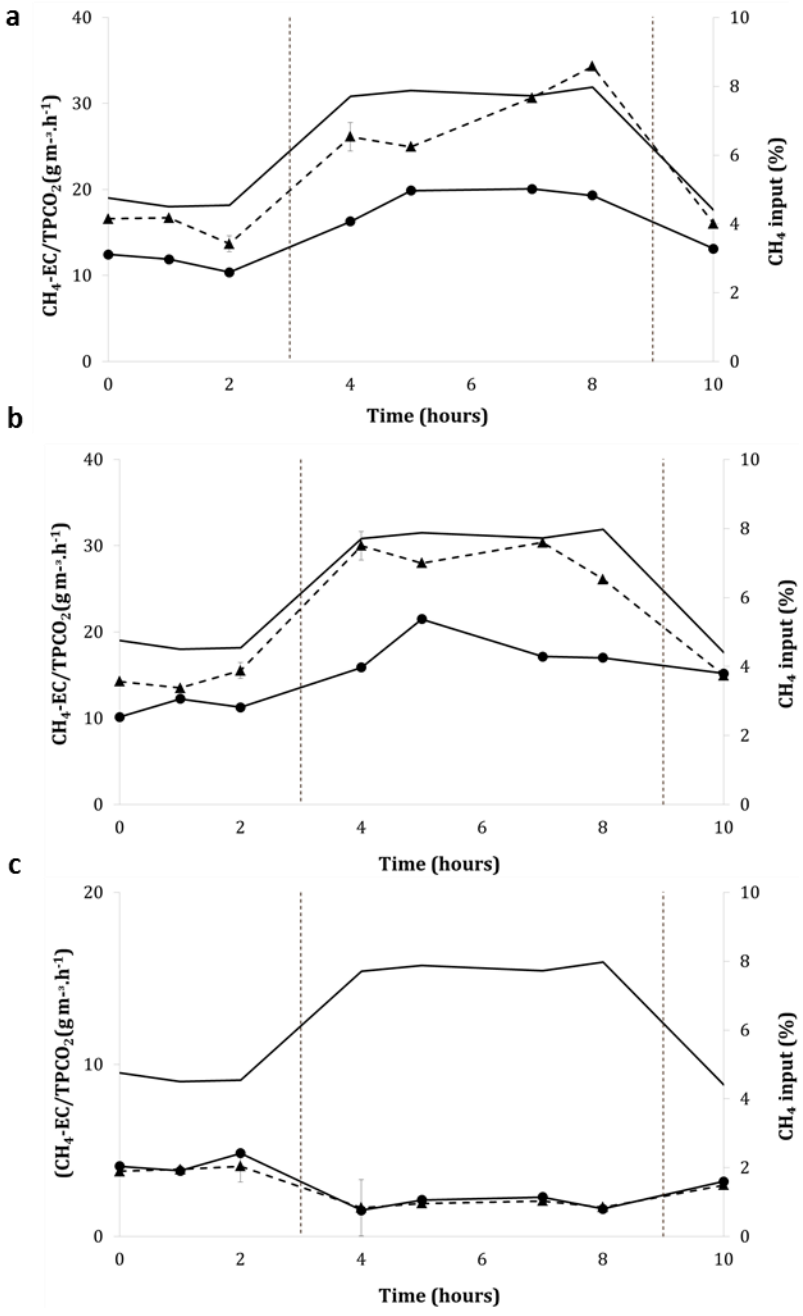

Figure S2. Time course of the inlet CH_4 concentration (black continuous line), EC (▲, dashed line) and the CO_2 production rate (●, dotted line) during the mass transfer limitation tests in a) experimental run 1 (3 % NaCl, low copper, 300 rpm) b) experimental run 2 (6 % NaCl, low copper, 300 rpm) and c) experimental run 4 (6% NaCl, low copper and 600 rpm).

Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-refineries into high added-value products.

Cantera S, Lebrero R, Rodríguez S, García-Encina P, Muñoz R. *Journal of Chemical Engineering Journal, 2017; 328: 44-48.*

Chapter 6

Ectoine *bio-milking* in methanotrophs: A step further towards methane-based bio-refineries into high added-value products.

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Abstract

This communication showed for the first time that the methanotrophic strain *Methylomicrobium alcaliphilum 20Z* (*M. alcaliphilum 20Z*) can efficiently synthesize and excrete (through a tailored *bio-milking process*) ectoine under continuous mode using methane (CH_4) as the sole energy and carbon source. First, three consecutive 50 h fed batch fermentations consisting of alternating high salinity (6 % NaCl for 24 h) and low salinity (0 % NaCl for 24 h) cultivation stages were carried out in triplicate to determine the influence of sudden modifications in media salinity on ectoine synthesis and excretion. The results demonstrated that *M. alcaliphilum 20Z* exhibited a rapid response to osmotic shocks, which resulted in the release of the accumulated ectoine under hyposmotic shocks and the immediate uptake of the previously excreted ectoine during hyperosmotic shocks. A second experiment was carried out under continuous cultivation mode in two sequential stirred tank reactors operated at NaCl concentrations of 0 and 6 %. Cells exhibited a constant intra-cellular ectoine concentration of 70.4 ± 14.3 mg g biomass^{-1} along the entire operation period when cultivated at a NaCl concentration of 6 %. The centrifugation of the cultivation broth followed by a hyposmotic shock resulted in the excretion of ~ 70 % of the total intra-cellular ectoine. In brief, this research shows the feasibility of the continuous bioconversion of diluted CH_4 emissions into high added-value products such as ectoine, which can turn greenhouse gas (GHG) abatement into a sustainable and profitable process.

Keywords: Ectoine, *bio-milking*, methane abatement, *Methylomicrobium*

1.Introduction

The steady rise in the average temperature of the Earth within the last decades and its associated detrimental impacts on the environment have resulted in an increasing concern on global warming, which is triggering the enforcement of political initiatives for greenhouse gas (GHG) abatement [1]. Today, methane (CH_4) is the second most important GHG emitted worldwide based on its high global warming potential (25 times higher than that of CO_2), representing 12 % of the total GHG emission inventory [1,2]. The CH_4 emitted by anthropogenic activities can be used for energy recovery when its concentration is higher than 30 % (v/v). However, more than 56 % of anthropogenic CH_4 emissions contain concentrations below 3 %, which are neither technical nor economically suitable for energy recovery [3,4]. These diluted emissions represent a threat to the environment [2,5,6] and their cost-efficient and environmentally friendly abatement is still unresolved [6–8]. In this context, the bioconversion of CH_4 into high added-value products such as ectoine has emerged as the most promising alternative for the control of diluted CH_4 emissions[9], which would turn GHG abatement into a sustainable and profitable process.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a compatible solute that maintains osmotic balance in a wide number of halotolerant bacteria [10]. Due to its high effectiveness as stabilizer of enzymes, DNA-protein complexes and nucleic acids, ectoine is likely one of the most valuable bioproducts synthesized by microorganisms. It retails in the pharmaceutical industry at approximately US$1000 kg^{-1} and its production at a global scale accounts for approx. 15000 tones $year^{-1}$ [11]. In this regard, biotechnologies for the production of ectoine have received increasing attention in the last two decades due to their simpler and highly specific product synthesis in comparison with chemical processes [10]. However, nowadays ectoine is only produced at industrial scale by the γ-*Proteobacteria Halomonas elongata*. The industrial ectoine production process -*bio-milking*- consists of a long fed-batch fermentation at varying NaCl concentrations. Thus, *H. elongata* is initially cultivated at low salinity to produce high density cultures, transferred to a high salinity medium to promote ectoine biosynthesis [12] and

finally exposed to a hyposmotic shock to induce the excretion of ectoine from the cell to the cultivation broth, where the product is collected for its downstream purification [10]. Nevertheless, this process is costly due to the need for high quality carbon sources (often glucose, yeast and sodium glutamate), which reduces its cost effectiveness [13,14].

Recent studies performed in both batch and continuous bioreactors have demonstrated the ability of the methanotrophic bacterium *Methylomicrobium alcaliphilum 20Z* (*M. alcaliphilum 20Z*) to synthesize ectoine using CH_4 as the sole carbon and energy source [15–17]. The promising results obtained hitherto with methanotrophs encourage further research devoted to develop biotechnologies capable of creating value from this greenhouse gas. This study constitutes, to the best of our knowledge, the first proof of concept of ectoine production by *M. alcaliphilum 20Z* under the current industrial fed-batch and osmotic downshock *bio-milking* process using diluted CH_4 emissions as a feedstock.

2.Experimental

A fed-batch fermentation, consisting of three consecutive 50 h two-stage cultivations at 25 ºC and 600 rpm (revolutions per minute), was initially carried out in triplicate in sterile 1.2 L gas-tight reactors containing 300 mL of a mineral salt medium (MSM) specific for alkalophilic methanotrophs [18] and inoculated at 0.13 ± 0.01 g TSS L^{-1} (Table S1). The two-stage *M. alcaliphilum 20Z* cultivation involved an initial incubation at high salinity (6 % NaCl) for 24 hours to promote bacterial growth and ectoine accumulation followed by a biomass harvesting step and a final bacterial incubation for 24 hours in the absence of NaCl to promote the excretion of ectoine (TS1). Cells were harvested by centrifugation at 9000g for 10 min, washed twice with a 0.85 % NaCl solution and re-suspended in a NaCl free MSM prior cultivation in NaCl free MSM, while 80 mL of sterile 22.5 % NaCl MSM were added to the NaCl free MSM prior cultivation at 6 % in TS2 and 3. The headspace CH_4 concentration at the beginning of each incubation stage (6 and 0 % NaCl) was 50 g CH_4 m^{-3} (8 %). The concentration of ectoine and biomass was monitored at time 0, 1, 3, 5, 7, 9, 12 and 24 hours by drawing 10 mL of cultivation broth. More details related to the fed batch

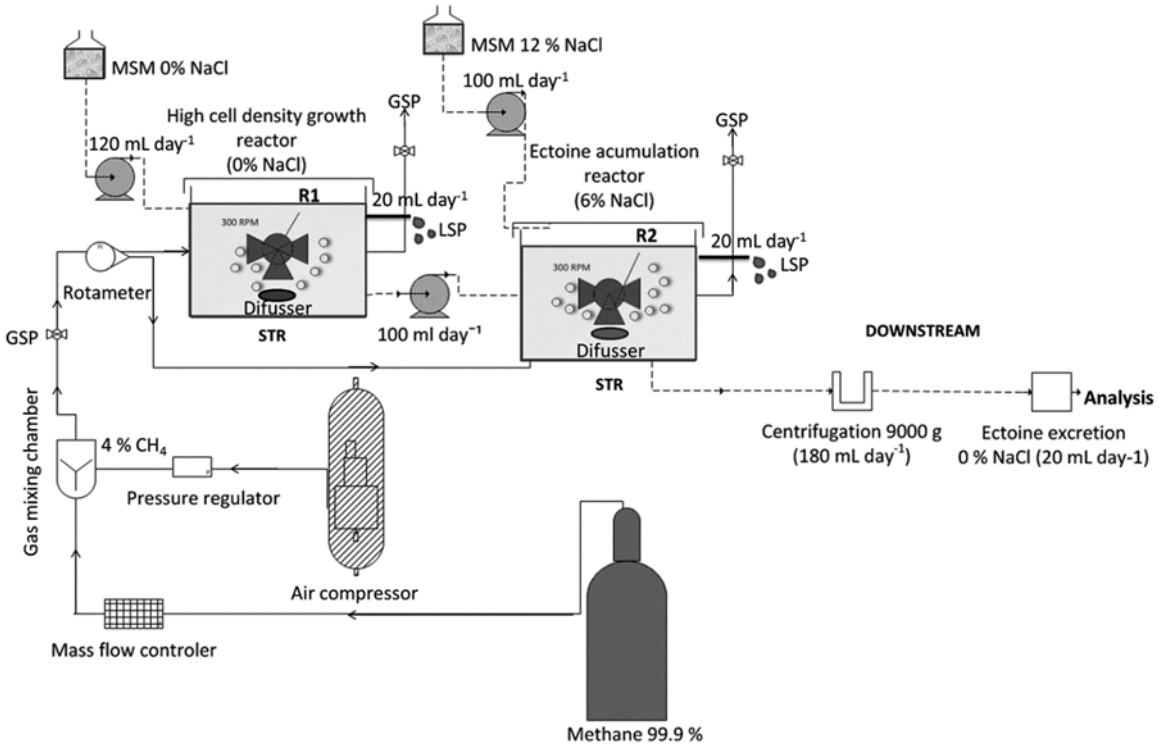

Figure 1: Schematic diagram of the experimental setup: high cell density growth reactor (R1), ectoine accumulation reactor (R2), mineral salt media storage tanks (MSM), Gas sampling port (GSP), Liquid sampling port (LSP), gas streams (continuous line), liquid streams (discontinuous line).

fermentation can be found in Text S1 and Table S1.

A second experiment targeting the continuous bioconversion of CH_4 into ectoine using a *bio-milking* approach was carried out in duplicate for 30 days in two 1-L stirred tank reactors agitated via a Rushton turbine (Figure 1). The high cell density growth reactor (R1) was operated at pH 9 and 0 % NaCl, while the ectoine accumulation reactor (R2) was operated at pH 9 and 6 % NaCl. Process operation based on two reactors was necessary to achieve high ectoine concentrations in R2 since a single stage cultivation at 6 % NaCl involves the adaptation of *M. alcaliphilum* with the subsequent reduction in the intracellular osmoprotectant concentration.

Preliminary tests showed that maximum ectoine accumulation occurred at biomass retention times of 5 days [17]. Both reactors were initially inoculated at a concentration of 0.34 g L^{-1} of *M. alcaliphilum 20Z* and operated at a CH_4 loading rate of 53 g m^{-3} h^{-1}. R1 and R2 were supplied with 120 and 100 mL d^{-1} of MSM at 0 and 12 % NaCl, respectively, while 100 mL of cultivation broth from R1 were daily transferred to R2. Thus, 180 mL of

cultivation broth from R2 were daily drawn for ectoine extraction by centrifugation (9000 g for 10 min) followed by biomass resuspension in 20 mL of NaCl-free medium and incubation for one hour at 300 rpm and 25 ºC. The concentrations of intra and extra-cellular ectoine and biomass were determined in R1, R2 and the concentrated biomass extraction broth. Gas samples for CH_4 and CO_2 analysis were daily drawn at the inlet and outlet of the bioreactors using 100 µl gas-tight syringes (HAMILTON, Australia). A more detailed description of the reactor setup, the analytical procedures and the statistical analysis used for data treatment is provided in Text S2, S3 and S4, respectively.

3.Results and discussion

The fed-batch fermentation conducted confirmed that NaCl concentration exerted a positive effect on the intra-cellular accumulation of ectoine in *M. alcaliphilum 20Z* (Figure 2) [15,16,19]. The specific intra-cellular ectoine concentrations reached 20.2 ± 4.2, 27.0 ± 2.0 and 25.8 ± 1.7 mg ectoine g biomass^{-1} at the end of the cultivation at high salinity in TS1, 2 and 3, respectively. The exposure of the cells to a low salinity medium resulted in a rapid excretion of the intra-cellular ectoine, which corresponded to 24.8 ± 2.2, 32.3 ± 2.7 and 29.1 ± 0.5 mg ectoine g biomass^{-1} in TS1, 2 and 3, respectively.

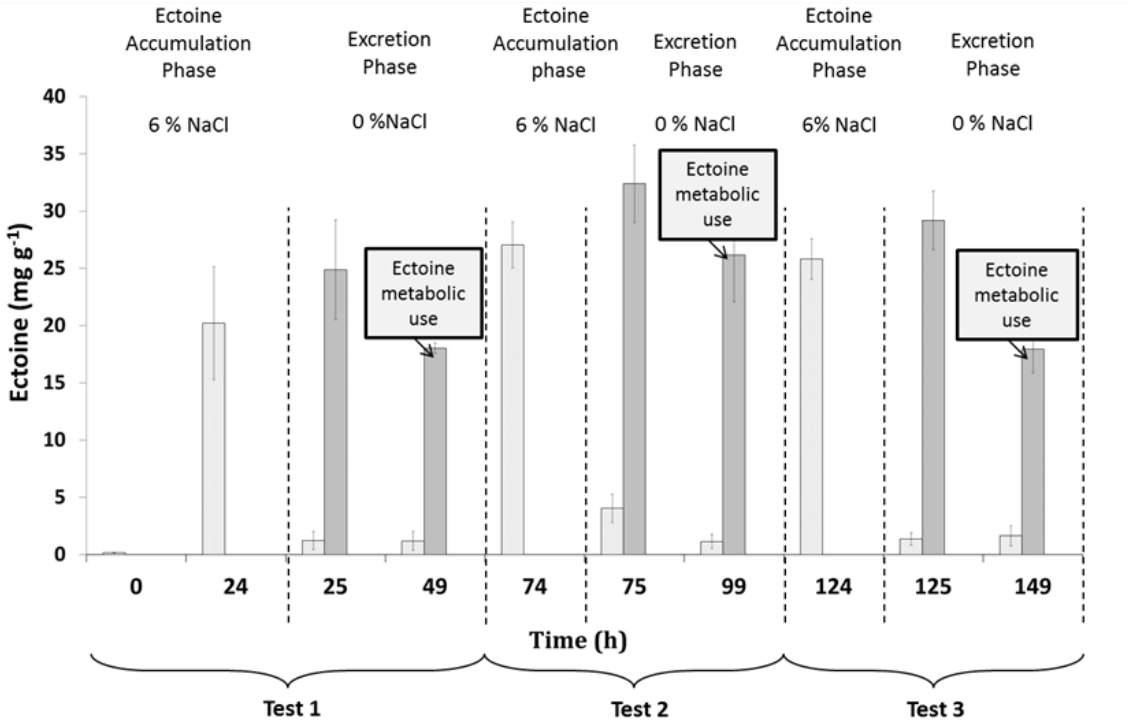

Figure 2: Time course of the average intra (light grey column) and extra (dark grey column) -cellular ectoine concentration during the 3 sequential two-stage cultivations carried out in triplicate in batch gas tight reactors.

Therefore, the hyposmotic shocks supported a complete ectoine excretion to the extracellular medium (Figure 2). A small fraction of the extra-cellular ectoine released to the cultivation broth (~27 %) was consumed by *M. alcaliphilum 20Z* during cultivation at 0 % NaCl (Figure 2). When NaCl was supplied again to the cultivation broth containing the extracellular ectoine, the compatible solute was actively transported back into the bacterial cytoplasm. The concentrations of total ectoine (the sum of extra and intra-cellular ectoine) detected in low and high salinity media were not significantly different among the three two-stage cultivations.

The evolution of the intra- and extra-cellular ectoine concentration during the first 64 h of experiment (Figure 3) revealed that *M. alcaliphilum 20Z* was able to accumulate intra-cellular ectoine within the first 24 hours as a compatible solute to protect the cell against the osmotic stress caused by the high salinity of the cultivation broth. The decrease in NaCl concentration (at 25 h) induced a rapid excretion of the cytoplasmic ectoine to the cultivation broth. This immediate release of osmoprotectant solutes has been previously observed in *Halomonas* sp. when exposed to sudden hyposmotic shocks [10,12,20]. Moreover, a slight

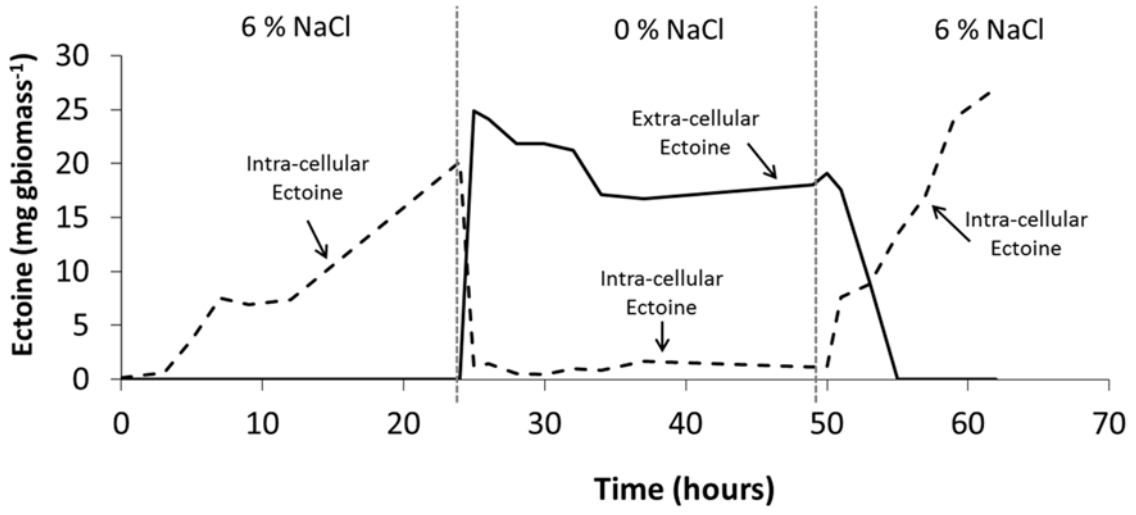

Figure 3: Time course of the average intra (dashed line) and extra (continuous line) -cellular ectoine concentration during the initial 64 hours of experiment in batch gas tight reactors.

Table 1. Methane abatement performance in R1 and R2

Reactor	Elimination capacity ($g\ m^{-3}\ h^{-1}$)	Total CO_2 Production ($g\ m^{-3}\ h^{-1}$)	Removal Efficiency (%)
R1 (0 % NaCl)	11.1 ± 1.6^{a}	13.6 ± 2.9^{b}	19.6 ± 2.9^{c}
R2 (6 % NaCl)	13.2 ± 1.2^{a}	14.1 ± 1.8^{b}	22.5 ± 2.5^{c}

Mean values within a column followed by different superscript letters (a.b and c) were significantly different at $p<0.05$. Means±SD.

decrease in extra-cellular ectoine concentration was observed during the first hours of cultivation under low salinity, while no significant variation in the intra-cellular ectoine content was detected in this period. This finding was attributed to the use of ectoine as an readily biodegradable nitrogen and carbon source by *M. alcaliphilum 20Z* [15,16]. The restoration of NaCl salinity resulted in a gradual re-assimilation of the extra-cellular ectoine within the first 10 h of cultivation under high salinity. This study confirmed that *M. alcaliphilum 20Z* can uptake the extra-cellular ectoine previously released to avoid the additional expenses of anabolic energy required for a *de-novo* ectoine synthesis.

3.2 CH_4 abatement in stirred tank reactors combined with ectoine *bio-milking*

Steady elimination capacities (ECs) and removal efficiencies (REs) of 11.1 ± 1.6 and 13.2 ± 1.2 g CH_4 m^{-3} h^{-1}, and 19.6 ± 2.9 and 22.5 ± 2.5 %, were recorded during the continuous CH_4 treatment in R1 and R2, respectively (Table 1). CH_4 has an inherently low solubility in water due to its hydrophobic nature (dimensionless CH_4 partition coefficient in water of 30 at 25 ºC[21,22]), which often entails a low CH_4 availability to the microbial community. Moreover, CH_4 solubility in water decreases at higher cultivation broth salinities, further limiting the mass transfer of CH_4 from the emission to the *M. alcaliphilum* culture [23,24]. However, no significant differences between the steady state CH_4-ECs and REs were recorded at low and high salt concentrations. Likewise, despite previous studies have observed an increase in the total CO_2 production

($TPCO_2$) at higher salinities [17], no statistical difference was encountered in this specific study (13.6 ± 2.9 and 14.10 ± 1.8 g CO_2 m^{-3} h^{-1} in R1 and R2, respectively). Thus, no significant effect of NaCl concentration neither on growth nor on CH_4 biodegradation activity was here recorded for *M. alcaliphilum 20Z*.

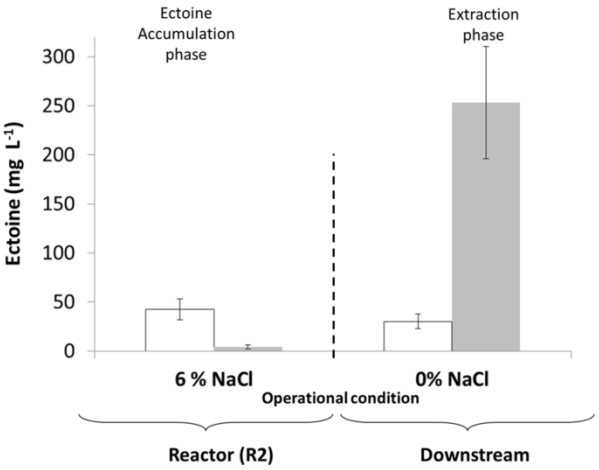

Figure 4: Steady state intra-cellular (white column) and extra-cellular (grey column) ectoine culture broth concentrations in R2 and in the extracted broth after *bio-milking*. Vertical lines represent standard deviations from replicates.

During the 20 days of steady state operation neither intra- nor extra-cellular ectoine were detected in R1. The hyperosmotic shock in R2 induced a constant intra-cellular ectoine yield along the operation of 70.4 ± 14.3 mg g biomass^{-1} (corresponding to 42.44 ± 10.3 mg L^{-1}). Extra-cellular ectoine was also detected in the cultivation broth of R2 (4.3 ± 2.2 mg L^{-1}), probably due to a cell disruption mediated by the hyperosmotic shocks and the stirring shear stress. The hyposmotic shock after biomass harvesting and concentration resulted in intra-cellular ectoine concentrations of 30.3 ± 7.5 mg L^{-1} and extra-cellular concentrations of 253.4 ± 55.1 mg L^{-1} (Figure 4). Therefore, the downstream operation was able to harvest 70.4 ± 24.6 % of ectoine accumulated in R2. This finding confirmed the hypothesis that *M. alcaliphilum 20Z* is able to release its compatible solutes in response to hyposmotic shocks similarly to the industrial process used with *H. elongata*. The *bio-milking* strategy here investigated did not cause a significant damage in cell integrity, as shown by the comparable biomass concentrations before (0.61 ± 0.05 g L^{-1}) and after the hyposmotic shock (0.58 ± 0.07 g L^{-1}), which represented a survival rate of ~100 %.

The production yields reported in literature are higher than those recorded for *M. alcaliphilum 20Z* in our study. For instance, the genus *Brevibacterium* accumulates 150-160 mg g biomass^{-1} depending on the specie, while ectoine yields of 155-200 mg g biomass^{-1} have been recorded in *Halomonas* sp.. Higher yields can be achieved by genetically

modified microorganisms (*Escherichia coli* DH5α can synthesize up to 400-540 mg g biomass^{-1}) [10,13,14,25–27]. Nevertheless, *M. alcaliphilum 20Z* exhibits a superior environmental performance based on its ability to produce ectoine from dilute CH_4 emissions, with a concomitant mitigation of climate change.

4.Conclusions

The experiments performed demonstrated that the methanotrophic strain *M. alcaliphilum 20Z* can efficiently synthesize and excrete ectoine into the medium using CH_4 as the sole energy and carbon source through the industrial process *bio-milking*. The batch wise study demonstrated that *M. alcaliphilum 20Z* was able to respond to hyposmotic shocks by rapidly releasing the accumulated solutes, and to hyperosmotic shocks by re-assimilating the excreted compatible solutes. Moreover, *M. alcaliphilum 20Z* was confirmed as a feasible strain for industrial ectoine production using fed batch fermentations and down-shocks in continuous, supporting a recovery of ~ 70 % of the total intra-cellular ectoine accumulated, and resulting in extracellular concentrations of 253.4 ± 55.1 mg L^{-1}. To the best of our knowledge, this study constitutes the first proof of concept of ectoine *bio-milking* coupled to CH_4 abatement from diluted emissions. The optimization of this innovative process will boost the development of a new generation of GHG biorefineries based on extremophile methanotrophs capable of creating value out of CH_4 mitigation.

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Supplementary material

Ectoine *bio-milking* in methanotrophs: A step further towards methane-based bio-refineries into high added-value products.

Short Communication

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Text S1. Chemicals and inoculum

A high-alkalinity mineral salt medium (MSM) with a final pH of 9.0 was used (Kalyuzhnaya et al. 2008). All chemicals and reagents were obtained from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (> 99.5 %) was purchased from Abello-Linde S.A (Barcelona, Spain. *Methylomicrobium alcaliphilum 20Z* was acquired from DSMZ (Leibniz-Institut, Germany).

Text S2. Reactor setup

The bioconversion of CH_4 into ectoine was carried out in 2 sequential stirred tank reactors (STR) with a working volume of 1 L (Afora S.A., Spain) and equipped with a Rushton turbine (300 rpm). The STRs were filled with 500 mL of MSM (containing 0% NaCl in R1 and 12 % NaCl in R2) and inoculated with 500 mL of a cultivation broth of *M. alcaliphilum* 20Z previously grown for one month in a STR at 0 % NaCl to a final biomass concentration of 0.66 g L^{-1}. A 0.033 L min^{-1} CH_4-laden air emission containing 26.8 ± 2.1 g CH_4 m^{-3} (≈ 4 %), corresponding to a methane load of 53 g m^{-3} h^{-1}, was fed to the STRs via 10 µm stainless steel porous diffusers located at the bottom of the reactors. This polluted air emission was obtained by mixing a pure CH_4 stream (regulated by a mass flow controller, Aalborg, USA) with a pre-humidified air flow, resulting in a gas empty bed residence time of 30 min. Temperature was maintained at 25 ºC in all experiments. The dilution rate in R1 was maintained at 0.12 d^{-1}, while R2 was operated at 0.2 d^{-1}. This entailed a biomass retention time of 5 days in R2, which corresponded to the maximum accumulation of ectoine under hyperosmotic conditions in previous tests in our lab [1,2]). Steady state conditions were achieved when the elimination capacities (CH_4-EC) and CO_2 production rates ($TPCO_2$) deviated <10 % from the mean for at least 20 days.

Text S3. Analytical methods

The intra-cellular ectoine contained in 2 mL of cultivation broth was extracted to the extra-cellular medium according to Cantera et al. (2016b) [2]. The specific intra-cellular ectoine concentration (g ectoine g $biomass^{-1}$) was calculated using the corresponding total suspended solids (TSS) concentration (g L^{-1}). An aliquot of 1 mL of cultivation broth was also drawn and filtered through 0.22 µM filters (Filter-lab, Barcelona) to determine the extra-cellular ectoine concentration. The concentration of ectoine was measured by HPLC-UV in a 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA) at 210 nm and 40 ºC using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, USA) and a C18 AQ + pre-column (Waters, Bellefonte, USA). A phosphate buffer, consisting of 0.8 mM $K_2HPO_4{\cdot}3H_2O$ and 6.0 mM $Na_2HPO_4{\cdot}12H_2O$, was used as a mobile phase at 25 ºC and 1 mL min^{-1} [3]. Ectoine quantification was carried out using external standards of commercially available ectoine ((S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %, Sigma Aldrich, Spain). The detection and quantification limits (DL and QL, respectively) were calculated via determination of the signal-to-noise ratio. In this sense, a signal-to-

noise ratio of 3:1 - 2:1 is considered acceptable for estimating the detection limit, while a signal-to-noise ratio of 10:1 is necessary to determine the quantification limit (ICH Expert working Group, 2005). The DL and QL of ectoine in our specific MSM were 0.4 mg L^{-1} and 0.65 mg L^{-1}, respectively.

CH_4 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) columns. The oven, injector and detector temperatures were maintained at 45, 150 and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

Culture absorbance measurements at 650 nm were conducted in a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). TSS concentration was measured according to Standard Methods [5].

Text S4. *Data analysis*

The statistical data analysis was performed using SPSS 20.0 (IBM, USA). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were determined by ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

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Table S1. Cultivation conditions during the fed batch fermentations.

Two-Stage cultivation (TS)	Stage (S)	Elapsed Time (hours)
TS1	6%	0-24
	0%	25-49
TS2	6%	50-74
	0%	75-99
TS3	6%	100-124
	0%	125-149

Multi-production of high added market value metabolites from methane emissions by an halotolerant methanotrophic consortia.

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Chapter 7

Multi-production of high added market value metabolites from methane emissions by an halotolerant methanotrophic consortia.

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Abstract

This study is the first probe of concept of a methane biorefinery based on the multi-production of high profit margin substances using methane as the only energy source. In this regard, the operation of two column bubble reactors is carried out with the aim of assess an innovative strategy for the valorization of dilute CH_4 emissions based on the continuous and simultaneous bioconversion of CH_4 into several products with a high market value such as ectoines (ectoine and hydroxyectoine), bioplastics (PHA) and exopolysaccharides, under different magnesium concentrations. The results obtained showed that higher magnesium concentrations promoted the accumulation of high ectoine concentrations (from 94.2 to 79.7 mg ectoine g biomass^{-1}), hydroxyectoine yields (up to 13 mg hydroxyectoine g biomass^{-1}) and exopolysacharides (up to 2.6 g EPS g biomass^{-1}) by a methanotrophic consortia consisted mainly on the genera *Halomonas*, *Marinobacter*, *Methylophaga* and *Methylomicrobium*. In the case of the PHAs, the values obtained were almost negligible under high concentrations of magnesium (0.2 g Mg^{2+} L^{-1}) and even if its yield increased at low magnesium concentrations the yields obtained were low (14.3 mg PHAs L^{-1}). The elimination capacity of methane by the methanotrophic consortia achieved values of ~ 20 g CH_4 m^{-3} h^{-1} even if the most representative genera have not been identified as methanotrophic bacteria to date. The promising results obtained in this preliminary study, support further research about these new methanotrophic strains in order to implement a feasible bioprocess capable of creating value out of GHG mitigation.

Keywords: Ectoines, Methane treatment, exopolysaccharide, *Halomonas* , CH_4-biorefinery.

1.Introduction

Methane (CH_4), with a global warming potential ~85 times greater than that of CO_2 (over a 20-y window), is the second most important greenhouse gas (GHG)[1]. The concentration of CH_4 increases at a yearly rate of 0.2-1 %, mainly due to anthropogenic activities (landfilling, agriculture, livestock farming, waste management and energy production[2,3]). More than 60 % of these anthropogenic CH_4 emissions contain this GHG at concentrations below 5 %, where current CH_4 treatment methods are neither efficient nor cost-effective [4].

This scenario has resulted in an increased concern for global warming, prompting the enforcement of political initiatives for GHGs abatement, while promoting intensive research on novel bio-technological strategies for CH_4 treatment. Among them, CH_4 bio-refinery into high added value products has emerged as the most promising one[5–7]. In this regard, under optimal operating conditions, CH_4-laden emissions can be used by microorganisms as feedstock to synthesize high added value products such as biopolymers, exopolysaccharides or ectoine. The valorisation of CH_4 emissions through their bioconversion into commodities with a high market value will turn their abatement into a sustainable and profitable process.

Ectoine and its hydroxylated derivative (hydroxyectoine) are one of the most valuable bioproducts synthesised by microorganisms, retailing in the pharmaceutical industry at approximately US$1000 kg^{-1}. These compatible solutes, which are synthesized by bacteria to survive in salt-rich environments, are an effective stabilizer for enzymes, DNA-protein complexes and nucleic acids[8]. Recent studies have demonstrated that *M. alcaliphilum* 20Z is able to reach ectoine concentrations from 37 up to 70 mg L^{-1} in a continuous fed batch fermentation process, similar to the one used in the commercial production of ectoine with heterotrophic bacteria[9]. However, the production of ectoine by *M. alcaliphilum 20Z* is still limited due to its low biomass growth, clearly compromised by the cell stress derived from the high agitation required in the bioreactors to ensure high methane mass transfer[10,11].

Polyhydroxyalkanoates (PHAs) are intracellular biopolyesters produced under nutrient-limiting and carbon-excess conditions by a wide range of microorganisms as carbon and energy

storage resources[12]. Their outstanding mechanical properties, along with their biodegradability and biocompatibility turns PHAs into an attractive and potential alternative to oil-based plastics[5,13]. Under nutrient-limited conditions (usually N-, P- or Mg-limitation), methanotrophic bacteria have been observed to reach PHAs accumulations ranging from 20 up to 50 % (wt) in both discontinuous[14,15] and continuous operation in suspended growth reactors [16].

Extracellular polysaccharides (EPS) constitute another potential high added value product resulting from this CH_4 bio-refinery. They are biopolymers in which biofilms are embedded, and comprise a wide variety of proteins, glycoproteins, glycolipids and polysaccharides[17]. EPS are usually excreted by bacteria under stressing conditions as a protective barrier or water retainer. These bioproducts are of interest due to their colloid and adhesive properties, and their effects on liquid rheology in the food, pharmaceutical, textile, and oil industries[18]. To date, some studies have demonstrated that methanotrophic bacteria are able to produce EPS in ranges of 0.03-0.43 g g^{-1}biomass[19].

This study assessed an innovative strategy for the valorization of dilute CH_4 emissions based on the continuous bioconversion of CH_4 into high added value products such as ectoine, PHAs and EPS simultaneously, under different magnesium concentrations.

2.Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used during the enrichment of alcalophilic and halotolerant methanotrophs, as well as during bioreactor operation, was a high-alcalinity medium with a final pH of 9.0 and 6 % NaCl according to Kalyuzhnaya et al. (2008). Magnesium (Mg^{2+}) was added to the MSM in the form of $MgSO_4$ at the 3 different concentrations tested (0.2, 0.02, 0.002 g L^{-1}). All chemicals and reagents were obtained from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (purity of at least 99.5 %) was purchased from Abello-Linde, S.A (Barcelona, Spain).

2.2. Microorganisms and inoculum preparation

Reactor 1 (R1) was inoculated with a pure strain of *M. alcaliphilum* 20Z acquired from DSMZ (Leibniz-Institut), an halophilic alcalitolerant methanotrophic strain able to produce ectoine[20].

A second inoculum of halotolerant and alcalophilic methanotrophs enriched from fresh aerobic activated sludge (≈ 6 g L^{-1}) from a wastewater treatment plant with sea water intrusion (Cantabria, Spain) and fresh cow manure from a dairy farm (Cantabria, Spain) was used in Reactor 2 (R2). The enrichment was carried out for 45 days in four glass bottles (1.2 L) containing 190 mL of MSM and 10 mL of the inoculum above described. The bottles were closed with gas-tight butyl septa and plastic screw caps. O_2 was supplied via air flushing of the bottle headspace prior to pure CH_4 injection to obtain a headspace concentration of 55 g CH_4 m^{-3}. This experimental protocol was periodically repeated to restore O_2 and CH_4 headspace concentrations following their depletion. After seven growth cycles, 10 mL of the inoculum were transferred to new 1.2 L bottles with 190 mL of MSM. These bottles were daily monitored for ectoine production and CH_4 degradation until CH_4 depletion. The methanotrophic bacteria able to produce ectoine were selected as the inoculum of R2.

Prior reactor inoculation, 10 mL of each inoculum were mixed with 190 mL of fresh MSM and added to 1.2 L glass bottles closed with gas-tight butyl septa and metallic caps. The inocula were grown for 20 days at 25 ºC and an initial headspace composition of 50 % air/CH_4 (v/v).

2.3 Experimental set-up and operating conditions

Two 2.5 L bubble column reactors (Afora S.A., Spain) were used for continuous CH_4 abatement combined with the production of ectoine, EPS and PHAs. Three different Mg^{2+} concentrations were tested in both reactors (C1 = 0.2 g L^{-1}, C2 = 0.02 g L^{-1}, and C3 = 0.002 g L^{-1}) to assess the effect of this nutrient in the production of ectoine, EPS and PHAs. A 0.066 L min^{-1} CH_4-laden air emission containing 25.9 ± 2.1 g CH_4 m^{-3} (≈ 4 %), corresponding to a methane load of 41.4 g m^{-3} h^{-1}, was fed into reactor 1 (R1) and reactor 2 (R2) via three 10 μm porous stainless steel diffusers located at the bottom of the reactors. The stream was obtained by mixing a pure CH_4 stream (controlled by means of a mass flow

controller, Aalborg, USA) with a pre-humidified air flow, resulting in a gas empty bed residence time (EBRT) of 30 min. Both reactors were operated at 25 ºC and a pH of 9.0 ± 0.3 was maintained via daily replacement of the MSM at a dilution rate of 0.05 day^{-1}. From the retrieved aqueous cultivation broth, 100 mL were used for the determination of the biomass concentration (measured as total suspended solids (TSS)), as well as ectoine, hydroxyectoine, EPS and PHAs content. Gas samples for CH_4 and CO_2 determination were also periodically taken from the sampling ports located at the inlet and outlet of the bioreactors using gas-tight syringes (HAMILTON, Australia). The elimination capacity (EC, g m^{-3} h^{-1}) and removal efficiency (RE, %) were calculated for both reactors. An operational steady state was achieved when neither the EC nor RE deviated >10% from the mean.

2.4 Analytical procedures

The intra-cellular ectoine and hydroxyectoine concentration was determined using 2 mL of cultivation broth according to Cantera et al. (2016b). The specific intra-cellular concentrations (g ectoine or g hydroxyectoine g biomass^{-1}) were calculated using the TSS concentration (g L^{-1}) of the corresponding cultivation broth. An aliquot of 1 mL of cultivation broth was also drawn and filtered through 0.22 μM filters (Filter-lab, Barcelona) to measure the extra-cellular ectoine concentration. The measurement was carried out by UV high performance liquid chromatography in a HPLC 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA detector) at 210 nm and 40ºC using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, EEUU) and a C18 AQ + pre-column (Waters, Bellefonte, EEUU). A phosphate buffer, consisting of 0.8 mM K_2HPO_4 and 6.0 mM Na_2HPO_4, was used as a mobile phase at 40 ºC and a flow rate of 1 mL min^{-1} [21]. Ectoine and hydroxyectoine quantification was carried out using external standards of commercially available ectoine and hydroxyectoine, with a purity of 95 % (Sigma Aldrich, Spain). Cultivation broth samples of 2 mL were centrifuged for 5 min at 13000 rpm and further processed according to López et al. (2014)[22]. The PHAs extracted from the samples were measured in a 7820A GC coupled with a 5977E MSD (Agilent

Technologies, Santa Clara, USA) and equipped with a DB-wax column (30 m × 250 µm × 0.25 µm). The detector and injector temperatures were maintained at 250 ºC. The oven temperature was initially maintained at 40 ºC for 5 min, increased at 10 ºC min^{-1} up to 200 ºC and maintained at this temperature for 2 min. Finally, the oven temperature was increased up to 240 ºC at a rate of 5º C min-[1]. The PHA content (wt %, wPHA/wTSS) of the samples was referred to the initial biomass concentration of the sample. After centrifugation for PHA analysis, 0.5 mL of supernatant were placed in a new dry and already weighted Eppendorf.

The EPS fraction from the bacterial supernatant was precipitated adding three volumes of ethanol 90 % (v/v) followed by an overnight incubation period. After incubation, centrifugation at 10000 rpm for 20 minutes was carried out and the supernatant was discarded. The sample was dried for 24 hours at 58 ^{0}C and dry weight was recorded [23].

CH_4, O_2 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were maintained at 45 ºC, 150 ºC and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

TSS concentration was measured according to standard methods [24].

2.6. Data analysis

The statistical data analysis was performed using SPSS 20.0 (IBM, USA). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were analysed by ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

2.7 Bacterial community analysis

Aliquots of 5 mL of both reactors were centrifuged at 9000 g for 10 min (table1). The resting pellet was used for DNA extraction with the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. DNA was quantified with a Nanodrop spectrophotometer (Nanodrop

Technologies, Wilmington, DE). The microbial composition of the 4 independently amplified reactor samples

Table 1. NGS samples analysed of the three stages of both reactors operated.

Reactor	Sample name	Mg^{2+} concentration	Sampling day	Sample extraction
R1	2R1	0.2 g Mg^{2+} L^{-1}	50	End stage 1
R1	2R1	0.02 g Mg^{2+} L^{-1}	50	Beginning stage 2
R1	3R1	0.02 g Mg^{2+} L^{-1}	100	End stage 2
R1	3R1	0.002 g Mg^{2+} L^{-1}	100	Beginning stage 3
R1	4R1	0.002 g Mg^{2+} L^{-1}	150	End stage 3
R2	1R2	0.2 g Mg^{2+} L^{-1}	0	Beginning stage 1
R2	2R2	0.2 g Mg^{2+} L^{-1}	50	End stage 1
R2	2R2	0.02 g Mg^{2+} L^{-1}	50	Beginning stage 2
R2	3R2	0.02 g Mg^{2+} L^{-1}	100	End stage 2
R2	3R2	0.002 g Mg^{2+} L^{-1}	100	Beginning stage 3
R2	4R2	0.002 g Mg^{2+} L^{-1}	150	End stage 3

was characterized by sequencing two variable 16S rRNA gene regions, V4 and V5, in three separate sequencing runs on Illumina's HiSeq2000 platform using the 515f/926r primer pair according to Waters et al., 2017[25]. Data analysis. 16S rRNA gene amplicons were filtered for quality (Q >25) and size (>200 bp) using NG-Tax, a pipeline for 16S rRNA gene amplicon sequence analysis level and phylogenetically classified[26]. The reference database used for taxonomic assignment was the SILVA database version 123 [27].

3. Results

3.1 Influence of cultivation conditions on ectoine production

The production of intra-cellular ectoine in both reactors was clearly influenced by the Mg^{2+} concentration under steady state. A Mg^{2+} concentration of 0.2 g L^{-1} (C1) was detected as the optimum value for the accumulation of intra-cellular ectoine, reaching average concentrations of 94.2 ± 10.1 and 79.7 ± 5.1 mg ectoine g $biomass^{-1}$ in R1 and R2, respectively, while lower Mg^{2+} concentrations supported lower ectoine yields (51.0 ± 3.9 and 54.9 ± 3.5 mg ectoine g $biomass^{-1}$ at 0.02 g Mg^{2+} L^{-1} (C2) and 35.0 ± 2.2 and 19.7 ± 0.8 mg ectoine g $biomass^{-1}$ at 0.002 g Mg^{2+} L^{-1} (C3) in R1 and R2, respectively). No

extra-cellular ectoine was detected in any condition or reactor, which was attributed to the low copper concentration used in the trace element solution.

3.2. Influence of cultivation conditions on hydroxyectoine production

Similar to ectoine, the Mg^{2+} concentration had also a significant influence on the production of this osmoprotectant (Figure 2). Mg^{2+} concentrations of 0.2 g L^{-1} (C1) promoted the accumulation of average concentrations of 13.3 ± 0.7 mg hydroxyectoine g $biomass^{-1}$ in R2, while lower Mg^{2+} concentrations supported lower hydroxyectoine yields (9.05 ± 0.3 and 3.8 ± 0.1 mg hydroxyectoine g $biomass^{-1}$ at 0.02 g Mg^{2+} L^{-1} (C2) and 0.002 g Mg^{2+} L^{-1} (C3) respectively in R2).

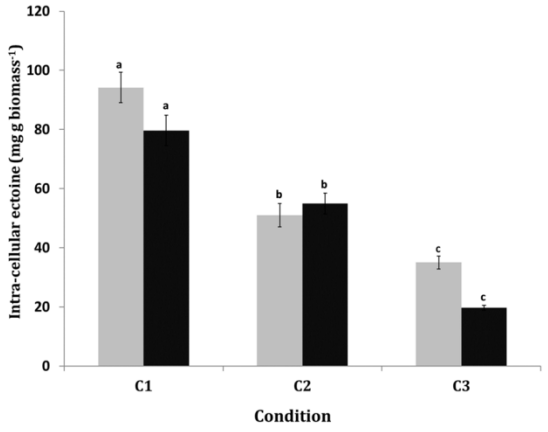

Figure 1: Intra-cellular ectoine yield detected in R1 (grey column) and in R2 (black column) under the three different operational conditions. C1 (0.2 g Mg^{2+} L^{-1}), C2 (0.02 g Mg^{2+} L^{-1}) and C3 (0.002 g Mg^{2+} L^{-1}). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

In the case of R1, hydroxyectoine was only recorded at the lowest Mg^{2+} concentration (2.5 ± 0.2 mg hydroxyectoine g $biomass^{-1}$).

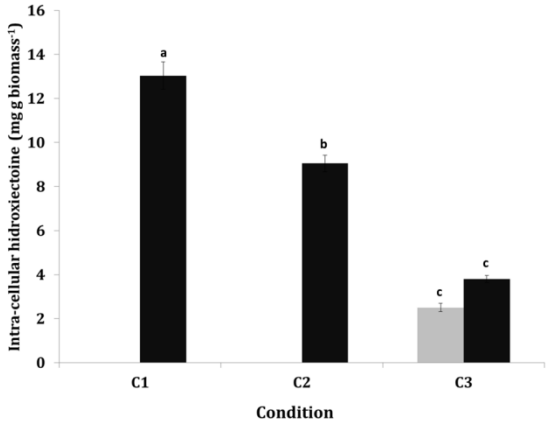

Figure 2: Intra-cellular hydroxyectoine yield detected in R1 (grey column) and in R2 (black column) under the three different operational conditions. C1 (0.2 g Mg^{2+} L^{-1}), C2 (0.02 g Mg^{2+} L^{-1}) and C3 (0.002 g Mg^{2+} L^{-1}). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

3.3 Influence of cultivation conditions on PHA production

PHAs were detected in both reactors at the three Mg^{2+} concentrations tested. As it was expected, higher Mg^{2+} concentrations negatively influenced the production of bioplastics in R1, since PHAs are produced under nutrient limiting conditions. Mg^{2+} concentrations of 0.002 g L^{-1} promoted the accumulation of PHAs up to average values of 14.6 ± 0.5 mg PHAs L culture $broth^{-1}$ in R1, while

higher Mg^{2+} concentrations supported lower PHAs concentrations (1.3 ± 0.1 and 1.8 ± 0.1 mg PHA L culture broth^{-1} at 0.2 g Mg^{2+} L^{-1} and 0.02 g Mg^{2+} L^{-1}, respectively in R1). In the case of R2, the Mg^{2+} concentrations tested did not affect PHAs accumulation (1.9 ± 0.1, 2.4 ± 0.1 and 2.4 ± 0.1 mg PHA L culture broth^{-1} at 0.2 g Mg^{2+} L^{-1}, 0.02 g Mg^{2+} L^{-1} and 0.002 g Mg^{2+} L^{-1}, respectively in R2).

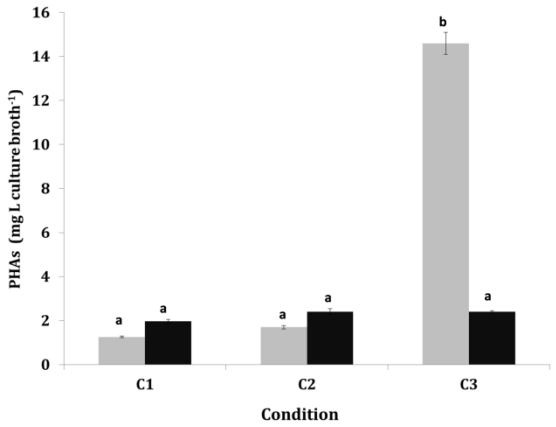

Figure 3: Intra-cellular PHAs yield detected in R1 (grey column) and in R2 (black column) under the three different operational conditions. C1 (0.2 g Mg^{2+} L^{-1}), C2 (0.02 g Mg^{2+} L^{-1}) and C3 (0.002 g Mg^{2+} L^{-1}). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

3.4. Influence of cultivation conditions on EPS production

The EPS obtained showed that Mg^{2+} influenced the production of EPS. A Mg^{2+} concentration of 0.2 g L^{-1} (C1) was detected as the optimum value for the accumulation of EPS, reaching concentrations of 1833 ± 87 and 2642 ± 83 mg EPS g biomass^{-1} in R1 and R2, respectively (Figure 4). Upon decreasing Mg^{2+} concentration, lower EPS yields were obtained (1000.2 ± 124.1 and 757.5 ± 53.2 mg EPS g biomass^{-1} at 0.02 g Mg^{2+} L^{-1} and 952.4 ± 61.7 and 476.8 ± 47.1 mg EPS g biomass^{-1} at 0.002 g Mg^{2+} L^{-1} in R1 and R2, respectively).

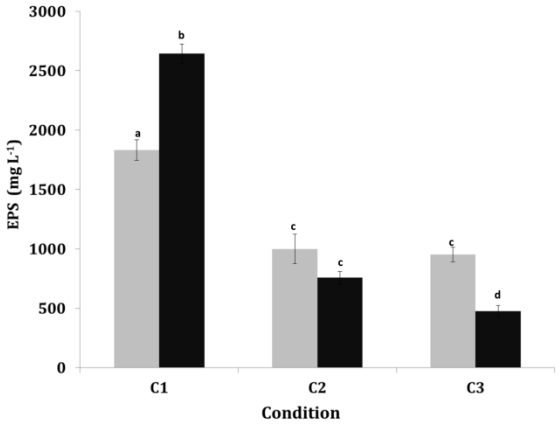

Figure 4: Exopolysaccharides yield detected in R1 (grey column) and in R2 (black column) under the three different operational conditions. C1 (0.2 g Mg^{2+} L^{-1}), C2 (0.02^{2+} g L^{-1}) and C3 (0.002^{2+} g L^{-1}). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

3.5 Comparative evaluation of the 6 stirred tank reactors for CH_4 abatement

The operation of both reactors under the three different Mg^{2+} concentrations tested was evaluated to compare their performance and the effect of nutrient limitation on the EC and the RE. Mass transfer tests were also carried out together with biomass concentration

studies to evaluate the type of limitation in each operational condition.

ECs of 16.1 ± 1.1, 18.9 ± 0.9 and 12.2 ± 0.3 g m^{-3} h^{-1} (corresponding to REs of 26.8 ± 1.8, 36.0 ± 1.9 and 20.8 ± 0.4 %) were recorded in R1 at 0.2, 0.02 and 0.002 g Mg^{2+} L^{-1}, respectively, while ECs of 16.2 ± 1.1, 15.05 ± 0.4 and 11.2 ± 0.7 g m^{-3} h^{-1} (corresponding to REs of 25.3 ± 1.1, 25.8 ± 0.6 and 18.5 ± 1.2 %) were recorded in R2 at 0.2, 0.02 and 0.002 g Mg^{2+} L^{-1}, respectively (Figure 5).

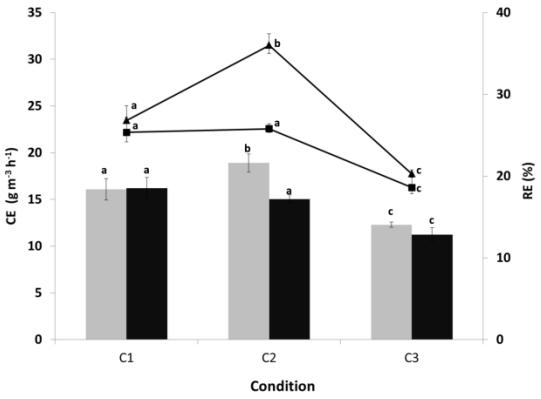

Figure 5: CH_4 elimination capacity (EC) (columns), removal efficiency (lines) detected in R1 (grey column; square) and in R2 (black column; triangle) under the three different operational conditions. C1 (0.2 g Mg^{2+} L^{-1}), C2 (0.02^{2+} g L^{-1}) and C3 (0.002^{2+} g L^{-1}). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

In the case of the mass transfer limitation tests at the three Mg^{2+} concentrations tested, they revealed that a ×2.2 ± 0.13 increase in the inlet load resulted in a ×1.8 ± 0.12 increase in the EC in both reactors. Therefore, the higher concentration gradient at increasing CH_4 concentrations in the gas phase supported higher ECs, thus confirming that the operation was always limited by mass transfer regardless the magnesium concentration tested[28]. Moreover, the TSS concentration did not show significant differences between the three conditions tested (average TSS of 1.5 ± 0.3 g L^{-1}).

3.6 Dynamic of bacterial population

Although R1 was inoculated with the pure strain *M. alcaliphilum* 20Z and operated under sterilized conditions, by the end of stage 1 (2R1), the genus *Methylomicrobium* only represented a 62.5 ± 2.3 % of the total population and other genera such as *Methylophaga* (11.9 ± 0.8 %), *Gelidibacter* (5.4 ± 0.3 %), *Marinobacter* (5.3 ± 1.0 %), and *Halomonas* (2.4 ± 0.3 %) also grow in R1. Furthermore, the reduction of magnesium concentration to 0.02 Mg^{2+} L^{-1} increasingly affected the population structure and the bacterial profile shifted by the end of the first stage, being the main genera *Methylophaga* (19.9 ± 1.7 %), *Gelidibacter* (18.4 ± 8.0 %), *Marinobacter* (5.3 ± 1.0 %), and *Halomonas* (4.2 ± 1.3 %), relegating

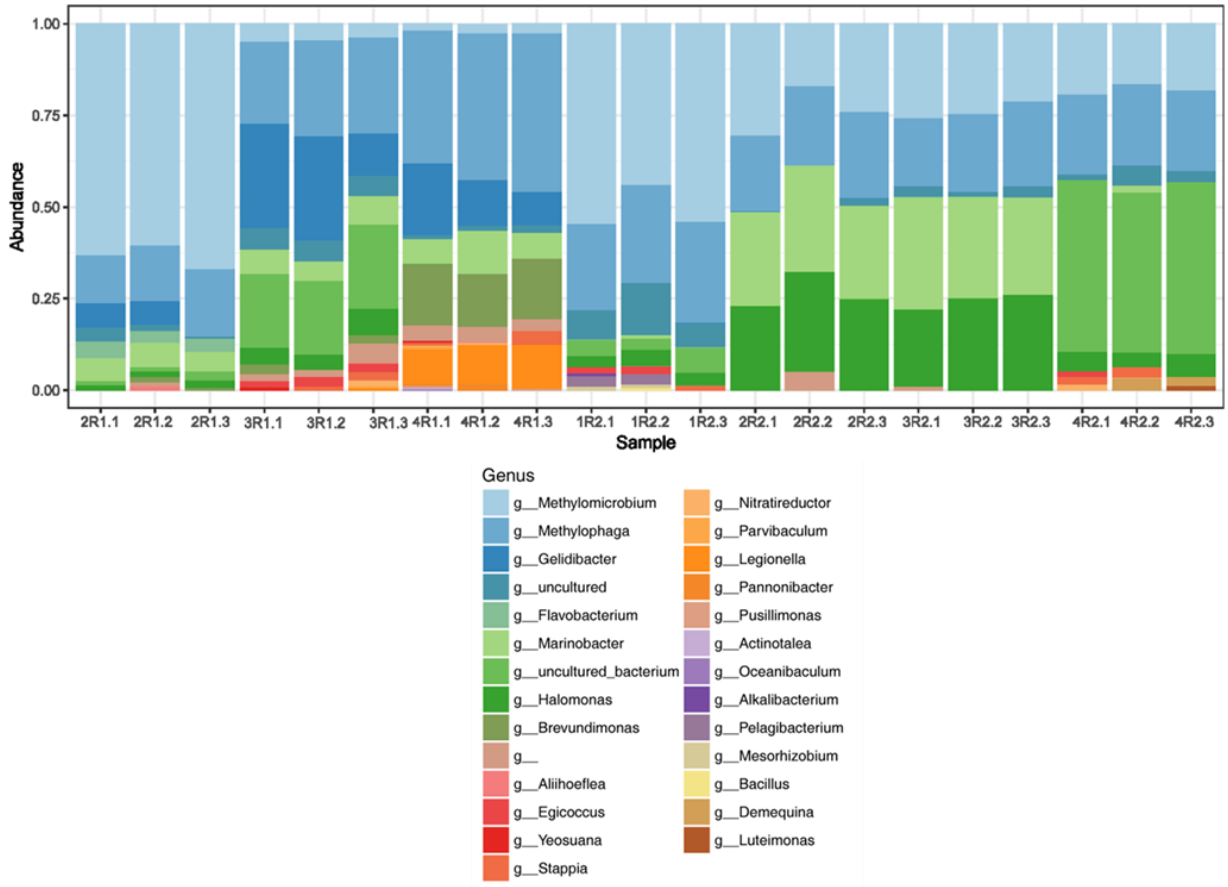

Figure 6: Community composition at a genus level across samples. The abundance is presented in terms of percentage in total effective bacterial sequences in a sample, classified using SILVA. Taxa represented occurred at a threshold abundance >0.01 in at least one sample.

Methylomicrobium to a 3.4 ± 0.5 % of the total bacterial population. Moreover, at the lowest magnesium concentration used *Methylophaga, Gelidibacter Marinobacter* and *Brevundimonas* were the most representative genera (31.9 ± 3.1, 11.5 ± 34.1, 7.7 ± 1.6, 7.7 ± 2.4 %, respectively).

In the case of R2, the inocula was constituted by mainly *Methylomicrobium and Halomonas* (40.7 ± 4.8 and 3.8 ± 0.7 %, respectively) and other genera such as *Methylophaga* and *Gelidibacter* microorganisms (20.7 ± 3.7 % and No Detected, respectively). However, at the end of the first stage *Methylomicrobium* represented only 15% of the total population, percentage that maintained constant until the end of operation. Three genera appeared to compete better in this alkaline and saline environment than *Methylomicrobium*. These three genera were *Methylophaga*, *Marinobacter* and *Halomonas* and their communities were

the most representative groups of the end of stage 1 and stage 2 (15.9 ± 2.1, 25.5 ± 4.1, 25.7 ± 2.6 %, respectively). In the last stage, the diminish of magnesium concentration produced a shift on the population and a novel uncultured family isolated from mug adapted better to the new conditions representing at the end of reactor operation (4R2) a 45.9 ± 2.5 % of the total bacterial community.

4. Discussion

Magnesium seemed to have a positive effect on the production of ectoine independently of the inoculum used, since in both reactors the ectoine production obtained was 3x times higher at the highest concentrations of magnesium tested and the main genera of both reactors did not shift much between stages. Other factors have been proved to boost ectoine production when increasing its concentration, such as NaCl, nitrogen, copper or the carbon source [29–31]. However, to the best of our knowledge, to date there is not research triggering the effect of magnesium in the ectoine pathway.

Regarding the type of ectoine producers, the statistical analysis revealed that the ectoine yield was not significantly different between reactors until the last stage which suggested that the operational conditions were more relevant for ectoine production than the source of the inoculum.

The majority of genera identified in R1 have been previously described as ectoine producers. Between them standed out the genus *Halomonas* which produces between 150-170 mg ectoine g biomass^{-}1. Moreover, other genera identified in both reactors have been previously described as ectoine producers, such as *Marinobacter* and *Methylophaga.* In this regard, the high concentrations of ectoine found in stage 1 of both reactors is probably due to the combination of different ectoine producers rather than to the individual action of *Methylomicrobium alcaliphilum*

The ability of a given microorganism to synthesize hydroxyectoine invariably depends on its ability to produce ectoine, since hydroxyectoine formation can occur either directly from ectoine or from one of its biosynthetic intermediates[32]. In this regard, Mg^{2+} seems to affect the biosynthetic route for ectoine production, while no influencing further steps implied in hydroxyectoine accumulation. It is noteworthy that hydroxyectoine was

detected in R2 under every condition tested, but only at low Mg^{2+} concentration in R1. Although no hydroxyectoine accumulation has been reported to date in *M. alcaliphilum* 20Z cells grown at salinities up to 9 % NaCl[33], a silent gene of ectoine hydroxylase (EctD) has been found in M. alcaliphilum 20Z [7,33]. Nevertheless, the conditions for *ectD* expression or hydroxyectoine accumulation in *M. alcalihilum* 20Z have not been thoroughly studied and the production of hydroxyectoine at low concentrations of magnesium was probably due to reactor contaminantion. This compatible solute is more common among gram-positive halophilic/halotolerant bacteria but it is often synthesized at lower amounts together with ectoine in many other ectoine-producing species (reported accumulations of 36.8 and 45 mg hydroxyectoine g $biomass^{-1}$ in *Halomonas boliviensis DSM 15516* and *Halomonas elongata KS3*, respectively)[29,34,35]. Interestingly, the genus *Halomonas* was detected in the three conditions tested in R2 but only at 0.002 g Mg^{2+} L^{-1}in R1. Thus, the concentration of *Halomonas* and *Marinobacter* genera maintained around 20 % in R2 during operation and they were probably contributing to ectoine hydroxylation. In the case of R1, new genera appeared in the last stage, such as Brevundimonas and Parvibaculum, which could be implied in ectoine hydroxylation.

This production of several ectoines by a methanotrophic consortia formed by *Halomonas*, *Marinobacter*, *Methylomicrobium* and *Methylophaga* combined with methane abatement open up new opportunities for methane bio-refinery, this time based on the use of a methanotrophic consortia to transform methane into several ectoines.

Other studies with methanotrophic bacteria have confirmed the positive effect of Mg^{2+} limitation in PHAs production. Wendland et al. (2001)[36] found PHAs concentrations of 28 % (g biomass) under Mg^{2+} limitation using the pure strain *Methylocystys sp. GB25*. Moreover, PHAs concenrtations up to 50-60 % (g biomass) have been recently reported using *Methylocystis parvus OBBP*[37,38]. However, the PHAs values found in this study are much lower than the ones found by other authors using *α-proteobacter* methanotrophs, since PHAs

synthesis is supposed to be linked with the serine cycle. Although, genera such as *Halomonas* and *Brevundimonas* have been described as producers, the rest of genera found in this study were not previously identified as PHAs producers.

According to previous studies, the RuMP-pathway *γ-proteobacter* methanotrophs are able to achieve high productions of EPS. Malashenko et al. (2001) observed EPS synthesis ranging from 300 to 450 mg g biomass^{-1} using *γ-proteobacter* methanotrophs, which can explain the high concentrations of EPS found in this study using *Methylomicrobium* or other *γ-proteobacteria*. Moreover, previous studies have demonstrated that stressing conditions such as low pH, high salinity and O_2 limitation increase the EPS concentration [39,40], which might explain the high EPS yields here obtained due to the high alkalinity and salinity of the medium. However, the limitation of magnesium negatively affected the production of exopolysaccharides.

According to these results, lower Mg^{2+} concentrations seemed to negatively affect the activity of the methanotrophic bacteria. However, the biomass found according to the TSS analysis, as well as the mass transfer test demonstrated that the main limitation of both reactors was due to the low solubility of methane in the aqueous phase. This result suggested that Mg^{2+} limitation probably affected physiological and biochemical activities of the microbial community, but the main limitation in the reactors were always the poor mass transfer of methane.

An interesting result, it is that the majority of genera here identified have not been previously related to the methane metabolism. In this regard, only the genus *Methylomicrobium* is considered a methanotrophic bacteria, however methane elimination was maintained during operation in C1 and C2 meaning that other microorganisms were able to use methane as the only energy source.

5. Conclusion

This study opens up a new perspective for CH_4 biorefinery based on the multi-production of high added market value metabolites from methane dilute emissions.

The identification of a new methanotrophic consortia able to produce both ectoines, ectoine and hydroxyectoine, using methane as the only feedstock, in addition to

exopolysacharides can make the process of methane elimination much more cost-effective. Moreover, the use of diverse microorganisms could give more resilience and stability to the process and could overcome the current problems of productivity proper of ectoine production from CH_4 by *Methylomicrobium alcaliphilum* 20Z.

In the case of magnesium limitation, it did not improved the cost-effectiveness of the process since it only enhanced PHA production, however the concentrations obtained were always low. Moreover, magnesium limitation diminished the production of ectoines and exopolysaccharides which are products with a higher profit margin.

The promising results obtained in this preliminary study, support further research about these new methanotrophic strains in order to implement a feasible bioprocess capable of creating value out of GHG mitigation.

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Novel haloalkalophilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery.

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Chapter 8

Novel haloalkalophilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery.

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Abstract

Methane bioconversion into products with a high market value, such as ectoine or hydroxyectoine can be optimized by the isolation of more efficient novel methanotrophic bacteria. The research here presented focused on the enrichment of methanotrophic consortia able to produce different ectoines while oxidizing CH_4. For this attempt, four different enrichments (Cow3, Slu3, Cow6 and Slu6) were carried out in basal media supplemented with 3 and 6 % NaCl, and adding methane as the single carbon and energy source. The highest ectoine production (~20 mg g of total biomass^{-1}) was found in the two consortia enriched at 6 % NaCl (Cow6 and Slu6). Moreover, hydroxyectoine was detected for the first time using methane as feedstock (~5 mg g of total biomass^{-1}). The majority of the haloalkalophilic bacteria found in both consortia by 16S rRNA community profiling have not been previously described as methanotrophos. From these enrichments, two novel strains (representing novel species) were isolated using methane as the only carbon and energy source: *Alishewanella* sp. strain RM1 and *Halomonas* sp. strain PGE1. *Halomonas* sp. strain PGE1 showed higher ectoine yields (70.2 to 91.7 mg ectoine g biomass^{-1}) than those previously described for other methanotrophs in continuous (around 37.5 to 70.4 mg ectoine g biomass^{-1}). The results obtained in this study show the potential of using novel methanotrophs to improve bioprocess based on using methane as the only feedstock and make it competitive in the ectoine industrial market.

Keywords: Ectoine, Methane treatment, *Halomonas*, *Alishewanella*, CH_4 biorefinery.

1. Introduction

Methanotrophic bacteria are receiving important scientific and technological interest due to their ability to convert methane (CH_4), the second most important greenhouse gas (GHG), into less harmful products (Hanson and Hanson, 1996) or even into products with a high profit margin in a cost efficient process (Khmelenina et al., 2015; Strong et al., 2016).

In recent years, methanotrophic bacteria have been implemented for the production of bioplastics, single cell proteins (SCP) and lipids. Companies such as Mango Materials, Calista Inc. and UniBio A/S, VTT Ltd. have started to distribute commercial SCP for animal feeding as well as polyhydroxyalkanoates for bioplastic production using methane as feedstock(Petersen et al., 2017; Pieja et al., 2017; Ritala et al., 2017). However, there is still a larger field of methane-based bioproducts unexploited, being undoubtedly one of the most profitable of them, the production of ectoines (hydroxyectoine and ectoine) (Cantera et al., 2016a).

Ectoines provide osmotic balance to a wide number of halotolerant bacteria (Lang et al., 2011; Pastor et al., 2010; Strong et al., 2016). Due to its high effectiveness as enzyme stabilizer, DNA-protein complexes and nucleic acids, they are used in medicine, cosmetology, dermatology and nutrition (Poli et al., 2017). Hydroxyectoine, despite being almost chemically identical to ectoine, is considered a more powerful bioprotectant due to its important role in heat stress protection (Pastor et al., 2010). In this regard, ectoines currently retail in the pharmaceutical industry at approximately US$1000 kg^{-1} and account for a global consumption of 15000 tones $year^{-1}$ (Strong et al., 2016).

Since 1997, some species of the genus *Methylomicrobium, i.e. M. alcaliphilum*, *M. buryatense*, *M. kenyense* or *M. japanense*, as well as *Methylobacter marinus* and *Methylohalobius cremeensis* have been shown to synthesize ectoine (Goraj and Stępniewska, 2016; Reshetnikov et al., 2011; Stępniewska et al., 2014). Due to its higher productivity in both, batch and continuous bioreactors, *M. alcaliphilum* has been the most studied one (Cantera et al., 2017a, 2016a). However, up to date hydroxyectoine accumulation was not shown for *M. alcaliphilum* and its ectoine productivity

cannot compete with those from heterotrophic bacteria currently used in the industry, such as *Halomonas elongate* and *Halomonas salina.* These *Halomonas* spp. achieved productivities from 5.3 to 7.9 g ectoine L^{-1} day^{-1}, while *M. alcaliphilum 20Z* achieves productivities in the range of 7.5 to 9.4 mg ectoine L^{-1} day^{-1} (Pastor et al., 2010; Salar-García et al., 2017). Moreover, the halophilic methanotrophs discovered to date are sensitive to mechanical stress which requires reducing the agitation rate of the system used, thus hampering the mass transfer of CH_4 to the microbial community (Cantera et al., 2016b).

To tackle the current mentioned limitations in the ectoine CH_4-based production, the aim of this research was to identify and isolate novel methanotrophic microorganisms able to produce higher quantities of ectoine combined with higher methane removal rates.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used during the enrichment and isolation of haloalkalophilic methanotrophs possessed high pH (9.0) and high-alkalinity according to the isolation medium of methane oxidizing bacteria from soda lakes, such as *M. alcaliphilum 20Z*(Kalyuzhnaya et al., 2008). During the enrichments NaCl was added to the MSM up to 3 and 6 %, for preparation of the solid media 1.5 % (g/v) agar was added to the MSM medium. All chemicals and reagents were obtained from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (purity of at least 99.5 %) was purchased from Abello-Linde, S.A (Barcelona, Spain).

2.2 Microorganisms and enrichment procedure

Fresh aerobic settled activated sludge (≈ 6 g L^{-1}(dry weight)) from a denitrification-nitrification wastewater treatment plant with seawater intrusion (Cantabria, Spain) and fresh cow manure and soil from a dairy farm at the sea cost (Cantabria, Spain) were used as separate inoculum for the enrichment of the halophilic methanotrophs. The cow manure and soil were dissolved in 10 mL of MSM to a final concentration of 10 g L^{-1}(dry weight). The enrichment was carried in four glass bottles (1.2 L) containing 190 mL of MSM and the two different concentrations of salt tested (3 and 6 %

NaCl). The bottles were closed with gas-tight butyl septa and plastic screw caps. O_2 was supplied via air flushing of the bottle headspace prior to CH_4 injection to obtain a gas headspace concentration of 55 g CH_4 m^{-3}. After sterilization, two of the bottles with different salinities were inoculated with 10 mL of the cow manure and soil mixture (Cow3 and Cow 6), and the other two bottles were inoculated with 10 mL of activated raw sludge (Slu3 and Slu6). Enrichments were transferred 7 times upon CH_4 depletion in the same conditions using 10% inocula. Enrichments were incubated at an agitation rate of 600 rpm and 25º C. The bottles were monitored daily for CO_2 and CH_4 concentrations in the headspace. Ectoine and optical density were measured daily dawning 5 mL of culture broth in the last growth cycle.

2.3 Enrichments community analysis

Aliquots of 5 mL of the last enrichment transfers were centrifuged at 9000 g for 10 min. The resting pellet was used for DNA extraction with the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. DNA was quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). DNA was added as template at a final concentration of 10−20 ng μL^{-1} for PCR amplification. PCR was carried out as described by Feng et al. (2017)(Feng et al., 2017) for both bacterial and archaeal 16S rRNA genes. The purified PCR products were then cloned into Escherichia coli XL1-Blue Competent Cells (Agilent Technologies, Santa Clara, CA) by using the p-GEM Easy Vector Systems (Promega, Madison, WI). Sanger sequencing was performed by GATC Biotech (Konstanz, Germany) using SP6 (5-ATTTAGGTGACACTATAGAA-3) as sequencing primer (Florentino et al., 2015). Vector contamination was removed from the sequences with DNA Baser software (version 4.20.0. Heracle BioSoft SRL, Pitesti, Romania), then they were aligned with SINA and merged with the Silva SSU Ref database (Heracle BioSoft, 2013; Pruesse et al., 2012; Quast et al., 2013). Phylogenetic trees were constructed in the ARB software package (v. 6) by using the maximum likelihood, neighbor-joining and maximum parsimony algorithms as implemented in the ARB package (Ludwig, 2004). Sequences deposited in NCBI under the accession numbers (MG956950 - MG957094).

Metabolic analysis were carried out with the KEGG and the Biocyc databases of metabolic pathways (Caspi et al., 2016; Kanehisa et al., 2016) with the available genomes of the platform.

2.4 Isolation and identification of novel haloalkaliphilic methanotrophs

Aliquots of 100 µL of each enriched culture were spread on to the surface of petri plates (5 replicates) and incubated in separate aerobic jars pressurized with air/CH_4 (80:20, v/v) at 30 °C until colony development was observed. A total of 400 individual colonies were transferred to sterile hungate tubes filled with 10 mL of MSM pressurized with air/CH_4 (80:20,v/v). Tubes where growth was observed were selected for further isolation by consecutive streaking on agar plates. Amplification and sequencing of the 16S rDNA gene was carried out with the primers 27-F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492-R (5-GYTACCTTGTTACGACTT- 3) for each isolate. The 16S rRNA gene sequences from the isolates´ were deposited in the NCBI database under accession numbers (MH042736-50 and MG958593-MG958594).

2.5 Physiological Tests.

Two of the three isolates were selected for further characterization based on novelty and methane degradation.

Substrate utilization of both strains was tested with API 50 CH and API 20 A (bioMerieux SA, Lyon, France) according to the manufacturer's instructions. API 50 CH is a standardized system, associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. The API 20 A system enables 21 tests to be carried out quickly and easily for the biochemical identification of anaerobes. Temperature range for growth of the isolates was assessed from 20 to 37 °C, pH range from 6 to 11 and NaCl range from 0 to 12 %. The analyses were carried out in biological triplicates and the results averaged.

2.6 Analytical procedures

The intra-cellular ectoine and hydroxyectoine concentration was determined using 2 mL of cultivation broth according to Cantera et al. 2016b (Cantera et al., 2016a; Tanimura et al., 2013). The specific intra-cellular concentrations (mg ectoine/hydroxyectoine g biomass^{-1}) were calculated using the total suspended solids (TSS) values (g L^{-1}) of the

corresponding cultivation broth. An aliquot of 1 mL of cultivation broth was filtered through 0.22 µM filters (Filter-lab, Barcelona) to measure the extra-cellular ectoine concentration. The measurement was carried out by UV high performance liquid chromatography in a HPLC 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA detector) at 210 nm and 40ºC using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, EEUU) and a C18 AQ + pre-column (Waters, Bellefonte, EEUU). A phosphate buffer, consisting of 0.8 mM K_2HPO_4 and 6.0 mM Na_2HPO_4, was used as a mobile phase at 40 ºC and a flow rate of 1 mL min^{-1} (Tanimura et al., 2013). Ectoine and hydroxyectoine quantification were carried out using external standards of commercially available ectoine ((S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %, Sigma Aldrich, Spain) and hydroxyectoine(4S,5S)-5-Hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4 carboxylic acid, purity 95 %, Sigma Aldrich, Spain). The detection and quantification limits (DL and QL) were calculated via determination of the signal-to-noise ratio performed by comparing the measured signals from samples with known low concentrations of the analyte with those of blank samples. This procedure allowed establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 and 2:1 is generally considered acceptable for estimating the detection limit while a signal-to-noise ratio of 10:1 is necessary to determine the quantification limit (ICH, 2005).

CH_4, O_2 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were maintained at 45 ºC, 150 ºC and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

Microbial growth was followed by absorbance measurements at 650 nm using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). TSS was measured according to standard methods (American Water Works Association, 2012).

3. Results and discussion

3.1 Enrichment of halotolerant methanotrophic bacteria

CH_4 degradation accounted for a longer lag phase at 6 % NaCl in the enrichments using fresh sludge and manure (Slu6 and Cow6). However, the degradation rates obtained were similar in the exponential phase (1.08, 1.09, 1.10 and 1.12 g CH_4 m^{-3} h^{-1} in Cow3, Slu3, Cow6 and Slu6 respectively). Ectoine maximum production was observed on the first days of growth in both saline conditions (Figure 1).

At 3 % NaCl maximum values of 9.10 ± 0.08 mg ectoine g biomass^{-1} were found when using the manure inoculum (Cow3), similar to those obtained (8.80 ± 0.03 mg ectoine g biomass^{-1}) when using the fresh sludge (Slu3). The enrichments at 6 % NaCl showed concentrations of ectoine 2x times higher than at 3 % NaCl: 19.95 ± 0.35 and 20.95 ± 0.38 mg ectoine g biomass^{-1} in Slu6 and Cow6, respectively. Moreover, 6 % NaCl promoted the accumulation of maximum concentrations of 4.30 ± 0.7 mg and 4.50 ± 0.10 mg hydroxyectoine g biomass^{-1} in Slu6 and Cow6, respectively. In the case of 3 % NaCl, hydroxyectoine concentrations were not recorded with none of the inocula tested.

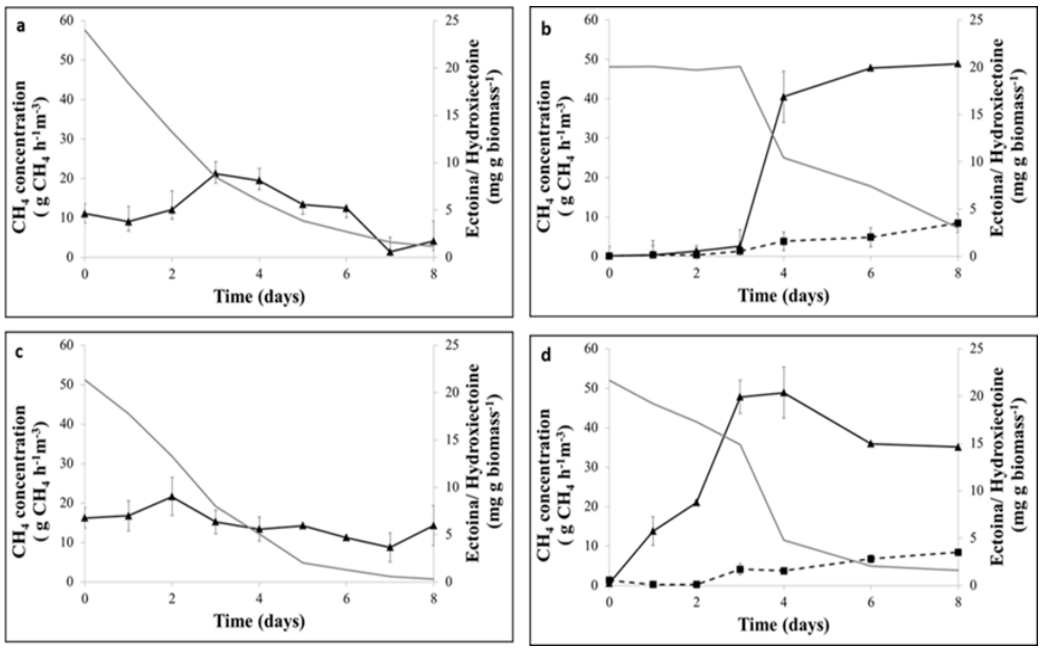

Figure 1: Time course of the concentration of CH_4 (continuous grey line), ectoine (▲) and hydroxyectoine (■) in the enrichment a) Cow3; b) Cow6; c) Slu3; d) Slu6 .

Although the ectoine yields obtained with the methanotrophic consortia enriched in this study were lower than the ones obtained with the pure strain *M. alcaliphilum* 20 Z (up to 70 mg g biomass^{-1}) (Cantera et al., 2017a, 2017b; Cantera et al., 2016b; Khmelenina et al., 2015), this consortia was also able to synthesis hydroxyectoine. No hydroxyectoine accumulation has been detected to date using methanotrophic cells grown at salinities from 0 up to 9 % NaCl (Khmelenina et al., 2015; Reshetnikov et al., 2011). This compatible solute is more commonly produced by gram-positive halophilic/halotolerant bacteria such as *Marinococcus* M52(134.8 mg hydroxyect oine g biomass^{-1}), but it is often synthesized at lower amounts together with ectoine in many other ectoine-producing species, such as *Halomonas boliviensis* DSM 15516 (36.8 mg hydroxyectoine g biomass^{-1}), and *Halomonas elongata* KS3 (45 mg hydroxyectoine g biomass^{-1}) (Fallet et al., 2010; Salar-García et al., 2017; Van-Thuoc et al., 2010).

In this regard, the methane abatement combined with the hydroxyectoine and ectoine production reported in this study opened up new opportunities for a novel and more profitable methane biorefinery based on the production of high added value products combined with methane removal by a determine extremophile consortia.

Moreover, the use of a consortium instead of a pure strain could be beneficial for ectoines industrial production. In this context, *M. alcaliphilum strain 20 Z* achieves really low average biomass concentrations (1.0 g L^{-1}), thus low productivities and CH_4 removals down to 4 times the ones obtained with other methanotrophic bacteria (43 to 67 % CH_4 removals) (Cantera et al., 2015; Cantera et al., 2017b; Cantera et al., 2017b). Furthermore, the purer a culture is, the higher are the restrictions to grow under environmental stress factors, while higher population richness and diversity promotes higher resilience and therefore, better reactor performance and bio-product recovery (Cabrol et al., 2012). Another consideration is that this was the first attempt to enrich for ectoine producers, however adaptive laboratory evolution could be carried out to gain insights into the adaptive changes that accumulate in microbial populations during long term selection: better ectoines

productivities and higher methane degradation rates (Dragosits and Mattanovich, 2013).

The microbial community analysis of the 4 enrichments yielded a total of 362 16S rDNA bacterial gene sequences, from which 144 passed the NCBI quality control with an average length of 1380 nucleotides. The individual phylotypes could be clustered (identity criteria of 0.97) mainly into 5 known phyla: *Proteobacteria*, *Bacteroidetes*, *Spirochaetes*, *Firmicutes* and *Planctomycetes*. No archaeal communities were detected in any of the enrichments.

At the genus level, sequences clustered into 14 genera (Figure 2). About 6.7 % of all the sequences could not be identified at the genus level and were classified at the next highest possible resolution level. In all enrichment conditions, some of the sequences analyzed belonged to known methane oxidizers such as *Methylomicrobium* spp. (36. 8, 16.3, 2.3 and 4.7 % in Cow3, Slu3 Cow6 and in Slu6, respectively), while other were identified as *Methylophaga* (26.3, 60.5 2.7 and 3.8 % in Cow3, Slu3 Cow6 and in Slu6, respectively), methylotrophic bacteria able to oxidize methanol, monomethylamines, dimthylsulfides, etc.

TAXA	Cow3	SLu3	Cow6	Slu6	Phylum
Thiotricales (Methylophaga)	26.5	60.5	2.7	3.8	*Proteobacteria*
Methylococcales (Methylomicrobium)	36.8	16.3	2.3	4.7	
Chromatiales (Uncultured)	ND	2.1	ND	ND	
Alteromonadales (Marinobacter)	15.7	4	ND	ND	
Alteromonadales (Alishewanella)	ND	5.2	ND	ND	
Unclassified Bacterium	5.5	2.7	ND	ND	
Xanthomonadales(Xanthomonas)	ND	ND	77.8	34.2	
Caulobacterales (Caulobacter)	ND	ND	2.8	11.5	
Caulobacterales (Brevundimonas)	ND	ND	ND	31.8	
Rhodobacterales (Pannonibacter)	ND	ND	4.6	ND	
Oceanospirilales (Uncultured)	ND	ND	9.8	4.9	
Rhizobiales (Aliihoeflea)	ND	ND	ND	9.1	
Sphirochaetales (Sphirochaeta)	5.2	ND	ND	ND	*Spirochaetes*
Clostridiales(Uncultured)	5.7	ND	ND	ND	*Firmicutes*
Flavobacteriales (Wandonia)	4.6	4.6	ND	ND	*Bacteroidetes*
Planctomycetales (Planctomyces)	ND	4.6	ND	ND	*Planctomycetes*

Figure 2. Heat-map of the main orders and genera determined in the four enrichments performed. ND: Not detected.

Remarkably the distribution of the microorganisms corresponded more to the percentaje of salinity used for the enrichments rather than to the source of inocula, probably due to the highly selective conditions imposed by high salinity and high pH.

At 3 % NaCl with both inocula, methane metabolism related microorganisms were the most abundant. Marine chemoorganotrophic microorganisms such as *Marinobacter* (15.7 and 4.0 % in Cow3 and Slu3 respectively) and *Wandonia* (4.6 and 4.6 % in Cow3 and Slu3 respectively) were detected as well as other broadly distribute bacteria belonging to the orders *Clostridiales, Sphirochaetales* and *Alteromonadales* (representing up to 10.5 and 5.2 % of the total bacteria in Cow3 and Slu3, respectively).

On the other hand, at 6 % NaCl, the majority of microorganisms identified belonged to widely distributed *Proteobacteria* such as *Xanthomas* spp. (77.8 and 34.2 % in Cow6 and Slu6 respectively), *Brevundimonas* spp. (31.8 % in Slu6), *Pannonibacter* spp. (4.6 % in Cow6) and *Caulobacter* spp. (2.8 and 11.5 % in Cow6 and Slu6, respectively). It has been previously observed that non-methanotrophic methylotrophs and oligotrophic, heterotrophic bacteria such as *Brevundimonas*, *Pannonibacter* or *Caulobacter* appear on primary isolation agar plates when enriching methanotrophic bacteria (Butterbach-Bahl et al., 2011). Although some species of the genera *Brevundimonas*, *Pannonibacter*, *Caulobacter* and *Xanthomonas* are identified as haloalkalotolerant aerobic bacteria, to the best of our knowledge, none of them has been described as methanotrophos. Moreover, according to current database resources, such as KEGG (Kanehisa et al., 2016) or Biocyc (Caspi et al., 2016), no proteins related to methane metabolism are present in the genomes of the mentioned genera. Some known ectoine producers were identified at 6 % NaCl, such as *Methylomicrobium*, the aforementioned methylotrophic *Methylophaga* and some marine microorganisms belonging to the orders *Oceanospirillales* (14.8 and 13.4 % in Cow6 and Slu6 respectively).

3.2 Isolation of new haloalkalophilic strains

Following the streaking procedure on agar plates, a total of 15 methanotrophic isolates were obtained and phylogenetically identified (Table 1).

All of the isolates obtained belonged to the *Proteobacteria* phylum, specifically to the *Gamma-proteobacteria* class while the original enrichments were constituted

Table 1. Isolated strains

Phylogentic affiliation	Identity (%)	Acc number of closest organism	Abundance within the isolates (%)	Salinity of the source of inocula (% NaCl)	Acc number
Methylomicrobium alcaliphilum	99	NR_074649	66.7	3-6	MH042736-45
Alishewanella sp.	98	NR_116499	20.0	3	MH042746-48
Halomonas sp.	97	NR_042812	13.3	6	MH042749-50
*Alishewanella sp.*strain RM1	*98*	NR_116499	Selected for phenotype description	3	MG958594
Halomonas sp. strain PGE1	98	NR_042812	Selected for phenotype description	6	MG958593

for 5 different genera, *Planctomycetes*, *Bacteroidetes*, *Proteobacteria*, *Firmicutes* and *S*pirochaetes (Figure 3). Within the isolates, 66.7 % were affiliated with *Methylomicrobium alcaliphilum* 20Z (99 % similarity). However, two other cluster of isolates belonged to the genera *Alishewanella* and *Halomonas*, from which, one isolate was chosen per genera. The isolate affiliated with *Alishewanella* sp., designated as strain RM1 (MG958594), was isolated from the enrichment Slu3 at pH 9. The closest phylogenetic relatives were *A. aestuari* (Roh et al., 2009) (98 % 16S rRNA gene similarity) and *A. agri* (Kim et al., 2010) (98 % 16S rRNA gene similarity) isolated from a tidal flat soil and a landfill cover soil, respectively (Figure 3). The other phylotype found was isolated from the enrichment Slu6.

The selected isolate was designated as *Halomonas* sp. strain PGE1 (MG958593), related with *H. ventosae* and *H. salina* with 98 %16S rRNA gene similarity on both cases, both species isolated from hypersaline soils (Figure 3).

According to the species definition of stablishing the cut-off for new species in 98.7% of 16S rRNA gene sequence identity (Yarza et al., 2014), *Alishewanella sp.* strain RM1 and *Halomonas* sp. strain PGE1 represent new species.

***Alishewanella sp.* strain RM1**

The genus *Alishewanella* was described for the first time by Vogel et al. (2000) (Vogel et al., 2000) and emended independently by Roh et al. (2009), Kim et al. (2010) and Sisinthy et al. (2017)(Kim et al., 2010; Roh et al., 2009; Sisinthy et al., 2017; Vogel et al., 2000). It belongs to the family *Alteromonadaceae* order *Alteromonadales* of the class *Gammaproteobacteria* (Xia et al., 2016). Cells stain Gram-negative. According to Sisinthy et al. (2017), *Alishewanella* genus contains a total of 8 species: *A. aestuarii*, *A. agri*, *A. fetalis*, *A. jeotgali*, *A. solinquinati*, *A. tabrizica*, *A. longhuensis* and *A. alkalitolerans* (Sisinthy et al., 2017).

Cells of *Alishewanella sp.* strain RM1 are coccoid-shaped, occurring either singly or in pairs (Table 2). Growth of strain RM1 occurred from 20 to 37 °C with an optimum at 30°C. Described members of this genus are mesophyles with temperature growth range between 4 to 44 °C depending on the species (Kim et al., 2010; Roh et al., 2009). Growth of *Alishewanella* sp. strain RM1 occurred at pH from 7 to 9 with an optimum growth at pH 8.

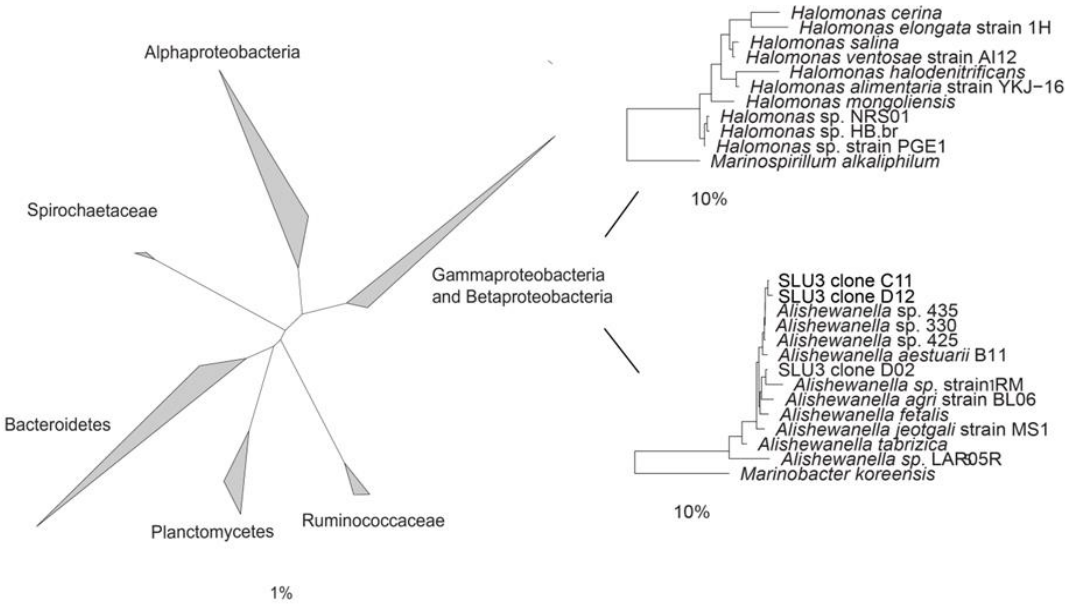

Figure 3: Phylogenetic affiliations of 16S rDNA sequences obtained (a) in the four enrichments obtained, (b) from the isolate of *Halomonas* sp. strain PGE1 and the closest phylogentic relatives and (c) *Alishewanella sp.* strain RM1. The trees display a consensus from neighbor-joining, maximum likelihood and maximum parsimony algorithms. Bars represent 10 (in b and c) changes per site or 100 % divergence in sequence.

Alishewanella spp. usually have a pH growth optimum of 6.0-8.0, although they are able to grow in broad pH ranges from 5.5 to 12, (Kim et al., 2010; Roh et al., 2009) (Table 2). *Alishewanella* sp. strain RM1 could use glycerol, erytritol, L-Arabinose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, esculin, cellobiose, raffinose and glycogen.

Phenotypic comparison with their closest relatives is shown in Table 2. No growth was detected in anaerobic conditions.

The optimum salt concentration was 3 % NaCl, although the strain was able to grow at a range from 0 to 6 %.

The genus *Alishewanella* is widely distributed and appears in sediments, saline environments or fresh water.

Table 2: Main characteristics of strain RM1 and the closest phylogenetic relatives of Alishewanella genus

Parameter	*Alishewanella* sp. strain RM1	*Alishewanella aestuarii* strain B11^T	*Alishewanella agri* strain BL06^T
Motility	-	-	+
Optimum:			
Optimum temperature (C)	30	37	30
Optimum NaCl (%)	3	3	2
pH optimum	8	NR	6-8
Ectoine production	-	NR	NR
Substrates			
CH_4 oxidation	+	NR	NR
Glycerol	+	-	-
erythritol	+	NR	-
L-Arabinose	+	-	-
D-xylose	+	NR	-
D-glucose	+	-	+
D-fructose	+	+	-
D-mannose	+	-	-
L-rhamnose	+	NR	-
D-mannitol	+	-	-
Esculin	+	-	+
Cellobiose	+	NR	-
Maltose	-	+	+
Sucrose	-	NR	+
Raffinose	+	-	-
Starch	-	-	+
Glycogen	+	-	-

Strains: 1 ***Alishewanella aestuarii***, otherwise indicated data from (Roh et al., 2009); and, ***Alishewanella agri***, otherwise indicated data from (Kim et al., 2010). + supported growth, - did not support growth, (NR) not reported. All strains are rods, Gram-negative. None of the strains use D-Arabinose, Myo-inositol, N-acetylglucosamine, Trehalose, Potassium Gluconate., D-ribose, L-xylose, D-adonitol, Methyl D-Xylopyranoside,**, L-sorbose, galactose, Dulcitol, D-sorbitol, Methyl D-mannopyranoside, Methyl D-Xylopyranoside,, Amygdalin, Arbutín, Salicin, D-lactose, Insulin, Melibiose, Melezitose,** Xylitol, Gentiobiose, Turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-Arabitol, L-Arabitol, 2-ketogluconate, 5-ketogluconate.

Depending on the isolation source of the strain, they have the ability to grow in the presence of high concentrations of NaCl such as *A. fetalis* (8 % NaCl), *A. agri (*6 % NaCl) *or A. alkalitolerants* (3 % NaCl), while *A. aestuarii* is not able to grow in the presence of more than 1 % NaCl content over a basal media (Kim et al., 2010; Roh et al., 2009; Sisinthy et al., 2017; Vogel et al., 2000).

The ability of *Alishewanella sp.* strain RM1 to use CH_4 as the only carbon source has not been reported for members of the *Alishewanella* genus. CH_4 degradation rates during the exponential growth at optimal growth conditions (pH 8, 3 % NaCl and 25 ºC) were 24.2 ± 3.1 mg CH_4 h^{-1} g $biomass^{-1}$ and CO_2 total production was 10 ± 0.5 mg CO_2 h^{-1} g $biomass^{-1}$. These values are similar to the ones obtained by other pure methanotrophic strains, such as *Methylomicrobium alcaliphilum* 20Z (Cantera et al., 2016c). *Alishewanella* genus is constituted by versatile microorganisms, known for their heterotrophic aerobic and anaerobic respiratory metabolism of a broad range of substrates (Sisinthy et al., 2017) which makes feasible their ability to grow in methane as the only carbon source (Vogel et al., 2000).

On the other hand, although a wide range of halotolerant bacteria including other genera inside the same order, such as *Marinobacter aquaeolei* and *Marinobacter hidrocarbonoclasticus* (86-85%) have been found to produce ectoine, when they are in the presence of salt, the strain isolated in our study was not detected as ectoine or hydroxyectoine producer(Pastor et al., 2010). However, to survive in a range of salt from 0 to 6 % and high pH, *Alishewanella sp.* strain RM1 should accumulate a soluble, low molecular weight molecule that could have a market value, such as polyols, sugars, amino acids, or betaines (Roberts et al., 2005).

***Halomonas sp.* strain PGE1**

The family *Halomonadaceae* belongs to the order *Oceanospirillales* within the *Gammaproteobacteria* and contains 13 genera (Vahed et al., 2018). *Halomonas* was defined as a genus in 1980 and includes around 100 different validly defined species (Vahed et al., 2018; Vreeland et al., 1980).

Halomonas cells stain Gram-negative and are able to grow in a wide range of salt

concentrations (0 to 25 % NaCl) and appearing in a wide range of habitats. *Halomonas* spp. perform aerobic and anaerobic respiration and they are able to use a great range of carbon sources (Mata et al., 2002; Tan et al., 2014; Vahed et al., 2018). *Halomonas* sp. strain PGE1 cells are rod-shaped, moderately halophilic being able of growing in salt concentrations of 3–10 % NaCl (w/v), whereas no growth occurs at 1 % NaCl (Table 3). Strain PGE1 grew within 20–37°C and at pH values between 6 and 11. They are aerobic microorganisms. *Halomonas* sp. strain PGE1 utilized esculin, d-glucose, melibiose, raffinose and glycogen. Phenotypic comparison with their closest relatives is shown in table 3.

Table 3: Phenotypic characteristics of strain PGE1 and the closest *Halomonas* species.

Parameter	*Halomonas sp. strain* PGE1	*Halomonas ventosae* AI12 ^{T}	*Halomonas elongate* IH15 ^{T}
Morphology	rod	rod	rod
Optimum:			
Optimum temperature (C)	30	15-50	30
Optimum NaCl (%)	6	6-9	1
pH optimum	9	3-15	5-9
Substrates:			
CH4 oxidation	+	NR	NR
D-galactose	-	+	-
D-glucose	+	+	+
D-mannose	-	-	+
D-sorbitol	-	+	-
Esculin	+	-	+
Cellobiose	-	-	+
maltose	-	+	-
D-lactose	-	-	+
Melibiose	+	-	-
Sucrose	-	-	+
Raffinose	+	-	-
Glycogen	+	-	-
Potassium Gluconate	-	+	+

Strains: 1 ***Alishewanella aestuarii***, otherwise indicated data from (Roh et al., 2009); and, ***Alishewanella agri***, otherwise indicated data from (Kim et al., 2010). + supported growth, - did not support growth, (NR) not reported. All strains are rods, Gram-negative. None of the strains use D-Arabinose, Myo-inositol, N-acetylglucosamine, Trehalose, Potassium Gluconate., D-ribose, L-xylose, D-adonitol, Methyl D-Xylopyranoside,**, L-sorbose, galactose, Dulcitol, D-sorbitol, Methyl D-mannopyranoside, Methyl D-Xylopyranoside,, Amygdalin, Arbutín, Salicin, D-lactose, Insulin, Melibiose, Melezitose,** Xylitol, Gentiobiose, Turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-Arabitol, L-Arabitol, 2-ketogluconate, 5-ketogluconate.

No member of *Halomonas* genus was reported as methane oxidizers, however they possess a high versatile metabolism and they are typically isolated from methane rich environments such as hydrocarbon reservoirs, methane seeps or methane rich sediments ((Niederberger et al., 2010; Piceno et al., 2014)). In optimal growth conditions (pH 9, 6 % NaCl and 30 ºC), maximum methane elimination capacities were 25.3 ± 1.2 mg CH_4 h^{-1} g $biomass^{-1}$ during the exponential growth, similar to other methanotrophic bacteria (S. Cantera et al., 2016; Gebert et al., 2003). However, after day 12 methane degradation and oxygen consumption stopped. This scenario was probably due to the accumulation of a toxic intermediate of the methane oxidation pathway, such as formic acid or methanol as described in other bacteria (Hou et al., 1979; Strong et al., 2015). Growth of the strain was recovered by medium replacement.

Hydroxyectoine production by *Halomonas sp.* strain PGE1 was not observed under any condition tested. However, 70.2 to 91.7 mg ectoine g $biomass^{-1}$ was produced when *Halomonas sp.* strain PGE1 grew at 6 % of NaCl and pH 8, 9 and 10. An extend number of *Halomonas* species are able to produce ectoine being *H. elongate* strain IH15 ^{T} the main strain used in the industry for the biotechnological production of ectoine through a fed-batch fermentation process called *biomilking* (total duration ~120 h)(Pastor et al., 2010; Sauer and Galinski, 1998). However, this process is costly due to the high quality of substrates required such as the carbon feedstock and the oxygen needed (Kunte et al., 2014; Lang et al., 2011; Pastor et al., 2010). Other *Halomonas* species such as, *H. salina* and *H. boliviensis* are able to produce high quantities of ectoine, and excret it naturally to the culture broth, creating a more efficient and less costly biotechnological process. The primary advantage of using members of the genus *Halomonas* is that its species have high ectoine production yields (170, 154 and 358 mg ectoine g $biomass^{-1}$ in *H. boliviensis, H. elongate* and *H. salina*, respectively), as well as high reactor productivities (3.4, 9.1 and 7.9 g ectoine L^{-1} day^{-1} in *H. boliviensis, H. elongate* and *H. salina*, respectively). The values of ectoine obtained in this first attempt by *Halomonas sp.* strain PGE1 are lower than the ones obtained by other *Halomonas* species; thus the carbon

source is fundamental on the quantity of ectoine produced (Chen et al., 2018). However the ectoine yields obtained in this study are higher than the ones accounted by *M. alcaliphilum* in the same culture conditions (30.4-66.9 mg g biomass^{-1}). Furthermore, *Halomonas sp.* strain PGE1 likely obtain higher productivities than *M. alcaliphilum* since it could achieve higher biomass concentrations due to its better resistance to stress and environmental perturbations. The enzyme used for the oxidation of methane could be different than the described methane monooxygenase which hampers the process due to the requirement of a high-energy electron donors, such as $NADPH^+$ as an energy input to activate the otherwise inert methane molecule (James C. Liao, Luo Mi, 2016). Despite the mentioned advantages, *Halomonas sp.* strain PGE1 has a serious handicap based on the production of a secondary metabolite toxic for the bacterial growth. The use of *continuos* bioreactors destined to methane treatment combined with ectoine production as a part of a determine consortia could entail high productivities and removal efficiencies overcoming this limitation.

Provided that the current economic and technical barriers for the operation of methane treatment by this new strain were overcome, a climate-change mitigating technology based on the use of the isolated novel *Halomonas sp*. strain PGE1 could be a cost-effectiveness solution for the continuous abatement of methane dilute emissions. A brief environmental and economic viability analysis of a methane bio-refinery producing ectoine, using real data from a landfill proper emission (5 % of CH_4) and productivities proper of *M. alcaliphilum* and *Halomonas sp.* strain PGE1is shown in Table 4. These analysis use a CH_4 feedstock typical of landfill emissions (30 g CH_4 m^{-3}), and a flow (Q) of 50 g CH_4 m^{-3} with a recirculation of 1 L min^{-1} appropriate for bubble column reactors. The daily feedstock of CH_4 would be of 1.3 kg of CH_4. In the case of *Halomonas* strain PGE, 70 % of carbon goes to produce biomass and members of the genus *Halomonas* are able to accumulate up to 15 % ectoine(Pastor et al., 2010). In the case of *M. alcaliphilum*, 20 % of carbon goes to biomass production and accumulates up to 7 % ectoine in continuous bioreactors according to Cantera et al. (2017b)(Cantera et al.,

Table 4: Economic and Environmental analysis

***Methylomicrobium alcaliphilum* strain 20Z**

REACTOR OPERATIONAL PARAMETERS

Landfill proper emission (4.5%) (g CH_4 m^{-3})	Q_{gas} + $Q_{recirculation}$ (m^{-3} h^{-1})	Methane feed (Kg CH_4 day^{-1})	Biomass produced from CH_4 (Kg day^{-1})	Ectoine yield (kg day^{-1}) (7 % of biomass)	Ectoine yield (kg $year^{-1}$) (7 % of biomass)	Ectoine ($ $year^{-1}$)
30	1.8	1.3	0.3	0.02	6.7	6 732.9

REACTOR ENERGY CONSUMPTION AND DOWNSTREAM PRICE

Liquid pressure (Ba)	Q_{gas} (m^3 s^{-1})	ΔP (kPa)	Blower efficiency	Air flow Energy consumption (KWh $year^{-1}$)	Air flow Energy consumption ($ $year^{-1}$)	Downstream consumption ($ $year^{-1}$)
0.09	0.0005	9.7	0.7	61.6	7.38	4 616.8

ECONOMICAL BALANCE

Total inversion ($ $year^{-1}$)	Maximum profit ($ $year^{-1}$)
4 624.2	2108.7

Halomonas sp. strain PGE1

REACTOR OPERATIONAL PARAMETERS

Landfill proper emission (4.5%) (g CH_4 m^{-3})	Q_{gas} (m^3 h^{-1})	Methane feed (Kg CH_4 h^{-1})	Biomass produced from CH_4 (Kg day^{-1})	Ectoine yield (kg day^{-1}) (7 % of biomass)	Ectoine yield (kg $year^{-1}$) (7 % of biomass)	Ectoine ($ $year^{-1}$)
30	1.8	1.3	0.9	0.14	50.5	50 497.0

REACTOR ENERGY CONSUMPTION AND DOWNSTREAM PRICE

Liquid pressure (Ba)	Q_{gas} (m^3 s^{-1})	ΔP (kPa)	Blower efficiency	Air flow Energy consumption (KWh $year^{-1}$)	Air flow Energy consumption ($ $year^{-1}$)	Downstream consumption ($ $year^{-1}$)
0.09	0.0005	9.7	0.7	61.6	7.38	16 159.0

ECONOMICAL BALANCE

Total inversion ($ h^{-1})	Maximum profit ($ h^{-1})
16 166.4	**34330.6**

Ψ and ectoine yields have been obtained from Cantera et al. (2017) and Pastor et al. (2010) based in experiments conducted with these microorganisms; The energy consumption [kw] for gas circulation was calculated as Q [m^3 s^{-1}] × ΔP [kPa] / Blower efficiency (~0.7) according to Estrada et al. (2012); (Kwh day^{-1}) was calculated according to Acien et al. (2012); The downstream process price was deduced from Kunte et al. (2014) and http://www.ectoin.net/

2017b). The energy consumption [kw] for gas circulation was calculated as Q_{gas} [m^3 s^{-1}] × ΔP [kPa] / Blower efficiency (~0.7) according to Estrada et al. (2012)(Estrada et al., 2012) as a function of the biomass. The downstream process price (48 \$ kg^{-1}) depends on the biomass accumulated and it was deduced from Kunte et al. (2014)(Kunte et al., 2014) and http://www.ectoin.net/. Following this equations, the use of *Halomonas* sp. strain PGE would be 16 times more cost-effective that the use of *M. alcaliphilum 20Z*.

4. Conclusions

New species of alkaliphilic and halophilic methanotrophic bacteria were isolated in this research with the aim of making the promising conversion of methane into ectoine a competitive and cost-effective process for the industry.

In a first attempt several consortia were enriched using methane as the only carbon source. The consortia enriched at 6 % NaCl were able to degrade methane efficiently and were able to produce, for the first time, both ectoines, ectoine and hydroxyectoine using methane as the only feedstock. In this context, the use of these enriched consortia for methane treatment could overcome the current limitations of methane bioconversion, throughout higher productivities, giving more resilience and stability to the process and throughout the multi production of substances with a high profit margin.

Additionally, two new species of haloalkalophilic methanotrophs were isolated in this research. From both, *Halomonas sp.* strain PGE1 showed higher ectoine productivities than those generally obtained by *M. alcaliphilum.* This result supports further research about the use of a novel *Halomonas* strain, capable of using methane as feedstock which could turn methane bio-refinery to a fairly competitive market process.

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Chapter 9

Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures

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Abstract

The environmental conditions during culture enrichment, which ultimately determine its maximum specific biodegradation rate (q_{max}) and affinity for the target pollutant (K_s), play a key role in the performance of bioreactors devoted to the treatment of methane emissions. This study assessed the influence of Cu^{2+} and CH_4 concentration and the effective CH_4 supply rate during culture enrichment on the structure and biodegradation kinetics of methanotrophic communities. The results obtained demonstrated that an increase in Cu^{2+} concentration from 0.05 to 25 μM increased the q_{max} and K_s of the communities enriched by a factor of ≈ 3, even if the Cu^{2+} concentration did not seem to have an effect on the enzymatic "copper switch" and only pMMO was detected. In addition, high Cu^{2+} concentrations supported lower diversity coefficients (Hs ≈ 1.5× lower) and apparently promoted the growth of more adapted methanotrophs such as *Methylomonas*. Despite no clear effect of CH_4 concentration on the population structure or on the biodegradation kinetics of the communities enriched was recorded at the two low CH_4 concentrations studied (1 and 8 %), a higher agitation rate increased the q_{max} by a factor of ≈ 2.3 and Ks by a factor of ≈ 3.1.

Keywords: biodegradation kinetics, Cu^{2+} concentration, CH_4 concentration, methanotrophs, microbial population structure.

1. Introduction

Methane (CH_4) is the second most relevant greenhouse gas (GHG) emitted by anthropogenic activities, representing more than 20 % of the total worldwide GHG emissions. CH_4 atmospheric concentration increases yearly at 0.2-1 % its current tropospheric level exceeding preindustrial concentrations by ≈150 %. Anthropogenic emissions are mainly attributed to agriculture, livestock farming, waste management and energy production, which combined represent over 60 % of the total CH_4 emissions worldwide (European Environmental Agency, 2013; IPCC, 2013; United States Environmental Protection Agency, 2013). In addition, the impact of CH_4 on climate change is ≈ 34 times more detrimental than that of carbon dioxide (CO_2) in a 100-y horizon (European Environmental Agency, 2013; IPCC, 2013; Scheutz et al., 2009). This scenario has caused a raised governmental and public awareness, which has promoted both the enforcement of multidisciplinary political initiatives and an increased research on CH_4 abatement technologies (European Environmental Agency, 2013; IPCC, 2013; Scheutz et al., 2009). Although emissions with CH_4 concentrations above 20 % are suitable for energy recovery by incineration or low-cost treatment by flaring, more than 50 % of anthropogenic emissions contain CH_4 at concentrations below 3 % (old landfills (0–20%), ventilated coal mines (0.1–1%), covered liquid manure storage tanks (0–3%), etc.) (Nikiema et al., 2007; Scheutz et al., 2009). CH_4 abatement at such low concentrations using conventional physical/chemical technologies is either inefficient or too costly, and often entails a large CO_2 footprint (Estrada et al., 2014; Nikiema et al., 2007). In this context, biological treatment technologies can become, if appropriately optimized, a low-cost and environmentally friendly alternative for the treatment of CH_4 due to their already proven effectiveness and low operating costs during the abatement of malodors and volatile organic compound emissions (López et al., 2013).

However, the cost-effective implementation of current biotechnologies for the treatment of CH_4 is still limited by the understanding of the communities underlying CH_4 biodegradation (Li et al., 2014; López et al., 2013). In this regard, there is still a need for studies assessing the effect of environmental factors during culture

enrichment on the performance and characteristics of methanotrophic communities. Among them, CH_4 gas concentration, O_2 gas concentration and Cu^{2+} concentration in the aqueous media have been identified as the main parameters influencing microbial CH_4 abatement due to their key role on the nature and level of expression of CH_4 monooxygenases (MMO), and therefore on the population structure of methane oxidizing bacteria (MB) (Li et al., 2014; Murrel et al., 2000; Semrau et al., 2010). However, most studies assessing the role of CH_4 and Cu^{2+} concentrations have focused only on the physiological and enzymatic aspects of CH_4 biodegradation (Murrel et al., 2000). On the contrary, studies on the macroscopic performance and characteristics of the microbial communities have not found a clear effect of these parameters on CH_4 abatement (Estrada et al., 2014; Ho et al., 2013; Li et al., 2014). Likewise, the influence of the effective CH_4 supply rate to the microbial communities during culture enrichment on the kinetics of CH_4 biodegradation, which can differ at similar gas CH_4 concentrations depending on the bioreactor configuration, has often been disregarded (López et al., 2014, 2013; Yoon et al., 2009). In this context, there is a limited understanding of the effect of CH_4 and Cu^{2+} concentration and of the effective CH_4 supply rate during culture enrichment on the characteristics and structure of methanotrophic communities (Semrau et al., 2010), which could shed light on the optimal operating conditions leading to an enhanced performance of CH_4 abatement biotechnologies.

The present study aims at systematically elucidating the influence of CH_4 and Cu^{2+} concentrations and of the effective CH_4 supply rate (governed by the CH_4 mass transfer to the microbial community) during the enrichment of MB communities on CH_4 biodegradation kinetic parameters (q_{max} and Ks) and community structure in order to identify the optimum environmental factors supporting an efficient CH_4 abatement and high biodiversity in full scale bioreactors.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) utilized during the enrichment of the methanotrophic communities and in the kinetic assays was a modified Brunner medium (Lopez et al., 2014) containing

(g L^{-1}): $Na_2HPO_4 \cdot 2H_2O$, 3.17; KH_2PO_4, 1.50; $NaNO_3$, 5.28 (used instead of $(NH_4)_2SO_4$ to avoid the inhibition of methanotrophs by NH_4 (Carlsen et al., 1991)); $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.05; EDTA, 0.005; $FeSO_4 \cdot 7H_2O$, 0.002; H_3BO_3, 0.0003; $CoCl_2 \cdot 6H_2O$, 0.00011; $ZnSO_4 \cdot 7H_2O$, 0.0001; $Na_2Mo_4 \cdot 2H_2O$, 0.00003; $MnCl_2 \cdot 4H_2O$, 0.00003; $NiCl_2 \cdot 6H_2O$, 0.00002. Cu^{2+} was added to the MSM as $CuCl_2 \cdot 2H_2O$ at two Cu^{2+} concentrations (0.05 µM and 25 µM). The final pH of the MSM was 7. All chemicals and reagents were procured from Panreac (Barcelona, Spain) with a purity higher than 99.0%. CH_4 was purchased from Abello-Linde, S.A. (Barcelona, Spain) with a purity of at least 99.5 %. Silicone oil 200 cSt was obtained from Sigma-Aldrich (Madrid, Spain).

2.2. Inoculum

The inoculum used for the enrichment of the methanotrophic communities was a mixture (50/50 %/% on a volume basis) of aerobic activated sludge (≈ 6 $g.L^{-1}$) from a denitrification-nitrification wastewater treatment plant (Valladolid, Spain) and fresh cow manure from a dairy farm (Cantabria, Spain) 10× diluted in MSM.

2.3. Microbial community enrichments

Eight enrichment series (45 days/enrichment) were carried out at two different mixing ratios of CH_4 (1 % and 8 %) and Cu^{2+} (0.05 µM and 25 µM) under two different magnetic stirring rates (300 rpm and 650 rpm). The series were performed in duplicate (Table 1).

Table 1. Cultivation conditions during microbial community enrichments

Test series (TS)	CH_4 headspace (%)	Cu^{2+} (µM)	Agitation (rpm)
TS 1	8	25	300
	8	25	650
TS 2	8	0.05	300
	8	0.05	650
TS 3	1	0.05	300
	1	0.05	650
TS 4	1	25	300
	1	25	650

In each enrichment series, 1.2 L batch gas-tight reactors containing 100 mL of MSM at its corresponding Cu^{2+} concentration and 50 mL of silicone oil (in order to support a high CH_4 mass transfer from the headspace to the microbial community), were inoculated with 10 mL of the inoculum previously described. The reactors were closed with gas-tight butyl septa and plastic screw caps, and monitored daily for O_2, CO_2 and CH_4 concentrations in the headspace. O_2 was daily supplied via air flushing of the reactor headspace prior injection of

pure CH_4 using a calibrated 100 mL gas tight syringe to obtain the desired headspace concentration (before methane injection the same volume of air was removed from the reactors to avoid overpressure). The enrichment batch reactors were maintained under agitation at 25 ºC. A dilution rate of 0.25 ± 0.05 day^{-1} was set in order to maintain the pH at ≈ 7.0, to replenish essential nutrients and, to remove any potential inhibitory metabolite accumulated in the medium. Silicone oil was not removed from the batch reactors during the enrichment series. Biomass samples from the aqueous phase were drawn with a liquid sampling syringe at the end of each enrichment series to determine the microbial population structure by denaturing gradient gel electrophoresis (DGGE)-sequencing, the presence of soluble or particulate methane monoxygenases (sMMO or pMMO, respectively), and the CH_4 biodegradation kinetics constants (q_{max} and Ks). The concentration of CH_4 and CO_2 in the headspace of the enrichment bottles was periodically measured by GC-TCD.

2.4. Structure of the enriched communities

The 16 samples from the 8 duplicate enrichment series stored at -20 ºC were thawed and centrifuged at 5000 rpm for 15 minutes. The biomass pellets were resuspended in 10 mL of phosphate buffered saline (PBS) medium at pH 7 by vortexing.

The total DNA of these samples was extracted by the Fast® DNA Spin Kit for Soil (MP Biomedicals, LLC) and its quantity and quality were evaluated by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) (English et al., 2006). The DNA was amplified with polymerase chain reaction (PCR) universal bacterial primers for 16S rRNA gene amplification (968-F-GC and 1401-R (10µM)) (Sigma- Aldrich, St. Louis, MO, USA) according to Lopez et al. (2014). The PCR products of the bacterial 16S rRNA fragments from the samples were separated by DGGE according to Lopez et al. (2014). The gels were stained for 60 minutes with GelRed Nucleic Acid Gel Stain (biotium). Specific PCR-DGGE bands were detected by a transilluminator, UV wavelength, 254 - 312 nm (Sigma-Aldrich) and carefully cut from the gel. The DNA contained in each band was extracted by

incubation in 50 mL of sterile water at 63 ºC for 70 minutes. The last PCR cycle was accomplished without the GC-clamp attached to the primer 968-F. The PCR products obtained were sequenced by Secugen S.L. (Madrid, Spain). RDP classifier tool was used to analyze the taxonomic classification of the DGGE bands sequenced (70% confidence level) (Wang et al., 2007). DECIPHER search tool was used to analyze the presence of chimeras (Wright et al., 2012). The NCBI BLAST search tool was used to compare the DNA sequences obtained with nucleotide sequences from the NCBI database (McGinnis and Madden, 2004). The sequences of the bacteria identified were deposited in the NCBI GenBank. The DGGE band pattern present in each gel was compared using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium) and the Pearson product-moment correlation coefficient as well as, the Shannon-Wiener diversity index (H_s) were calculated according to Lopez et al. (2014). In addition, a multiple sequence alignment was performed. Sequences alignment (Clustal X 2.1) and phylogenetic analysis were obtained using the MEGA software (version 6.06). The phylogenetic trees were constructed using the neighbor-joining method (1000-fold bootstrap analysis) (Hall, 2013).

2.5 Detection of pMMO and sMMO

After DNA extraction, the specific primer set *A189f/mb661r*, targeting the *pmoA* gene (which encodes the *β-subunit* of particulate MMO) (Costello and Lidstrom, 1999; Erwin et al., 2005), and the primer set *mmoX* f882/*mmoX* r1403, targeting the *mmoX* gene (which encodes the *α-subunit* of the hydroxylase component of sMMO) (McDonald et al., 1995; Newby et al., 2004) were used for PCR amplification. The PCR mixture (50 µL) consisted of 25 µL of BIOMIX (Bioline, Ecogen), 2 µL of the extracted DNA, 2 µL of the specific primer set depending of the gene amplified and Milli-Q water up to a final volume of 50 µL. The annealing temperature used was 58 ºC for *mmoX* and 56 ºC for *pmoA*. The PCR conditions were as follows: 94 ºC for 5 min; *Taq* polymerase added; 58/56 ºC for 1 min; 72 ºC for 1 min; 40 cycles consisting of 94 ºC for 1 min, 58/56 ºC for 1 min, and 72 ºC for 1 min; and a final cycle consisting of 94 ºC for 1 min, 58/56 ºC for 1 min, and 72 ºC for 5 min.

2.6. CH_4 biodegradation kinetics

Kinetic assays were performed in duplicate to elucidate the influence of CH_4 and Cu^{2+} concentration and the agitation rate (i.e. the effective CH_4 supply rate) during culture enrichment on the maximum specific CH_4 biodegradation rate q_{max} (g CH_4 $g^{-1}_{biomass}$ h^{-1}) and the Michaelis-Menten half-saturation constant, K_s (g m^{-3}) for CH_4 of the enriched microbial communities. In-vitro kinetic assays were performed in 120 mL glass serum bottles. Each bottle contained 20 mL of MSM and was inoculated with the corresponding enriched methanotrophic community at a concentration of 98 ± 34 g $_{biomass}$ m^{-3}. Preliminary assays carried out under the same conditions in our laboratory (CH_4 concentration =25.8 ± 2.3 g m^{-3}) showed that even a total suspended solids (TSS) concentration of 900 ± 70 g $_{biomass}$ m^{-3} ensured the absence of CH_4 mass transfer limitations (Data non published).

The glass bottles were sealed with butyl septa and aluminum crimp seals. CH_4 was initially added at headspace concentrations of 131 ± 6 g m^{-3}, 99 ± 6 g m^{-3}, 60 ± 5 g m^{-3}, 21 ± 0.9 g m^{-3} and 2.4 ± 0.3 g m^{-3}.CH_4 aqueous concentrations were 4.3 ± 0. 2 g m^{-3}, 3.3 ± 0.8 g m^{-3}, 2.0 ± 0.2 g m^{-3}, 0.7 g m^{-3} ± 0.03 and 0.08 ± 0.001 g m^{-3}, according to a CH_4 Henry's law constant at 25 ºC and 1 atm of 29.4. The glass bottles were agitated at 650 rpm (25 ºC) for 13 h. A GC-TCD was used to monitor the headspace concentration of CH_4 and CO_2 every 1 hour. The kinetic parameters K_s and q_{max} were obtained by fitting the initial specific CH_4 biodegradation rate (q, g CH_4 $g^{-1}_{biomass}$ h^{-1}) at each aqueous CH_4 concentration ($[CH_4]$, g m^{-3}) to the Michaelis-Menten equation (Equation 1) by nonlinear regression using Microsoft Excel Solver (Microsoft Office Professional Plus 2013) (Sakač and Sak-Bosnar, 2012):

$$q = q_{max} \frac{[CH_4]}{[CH_4]+K_s}$$

2.7. Analytical procedures

CH_4, O_2 and CO_2 headspace concentrations were measured in a Bruker 430 GC-TCD (Palo Alto, USA) according to Estrada et al. (2014). The culture optical density was measured at 650 nm with a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan) and correlated to dry biomass concentrations, which were determined as TSS (American Water Works Association, 2012).

2.8. Statistical analysis

The statistical data analysis was performed using SPSS 20.0 (IBM). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were analyzed by Student's t-test for two group comparisons, and ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

3. Results

3.1 Microbial population structure

The Shannon-Wiener diversity index revealed that Cu^{2+} was the main factor determining bacterial diversity (Figure 1). Hence, an increase in Cu^{2+} concentration from 0.05 to 25 µM at a CH_4 concentration of 8% entailed a statistically significant decreased in bacterial diversity from 2.7 ± 0.05 to 1.4 ± 0.15 at 300 rpm and from 2.8 ± 0.1 to 1.8 ± 0.15 at 650 rpm. Similarly, H_s decreased from 2.1 ± 0.05 to 1.9 ± 0.05 at

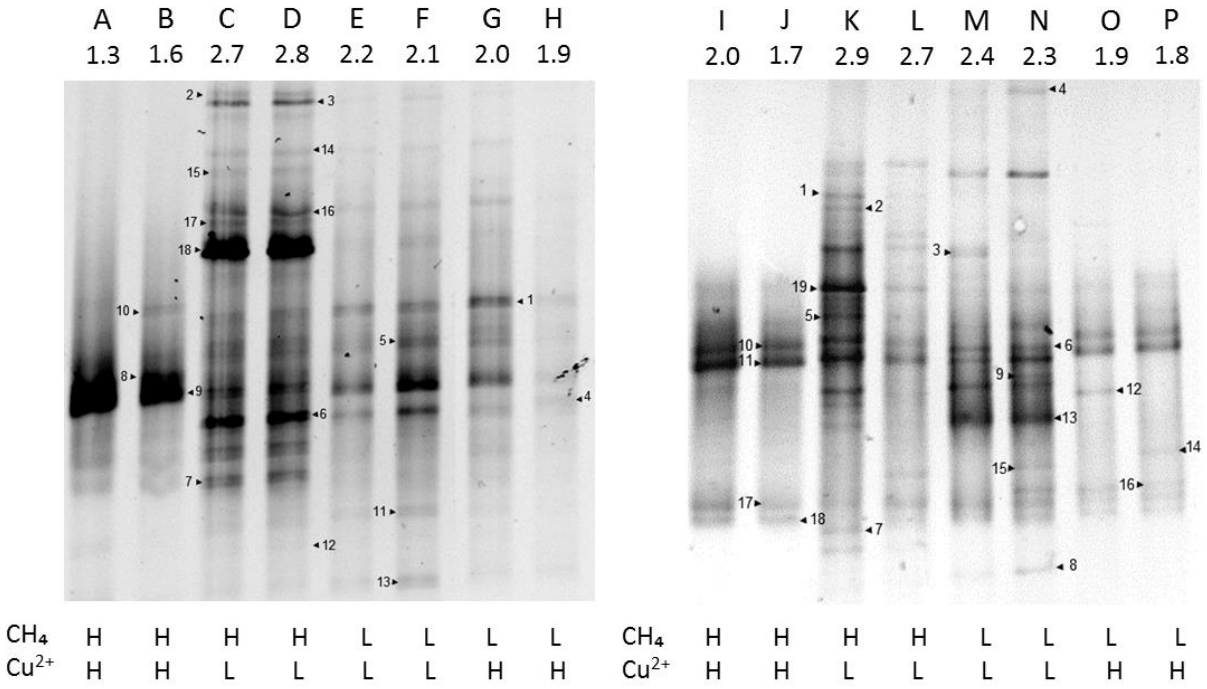

Figure 1: DGGE profile of the cultures enriched at 300 rpm (A, B, C, D, E, F, G, H) and 650 rpm (I, J, K, L, M, N, O, P). *L* and *H* refer to low and high CH_4 and Cu^{2+} concentrations, respectively. The Shannon-Wiener diversity indexes are indicated in the upper part of the gel. The sequenced bands are indicated by "►" and the corresponding number of each band.

300 rpm and from 2.3 ± 0.05 to 1.8 ± 0.05 at 650 rpm when Cu^{2+} concentration was increased from 0.05 to 25 μM at a CH_4 concentration of 1%.

The influence of Cu^{2+} on the Pearson similarity coefficients was consistent with the effect on the Shannon-Wiener indexes. Thus, the similarities found between the communities enriched at 0.05 and 25 μM at a CH_4 concentration of 8 % were 42.5 ± 3.7 % and 37.5 ± 3.8 % at 300 and 650 rpm, respectively, while the similarities obtained in the cultures grown at 1% were slightly higher (76.0 ± 4.0 % at 300 and 65.8 ± 6.9 % at 650 rpm).

The phylogenetic analysis based on the 16S rRNA sequences obtained from the DGGE gels at 650 rpm represented by the phylogenetic tree (Figure 2) further supported the effect of Cu^{2+} on the similarity among the bacterial populations enriched.

3.2 Microbial community profile

A total of 37 bands were sequenced from all conditions tested (18 for 300 rpm; 19 for 650 rpm). These 16S rRNA sequences were classified in three different phyla:

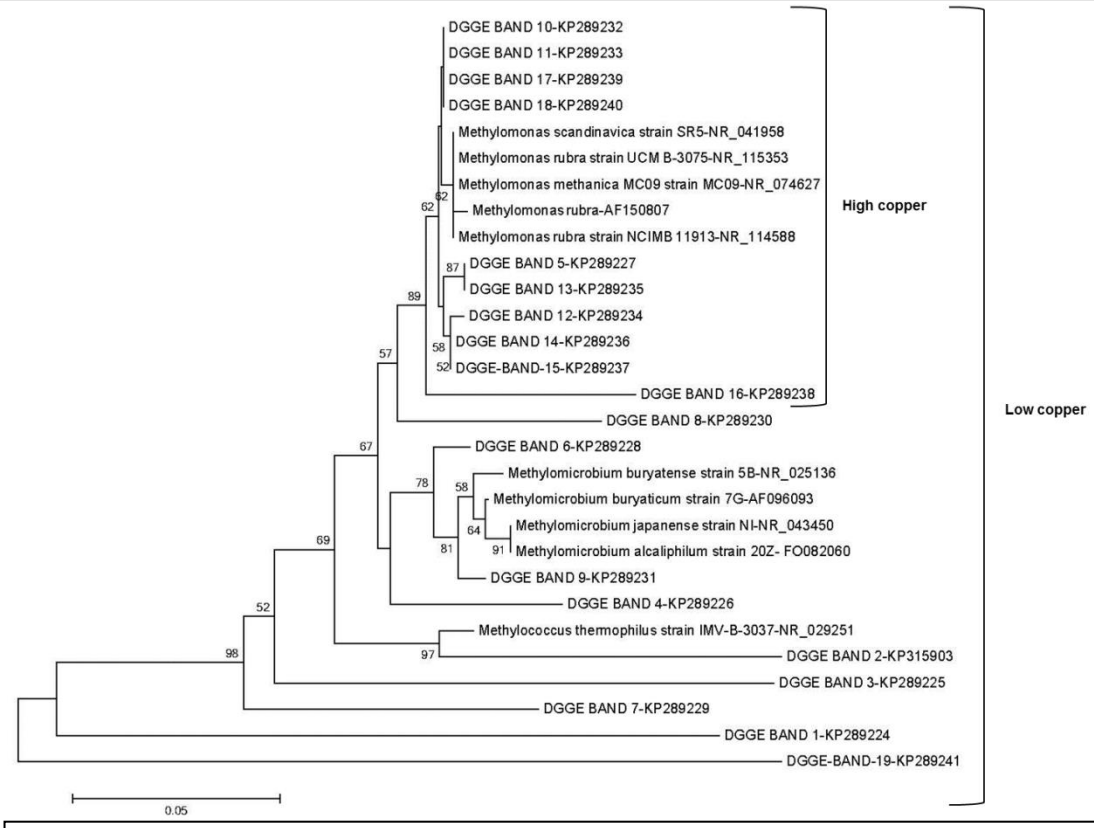

Figure 2. Bacterial phylogenetic tree based on neighbour-joining analysis of the 16S rRNA sequences from the enriched populations at 650 rpm detected by PCR-DGGE and their closest relatives (similarity > 96%) in GenBank obtained by the Blast search tool. Accession numbers are indicated. Numbers on the nodes indicate bootstrap values (1000 replicates). The scale bar indicates 10% sequence difference.

Proteobacteria, *Lentisphaerae* (at 650 rpm) and *Chlamydiae* (at 300 rpm). The closest matches for each band, and its similarity percentage and sources, are shown in Tables 2 and 3. The phylum *Proteobacteria* was predominant in all cultures enriched (DGGE bands 2-13, Table 2, and DGGE bands 1–18, Table 3). Most of these *Proteobacteria* were closely related (> 90 % sequence identity) to aerobic CH_4 oxidizing bacteria within the class *Gammaproteobacteria*. They belonged to the type I methanotroph genera *Methylomonas*, *Methylobacter* and *Methylomicrobium* (which were always present in the microbial community regardless of the enrichment conditions) and to the type X methanotroph genus *Methylococcus.* A Cu^{2+} concentration of 25 μM supported the dominance of *Methylomonas* (particularly at a CH_4 concentration of 8 %), while *Methylobacter* was never detected in the cultures enriched at the highest Cu^{2+}concentration. On the other hand, despite being more representative in the presence of 0.05 μM Cu^{2+}, *Methylomicrobium* appeared in the enrichments conducted at both Cu^{2+} concentrations at a CH_4 concentration of 8 % (Figures 1 and 2). These results further confirmed the influence of Cu^{2+} concentration on the community profile. The type X genus *Methylococcus* was found at 650 rpm under low Cu^{2+} and high CH_4 concentrations (Figure 1).

Non methanotrophic genera of the phylum *Proteobacteria* such as *Dokdonella* (bands 12-13) and *Bdellovibrio* (bands 2-3) were also detected at 300 rpm. However, while *Dokdonella* was present in all enrichments, *Bdellovibrio* was only detected at 0.05 μM Cu^{2+}. In addition, in the cultures enriched at 300 rpm, bands 14-18 were affiliated with the phylum *Chlamydiae*. All of them were closely related with the genus *Neochlamydia* (> 90 % sequence identity) and were found under all enrichment conditions but 8 % CH_4 and 25 μM Cu^{2+}. Finally, all bacteria enriched at 650 rpm detected by the DGGE were methanotrophs regardless of the Cu^{2+} and CH_4 concentration, except for band 19 which was an associated heterotroph classified within the phylum *Lentisphaerae*. Bacteria corresponding to band 19 were classified in the genus *Victivallis* (> 90 % sequence identity) and observed under all enrichment conditions regardless of the concentration of Cu^{2+} and CH_4.

Table 2. RDP classification of the bacterial DGGE bands, sequences and corresponding matches (Standard Nucleotide BLAST) of cultures enriched at 300 rpm using the NCBI database, including similarity percentages and sources of origin.

Taxonomic placement (70% confidence level)	Band n°	Acc. Nº	A	B	C	D	E	F	G	H	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
Kingdom Bacteria	1	KP289206			x	x	x	x	x	x	*Uncultured bacterium YC7*(KJ734895)	95	Compost of cow dung
Phylum *Proteobacteria*													
Class *Deltaproteobacteria*													
Order *Bdellovibrionales*													
Family *Bdellovibrionaceae*													
Genus Bdellovibrio	2	KP289207			x	x					*Bdellovibrio bacteriovorus*(CP002190)	97	High loaded MBR
											Bdellovibrio sp. (DQ302728)	97	Soil
	3	KP289208			x	x	x	x			*Uncultured bacterium* (EU050708)	99	Production water of an oil field
											Bdellovibrio sp. (DQ302728)	98	Soil
Class *Gammaproteobacteria*	4	KP289209							x	x	*Methylomonas rubra* (AF150807)	92	Lake sediment
											Methylomonas methanica (NR074627)	93	Marine sediment
	5	KP289210			x	x	x	x	x		*Uncultured Methylococcaceae* (JX505396)	94	Soil
Order *Methylococcales*													
Family *Methylococcaceae*	6	KP289211			x	x	x	x	x	x	*Methylomicrobium agile* (NR_116197)	94	Soil
	7	KP289212			x	x					*Uncultured Methylomicrobium sp.* (HQ044145)	90	Tailing pond
Genus *Methylomonas*	8	KP289213	x	x							*Methylomonas rubra* (NR_114588)	99	Sea sample
											Methylomonas methanica (NR074627)	99	Marine sediment
	9	KP289214	x	x	x	x	x	x	x	x	*Methylomonas rubra* (NR_115353)	99	Coal mine drainage
											Methylomonas methanica (NR074627)	99	Marine sediment
	10	KP289215		x							*Methylomonas methanica* (NR074627)	99	Marine sediment
											Methylomonas rubra (AF150807)	99	Lake sediment
Order *Xanthomonadales*													
Family *Xanthomonadaceae*	11	KP289216	x		x	x	x	x	x	x	*Uncultured Gammaproteobacteria* (HM238179)	96	Biofilter treating waste gas
Genus *Dokdonella*	12	KP289217			x		x	x			*Uncultured bacterium* (KM886284)	98	Biofilter for CH_4 abatement
											Uncultured Dokdonella sp. (KJ486363)	97	Sludge from membrane bioreactor
	13	KP289218	x		x	x	x	x	x	x	*Uncultured Dokdonella sp.* (KJ486363)	97	Sludge from membrane bioreactor
Phylum *Chlamydiae*													
Class *Chlamydiia*													
Order *Chlamydiales*													
Family *Parachlamydiaceae*													
Genus Neochlamydia	14	KP289219			x	x	x	x	x		*Neochlamydia hartmannellae*. (NR_025037)	99	Water public conduit system
											Neochlamydia endosymbiont of Acanthamoeba (KF924593)	97	river sediment
	15	KP289220									*Neochlamydia hartmannellae* (NR_025037)	91	Water public conduit system
	16	KP289221			x	x	x	x	x	x	*Neochlamydia hartmannellae* (NR_025037)	95	Water public conduit system
	17	KP289222			x	x					*Neochlamydia hartmannellae* (NR_025037)	98	Water public conduit system
											Neochlamydia endosymbiont of Acanthamoeba (KF924590)	97	river sediment
	18	KP289223			x	x	x	x			*Neochlamydia hartmannellae* (NR_025037)	99	Water public conduit system
											Neochlamydia sp. (EU683885)	97	Water reservoir sediment

Table 3. RDP classification of the bacterial DGGE bands sequences and corresponding matches (Standard Nucleotide BLAST) of cultures enriched at 650 rpm using the NCBI database with indication of the similarity percentages and sources of origin.

Taxonomic placement (70% confidence level)	Band n°	Acc. N°	I	J	K	L	M	N	O	P	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
Kingdom Bacteria													
Phylum *Proteobacteria*	1	KP289224			x	x	x	x			*Uncultured bacterium* (EF648097)	98	Aerobic activated sludge
Class *Gammaproteobacteria*	2	KP315903			x	x					*Methylococcus thermophilus* (NR_029251)	97	Soil
Order *Methylococcales*													
Family *Methylococcaceae*	3	KP289225					x	x			*Methylobacter sp.* (HF565143)	91	Landfill cover soil
	4	KP289226					x	x			*Uncultured bacterium* (JX434234)	93	Water mine
	5	KP289227			x				x	x	*Uncultured Methylomicrobium sp*. (HQ044206)	94	Tailing pond
	6	KP289228					x	x			*Methylomicrobium buryatense* (NR_025136)	96	Soda lake
											Methylomicrobium japanese (NR_043450)	96	Marine sediment
	7	KP289229			x	x	x	x			*Methylomicrobium sp*. (DQ496230)	90	Soda lake
	8	KP289230					x	x			*Methylomonas methanica* (NR_074627)	93	swamp soil
											Methylobacter sp. (AF131868)	91	
Genus Methylomicrobium	9	KP289231						x			*Methylomicrobium buryaticum* (AF096093)	97	Soda lake
											Methylomicrobium alcaliphilum (FO082060)	96	Saline environment
Genus *Methylomonas*	10	KP289232	x	x	x	x			x	x	*Methylomonas methanica* (NR_074627)	99	Marine sediment
											Methylomonas rubra (AF150807)	98	Lake sediment
	11	KP289233	x	x	x	x	x	x	x	x	*Methylomonas rubra* (NR_115353)	99	Coal mine drainage
											Methylomonas methanica (NR_074627)	99	Seawater
	12	KP289234							x		*Methylomonas rubra* (NR_114588)	98	Sea sample
											Methylomonas methanica (NR_074627)	98	Marine sediment
	13	KP289235	x		x		x	x			*Methylomonas methanica* (NR_074627)	98	Marine sediment
											Methylomonas rubra (NR_115353)	98	Coal mine drainage
											Methylomonas scandinavica (NR_041958)	97	Igneous rocks
	14	KP289236	x	x						x	*Methylomonas methanica* (NR_074627)	98	Marine sediment
											Methylomonas rubra (AF150807)	97	Lake sediment
	15	KP289237	x			x		x			*Methylomonas rubra* (AF150807)	94	Lake sediment
											Methylomonas methanica (NR_074627)	94	Marine sediment
	16	KP289238						x	x	x	*Methylomonas rubra* (AF150807)	95	Lake sediment
											Methylomonas methanica (NR_074627)	95	Marine sediment
	17	KP289239	x	x	x	x	x	x	x	x	*Methylomonas rubra* (AF150807)	99	Lake sediment
											Methylomonas methanica (NR_074627)	99	Marine sediment
	18	KP289240	x	x			x	x	x	x	*Methylomonas rubra* (AF150807)	99	Lake sediment
											Methylomonas methanica (NR_074627)	99	Marine sediment
Phylum Lentisphaerae													
Class *Lentisphaeria*													
Order *Victivallales*													
Family *Victivallaceae*													
Genus Victivallis	19	KP289241	x	x	x	x	x	x	x	x	*Uncultured Victivallaceae* (JQ724358)	91	Biofilm in a microbial cell fuel

3.3 Detection of mmoX and pmoA genes

The gene *pmoA* was present in the enrichments obtained under all tested conditions regardless of the concentration of Cu^{2+} or CH_4 or the agitation rate (Figure. 3).The gene *pmoA* was more abundant at 650 rpm due to the higher concentration of biomass and DNA extracted under this enrichment condition (Figure 4). The gene *mmoX* was only detected in the inoculum and the cultures enriched at 8 % of CH_4 and 0.05 µM Cu^{2+} at 650 rpm (Figure 3).

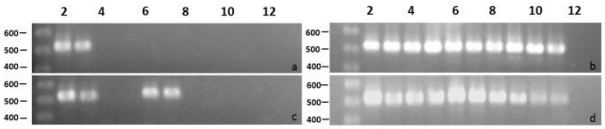

Figure 3. PCR profile for the gene *mmoX* of the cultures enriched at 300 (a) and 650 rpm (c). Lanes: 1) HiperLadder II (50-2000bp), 2) *Methylosinus sporium*, *Methylocystis* sp. (commercial strains mixed), 3) Inoculum (A mixture (50/50 %/% on a volume basis) of fresh aerobic activated sludge and fresh cow manure), 4/5) High CH_4-High Cu^{2+}, 6/7) High CH_4-Low Cu^{2+}, 8/9) Low CH_4-Low Cu^{2+}, 10/11) Low CH_4-High Cu^{2+}, 12) C^-(negative control without DNA). PCR profile for the gene *pmoA* of the cultures enriched at 300 (b) and 650 rpm (d). Lanes: 1) HiperLadder II (50-2000bp), 2) *Methylomonas* and *Methylobacter* sp. mixed culture, 3) Inoculum, 4/5) High CH_4-High Cu^{2+}, 6/7) High CH_4-Low Cu^{2+}, 8/9) Low CH_4-Low Cu^{2+}, 10/ 11) Low CH_4-High Cu^{2+}, 12) C^- (negative control without DNA).

3.4 Biodegradation Kinetics

The kinetic parameters q_{max} and K_s differed up to one order of magnitude among the cultures enriched at 300 and 650 rpm (Table 4), with the highest q_{max} and K_s obtained at 650 rpm. No statistical differences among the kinetic parameter values were found in the cultures enriched at 1 % and 8 % of CH_4 under similar Cu^{2+} concentrations regardless of the agitation rates. However, a higher Cu^{2+} concentration increased both q_{max} and K_s in the low range of CH_4 concentration tested (Table 4). Hence, the highest values of q_{max} were recorded at 25 µM Cu^{2+} regardless of the CH_4 concentration during enrichment. The q_{max} ranged from $7.7 \pm 0.21 \times 10^{-4}$ to $6.3 \pm 0.4 \times 10^{-4}$ g CH_4. $g_{biomass}^{-1}$ h^{-1} at 650 rpm and from $3.6 \pm 0.3 \times 10^{-4}$ to $2.4 \pm 0.24 \times 10^{-4}$ g CH_4. $g_{biomass}^{-1}$ h^{-1} at 300 rpm (Table 4). Likewise, the highest half saturation constants were also recorded at 25 µM Cu^{2+} regardless of the CH_4 concentration and the agitation rates during enrichment. The average Ks values of the cultures enriched at a CH_4 concentration of 1 and 8 % increased from 8×10^{-5} to 1.7×10^{-4} at 650 rpm and from 2.3×10^{-5} to 6.0×10^{-5} at 300 rpm when Cu^{2+} increased from 0.05 to 25 µM (Table 4).

4. Discussion

4.1 Microbial community profile

This study demonstrated the key role of Cu^{2+} concentration on the diversity and bacterial composition during the enrichment of CH_4 degrading microbial communities. CH_4 concentration did not entail significant differences on either the microbial diversity or community structure.

Table 4. Kinetic parameters of the communities obtained at the different conditions tested.

	300 rpm		
	K_s (M)	q_{max} (g CH_4·g biomass^{-1}·h^{-1})	K_s (M)
TS1			
8% CH_4, 25µM Cu^{2+}	$6.4\times10^{-5} \pm 1.2\times10^{-6a}$	$3.6\times10^{-4} \pm 3.0\times10^{-5a}$	$1.9\times10^{-4} \pm 5.$
TS2			
1% CH_4, 0.05µM Cu^{2+}	$1.7\times10^{-5} \pm 1.2\times10^{-6b}$	$9.7\times10^{-5} \pm 7.1\times10^{-6b}$	$6.6\times10^{-5} \pm 6.$
TS3			
8% CH_4, 0.05µM Cu^{2+}	$2.9\times10^{-5} \pm 4.4\times10^{-7bc}$	$9.3\times10^{-5} \pm 4.7\times10^{-7b}$	$9.4\times10^{-5} \pm 3.5$
TS4			
1% CH_4, 25µM Cu^{2+}	$5.7\times10^{-5} \pm 3.5\times10^{-6ac}$	$2.4\times10^{-4} \pm 2.4\times10^{-5a}$	$1.5\times10^{-4} \pm 2.$

Mean values and standard deviations for each duplicate. Kinetic parameters followed by different superscript letters were significantly different at $p<0.05$.

The Shanon diversity index revealed that low Cu^{2+} concentrations at similar CH_4 supply rates resulted in a higher diversity. This result was mainly due to a negative effect of high Cu^{2+} concentrations in some of the ecosystem communities.

The analysis of the Pearson similarity coefficients and the phylogenetic trees also confirmed that the enriched methanotrophic bacteria shifted differently depending on the Cu^{2+} concentration. While *Methylomonas*, *Methylobacter* and *Methylomicrobium* were always present in all enrichments, a clear influence of Cu^{2+} concentration on the relative abundance and dominance of these species was observed. This was more evident in *Methylomonas*, which became dominant in the presence of high Cu^{2+} concentrations and outcompeted other microorganisms. Indeed, this genus was present at different positions in the DGGE gel and the phylogenetic tree, thus representing either different species of *Methylomonas* or distinct fragments of the same rich species.

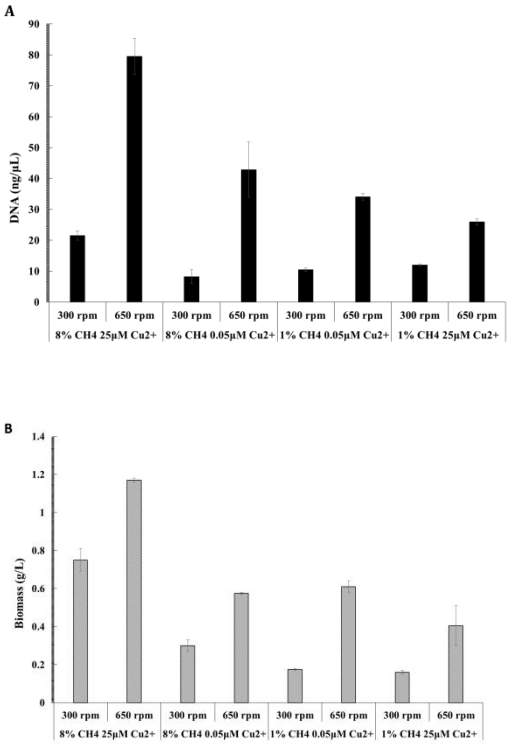

Figure 4. Extracted DNA (A) and biomass (B) concentration from the cultures enriched under each particular condition tested. Error bars represent the standard deviations from duplicate samples

This fingerprint pattern was comparable with that obtained in batch bioreactor cultures operated at low CH_4 concentrations, where *Methylomonas* became the dominant microorganism

when Cu^{2+} concentration was increased by a factor of 10 (Estrada et al., 2014; Van Der Ha et al., 2013).

On the contrary, the DGGE profiles of the communities enriched under the 8 conditions tested showed that both Cu^{2+} concentration and the cross effect of CH_4 and Cu^{2+} concentration exhibited a minor impact on the structure of the associated heterotrophs. In this sense, the genus *Bdellovibrio* was the single identified genus associated to a low copper concentration at 300 rpm. Interestingly, other authors have found using stable isotope probing (SIP) that *Bdellovibrio* is labeled in methanotrophic enrichments, suggesting that these might be parasites or predators of methanotrophs (McDonald et al., 2005; Semrau et al., 2010). In this regard, the high Cu^{2+} concentration used could have been toxic for *Bdellovibrio*, which suggest that the addition of Cu^{2+} could be used as a potential predator-parasite control strategy. Despite previous studies suggested that changes on Cu^{2+} concentration can induce a positive effect on the dominant species, to the best of our knowledge no clear influence of Cu^{2+} on the main bacterial communities and on the microbial diversity was reported to date. This is probably due to the narrow range of concentrations tested in environmental studies simulating natural soils but not taking into account the possible biostimulation effect derived from the addition of copper in landfill cover soils (Estrada et al., 2014; Ho et al., 2013; Nikiema et al., 2013).

The effective CH_4 supply rate was determined by the agitation rate set under similar CH_4 headspace concentrations, the lower stirring rates supporting a limited transfer of CH_4 to the microbial community. The agitation rate exhibited a strong influence not only on the structure of the methanotrophic community (*Methylomonas* presence was significantly higher at 650 than at 300 rpm) but also on that of the associated heterotrophic population, which can play a key role on CH_4 oxidation by avoiding a metabolite-mediated inhibition of MB or by excreting substances that stimulate MB activity (Hrsak and Begonja, 2000; Van Der Ha et al., 2013). The diversity of associated heterotrophs was higher at 300 rpm. In this context, *Dokdonella* (bands 12-13) and *Bdellovibrio* (bands 2-3), two heterotrophs belonging to the *Proteobacteria* phylum that have been retrieved in bioreactors treating waste

gases (Kallistova et al., 2014; Lebrero et al., 2013), were detected. In addition, the phylum *Chlamydiae*, typically found in bioreactors inoculated with aerobic activated sludge, was present under low agitation rates (Estrada et al., 2014). However, the main bacteria present in the cultures enriched at 650 rpm were methanotrophic bacteria. Therefore, a more efficient transfer of CH_4 favored the presence of dominant methanotrophs, while a lower CH_4 effective supply rate promoted the occurrence of non-specialized organisms.

4.2 Methane monoxygenase

Copper is one of the most important factors controlling the relative expression of the two forms of monooxygenases in methanotrophs, sMMO and pMMO, and therefore influences the type of MB enriched (Murrel et al., 2000; Semrau et al., 2010). Previous enzymatic assays have shown that sMMO is synthesized at low Cu^{2+} concentrations (below 0.8 μM, (Semrau et al., 2010)), while *pmoA* is always expressed at a significant level regardless of the Cu^{2+} concentration and such expression increases at increasing copper concentrations (Hakemian and Rosenzweig, 2007; Murrel et al., 2000). However, other studies assessing the influence of copper on MB structure and MMO expression in natural ecosystems did not observe sMMO or type II MB at concentrations as low as 0.1 μM (Chen et al., 2007; Ho et al., 2013; Semrau et al., 2010). Thus, the mechanisms governing both the environmental distribution of both enzymes and the cross-effect of multiple parameters on the type of methanotrophs enriched or the enzyme expression are still unclear. In fact, additional factors such as the inorganic nitrogen concentration have been claimed to determine the type of methanotroph enriched. For example, Type II methanotrophs are often dominant in environments exposed at high CH_4 concentrations and low inorganic nitrogen levels, due to their nitrogen fixing ability (Graham et al., 1993; Li et al., 2014; López et al., 2013). In this study, the high concentration of nitrogen (62 mM) together with the low CH_4 concentrations (<8%) treated could explain the enrichment of type I methanotrophs.

On the other hand, the presence of both *mmoX* and *pmoA* genes in the inoculum confirmed that both enzymes were initially present, which suggests that the three types of methanotrophs were present in the inoculum but were

gradually overcame by type I MB (Fig. 3A), with only the type X *Methylococcus* being detected at high CH_4 and low Cu^{2+} concentrations. pMMO was thus the main enzyme present in the cultures enriched, even at lower Cu^{2+} concentrations than the thresholds set by previous enzymatic studies (Hanson and Hanson, 1996; Murrel et al., 2000; Semrau et al., 2010). This finding could be due to the presence of a Cu^{+2} uptake mechanism, such as that driven by methanobactin, that sensed and concentrated Cu^{2+}, thus supporting the correct activity of pMMO (Kalidass et al., 2015; Semrau et al., 2010). At this point it should be noted that despite some research provided insight into the role of copper using enzymatic and biochemistry studies, real scale operation is far more complex and requires the elucidation of the role of Cu^{2+} on the enzyme expression in natural mixed cultures.

4.3 Biodegradation kinetics

No significant effect of Cu^{2+} concentration on the CH_4 biodegradation rates were reported to date in literature for methane abatement technologies (Estrada et al., 2014; Nikiema et al., 2013). The results obtained in this study showed that differences in Cu^{2+} concentration of 500× supported significant variations on CH_4 biodegradation kinetics. In this sense, the effect of Cu^{2+} on the population was consistent with the changes observed on the biodegradation kinetics parameters. Interestingly, the values of q_{max} at 25 μM of Cu^{2+} were higher than those previously reported in the literature (typically ranging from 4.2×10^{-5} to 1.3×10^{-4} $gCH_4\ g_{biomass}^{-1}\ h^{-1}$ (Bender and Conrad, 1992; Gebert et al., 2003). However, K_s values were within the ranges determined for low-term culture exposure to diluted CH_4 emissions (López et al., 2014). In addition, a higher mass transfer of CH_4 during bacterial enrichment (resulting in a higher CH_4 availability) favored the growth of those communities with lower affinity for CH_4 but with higher specific degradation rates. The superior specific CH_4 biodegradation rates of the communities grown under higher CH_4 transfer rates (650 rpm) was also confirmed by the higher biomass and DNA concentration obtained (Figure 4).

In summary, high copper concentrations and an enhanced methane transfer (higher agitation rate) to the community during microbial enrichment resulted in communities with superior specific methane biodegradation rates (based on a

different specialization of the MB community). Despite the large differences in Cu^{2+} concentration, only Type I methanotrophs were preferentially enriched as confirmed by the absence of sMMO. In addition, the present study demonstrated the key role of enrichment cultivation conditions to develop microbial communities capable of maintaining a high efficiency and robustness, overcoming the limitations of current biotechnologies and improving methane abatement performance.

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Conclusions and future work

Chapter 10

A battery of novel biotechnological strategies was investigated during this thesis with the global aim of enhancing the cost-effectiveness of the treatment of dilute methane emissions from a technical and microbiological perspective. The results obtained opened up a new door of possibilities for methane treatment biotechnologies from a multidisciplinary approach:

Chapter 3 focused on the comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors for methane abatement. This study showed, for the first time, the ability of hydrophobic methanotrophs to grow and maintain their viability inside a non-aqueous phase. Moreover, this work validated a systematic method to enrich hydrophobic bacteria that can be used for the treatment of different hydrophobic toxic compounds. Contrary to what it was expected, the addition of a NAP at high biomass concentrations did not result in an enhancement in methane abatement. According to this study and the controversial results previously found by other authors using two-liquid phase bioreactors for methane treatment, it was concluded that two phase partitioning stirred tank reactors using silicone oil as the NAP and with hydrophobic biomass growing inside the silicone oil are not a cost-effective solution for methane treatment. This might be due to the low affinity of silicone oil for methane, which is only 10 times higher than that of CH_4 for water, as well as the limitation in nutrients or water activity inside the hydrophobic phase, which causes limitations in biological activity and therefore lower removal rates. In this regard, throughout the development of the present thesis, innovative advanced technologies were developed, implemented, and evaluated with a focus on technologies that could make this process environmentally friendly and cost-effective.

These novel technologies were focused on the biological transformation of CH_4 into products with a high profit margin. In this context, a first attempt was carried out in **Chapter 4**, where an innovative strategy for the valorization of dilute methane emissions was assessed, based on the bio-conversion of CH_4 into ectoine by the methanotrophic ectoine-producing strain *Methylomicrobium alcaliphilum 20 Z*. This study demonstrated that a proper selection of the environmental parameters (temperature, CH_4, Cu^{2+} and NaCl concentration) during *M. alcaliphilum 20Z* cultivation was crucial to simultaneously maximize both, the intra-cellular production and excretion of ectoine and CH_4 abatement. In this regard, concentrations of 20 % CH_4, 6 % NaCl, 25 µM Cu^2 and a temperature of 25

ºC supported a maximum intra-cellular ectoine production yield of 66.9 ± 4.2 mg g biomass^{-1}. On the other hand, extra-cellular ectoine concentrations of up to 4.7 ± 0.1 mg L^{-1} were detected at high Cu^{2+}concentrations (50 μM), despite this methanotroph has not been previously classified as an ectoine-excreting strain. These promising results supported further research in order to implement the bio-conversion of CH_4 into ectoine in a continuous system capable of creating value out of GHG mitigation. In this regard, firstly a study of ectoine production during the continuous abatement of diluted emissions of methane by *M. alcaliphilum 20Z* in stirred tank reactors under non-sterile conditions was carried out in **Chapter 5**. NaCl concentration was identified as the main factor influencing the accumulation of intra-cellular ectoine, with high salt concentrations (6% NaCl) inducing average intra-cellular ectoine yields of 37.4 ± 4.2 mg g biomass^{-1} without compromising methane abatement. Moreover, it was observed that process operation at high agitation rates (600 rpm) damaged the bacterial population, with a subsequent decrease in both CH_4 removals and ectoine yields. The promising results obtained in this study, under continuous mode, encouraged further research in order to implement CH_4 biorefineries for the production of ectoine under a fed-batch "bacterial milking" approach similar to the one currently used in the industry for ectoine production. "Bacterial milking" consists of a fermentation technology procedure for large-scale internal metabolite extraction varying NaCl concentrations to firstly promote ectoine biosynthesis at high salinities, and secondly to induce the excretion of ectoine from the cell to the cultivation broth at low salinities, where the product is collected for its downstream purification. Following this idea, **Chapter 6** showed, for the first time, that the methanotrophic strain *M. alcaliphilum 20Z* exhibited a rapid response to osmotic shocks under batch and in continuous modes, which resulted in the release of the accumulated ectoine under hyposmotic shocks and the immediate uptake of the previously excreted ectoine during hyperosmotic shocks. The ectoine yield obtained in this study, after determining the best operational conditions in the previous studies, exhibited a constant high intra-cellular concentration of 70.4 ± 14.3 mg g biomass^{-1} along the entire operation period and constituted the first proof of concept of ectoine *bio-milking* coupled to CH_4 abatement from diluted emissions.

Although at this point the prospect of a near future methane based bio-refinery was promising, the implementation of these CH_4 bioconversion processes was still scarce due to the occurrence of physical and biological limitations. Thus, the low aqueous CH_4 solubility limited the gas-liquid mass transport of this greenhouse gas and entailed low growth rates during *M. alcaliphilum* 20Z cultivation, while its fragility against mechanical shear stress hampered the process productivities obtained. In this context, to improve both the mass transfer of CH_4 to the methanotrophic community as well as the cost-effectiveness of the process, a bubble column bioreactor configuration supporting multiple metabolite co-production (ectoine, hydroxyectoine, bioplastics and exopolyshaccarides) was evaluated using the pure strain *M. alcaliphilum* 20Z and an enriched methanotrophic consortium in **Chapter 7**. In this study, higher concentrations of ectoine were accumulated than in the previous studies, likely due to a superior mass transfer of methane from the gas to the liquid phase. On the other hand, the enriched methanotrophic consortium was capable of accumulating the same concentration of ectoine than *M. alcaliphilum*, in addition to co-producing high quantities of hydroxyectoine and EPS. Nevertheless, bioplastics were not obtained in either conditions because there was not a fasting period that allowed the bacterial synthesis of PHAs.

Additionally, novel methanotrophic extremophiles were isolated in **Chapter 8** with the aim of optimizing methane bioconversion into high added value products. In this regard, two genera that were not previously identified as methanotrophic bacteria (*Alishewanella* and *Halomonas*) were demonstrated to efficiently degrade methane. Due to a dissimilarity in the 16SrDNA sequences with their closest relatives of at least 98 %, they were described as two novel strains (representing novel species): *Alishewanella sp.* strain RM1 and *Halomonas sp.* strain PGE1. *Halomonas sp.* strain PGE1 presented higher ectoine yields (70.2 to 91.7 mg ectoine g biomass^{-1}) than those generally obtained by *M. alcaliphilum* 20Z (37.4 to 70.0 mg ectoine g biomass^{-1}), although *Halomonas sp.* strain PGE1 seems to be inhibited by the production of a secondary toxic metabolite for bacterial growth. In this context, medium replacement could eventually overcome this process limitation and ultimately support high ectoine productivities and CH_4 removal efficiencies.

A final study was carried out to increase the efficiency of the process through the improvement of the physiology as well as the biochemistry of the microbial community. In

this context, a study investigating the role of copper and CH_4 concentration during culture enrichment on the microbial community structure and biodegradation kinetics of methanotrophic cultures was conducted in **Chapter 9**. In this study, no clear effect of CH_4 concentration on the population structure or on the biodegradation kinetics of the communities enriched was recorded. However, it was determined that high copper concentrations resulted in communities with superior specific methane biodegradation rates (based on a different specialization of the methanotrophic community). Overall, this work demonstrated the key role of environmental conditions during culture enrichment to develop microbial communities capable of maintaining a high efficiency and robustness during CH_4 abatement, overcoming some of the current limitations of state-of-the art biotechnologies.

Despite the advances achieved in this thesis towards the widespread implementation of biological technologies for CH_4 treatment, their optimization in terms of enhanced gas-liquid mass transfer and co-production of multiple commercial bioproducts entails niches for future research. Based on the outcomes and limitations encountered throughout the course of the present thesis, the following future lines of research in the field are proposed:

- The investigation in new NAPs with higher affinities for CH_4 than silicone oil, and the elucidation of the differential microbial growth and specific strategies that enable some methanotrophs to be viable inside the NAP will be of major interest for the optimization of TPPBs with the biocatalytic activity confined in the NAP.
- The performance of new bioreactor configurations, such as suspended-growth membrane diffusion and pressurized bioreactors, which operate at low-moderate energy demands, must be explored in order to improve the mass transfer of methane and subsequently the cost-competitiveness of CH_4 biorefineries. In addition, the performance of these innovative high-mass transfer bioreactors can be boosted via internal gas recirculation, which allows decoupling the $K_la_{G/A}$ from the overall gas residence time.
- The standardization of culture conditions to maximize the synthesis of a target bioproduct must be further studied. For instance, the effect of adding co-substrates, such as methanol, during methane bioconversion must be investigated in order to tailor

the characteristics of the bioproducts synthesized and to obtain better productivities, as well as higher bioproduct concentration.

- The use of new "omics" will be of the utmost importance for a better understanding of the microbial ecology and its role in the methane bioconversion processes, for improving the enzymatic activation and function, as well as for further studying the novel isolated methanotrophs with ability to synthesize bioproducts of commercial interests.
- Ectoine production from methane and its further downstream must be evaluated at pilot scale in order to confirm its cost-competitiveness based on the promising results obtained at laboratory scale.
- The potential of biogas to support ectoine production must be also explored based on the widespread availability of this point source CH_4-laden residual streams.

About the author

Chapter 11

Sara Cantera has always considered herself a person with a high sense of environmental awareness, which led her to initiate an academic career with the main goal of finding different biotechnological alternatives that minimize the environmental destruction of the planet, and that directly affects social and animal welfare.

Sara Cantera obtained a Degree in Biology at the Autonomous University of Madrid (a 5-year program equivalent to BS+MS) with a specialization in Molecular Biology in 2010. Between 2010 and 2011, she worked for 1 year at Prof. María Ángeles Martín-Cabrejas's lab at the Department of Agricultural Chemistry and Food Science of the Autonomous University of Madrid. In September 2011, she started a master's program in Environmental Microbiology at the Autonomous University of Madrid. She completed her master's thesis at the Civil Engineering School of the University of Cantabria, supervised by Prof. Iñaki Tejero and Prof. José Luis Sanz in 2012, where she developed her expertise in reactor operation and biological waste treatments. In January 2013, Sara moved to Scotland and carried out animal care volunteering in several centers until December 2013.

In February 2014, Sara joined the VOCs and Odours Treatment Group headed by Professor Raúl Muñoz in the Environmental Technology Research Group (Department of Chemical Engineering and Environmental Technology - University of Valladolid). During this time, she was awarded a JCyL pre-doctoral Grant by the Spanish Ministry of Science and Innovation (October 2014-October 2018). Since 2014, her PhD studies adopted a multidisciplinary approach combining environmental engineering, biology and biotechnology. These approaches were implemented throughout the set up and operation of reactors, microbial studies and research about the possible paths towards an effective CH_4 destruction or bioconversion into valuable bio-products. Sara also carried out a 3-month research stay at the Department of Agrotechnology and Food Sciences (Microbiology subdivision) of Wageningen University (2016, The Netherlands) under the supervision of Dr. Irene Sánchez-Andrea and Dr. Alfons Stams.

Scientific Production

Publications in peer review journals

10. **Cantera S, Sánchez-Andrea I, Lebrero R, García-Encina P.A, Stams S.J.M, Muñoz R (2018).** *Novel haloalkalophilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery.* Waste Management (Under revision).

9. **Cantera S, Muñoz R, Lebrero R, Lopez J.C, Rodríguez, Y, García-Encina P (2017).** *Technologies for the bioconversion of methane into more valuable products*. Current Opinion in Biotechnology 50: 128-135.

8. **Cantera S, Lebrero R, Rodríguez S, García-Encina P, Muñoz R (2017).** *Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-refineries into high added-value products***.** Chemical Engineering Journal 328: 44-48.

7. **Frutos O, Cortes I, Cantera S, Arnaiz E, Lebrero R, Muñoz R (2017).** *Nitrous oxide abatement coupled with biopolymer production as a model GHG biorefinery for cost-effective climate change mitigation*. Environmental Science & Technology 51 (11), 6319-6325.

6. **Toledo-Cervantes A, Madrid-Chirinos C, Cantera S, Lebrero R, Muñoz R (2017).** *Influence of the gas-liquid flow configuration in the absorption column on photosynthetic biogas upgrading in algal-bacterial photobioreactors***.** Bioresource Technology 225: 336-342.

5. **Cantera S, Lebrero R, Rodríguez E, García-Encina P, Muñoz R (2017).** *Continuous abatement of methane coupled with ectoine production by Methylomicrobium alcaliphilum 20Z in stirred tank reactors: a step further towards greenhouse gas biorefineries.* Journal of Cleaner Production 152:134-151.

4. **Cantera S, Sadornil L, Lebrero R, García-Encina P, Muñoz R (2016*).*** *Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement*. Journal of Environmental Management 182: 160-165.

3. **Cantera S, Lebrero R, García-Encina P.A, Muñoz R (2016).** *Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures*. Journal of Environmental Management 171 (15): 11-20.

2. **Cantera S, Estrada J.M, Lebrero R, García-Encina P, Muñoz R (2015).** *Comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors*

for methane abatement: Mass Transfer and Biological Considerations. Biotechnology and Bioengineering 113(6): 1203-1212.

1. **Benítez V, Cantera S, Aguilera Y, Mollá E, Esteban R, Díaz M.F, Martín-Cabrejas M.A (2012).** *Impact of germination on starch, dietary fiber and physicochemical properties in non-conventional legumes.* Food Research International 50: 64–69.

- In preparation

Cantera S, Sánchez-Andrea I, Lebrero R, García-Encina P.A, Muñoz R (2018). *Multi-production of high added market value metabolites from methane emissions by an halotolerant methanotrophic consortia. bio-reactors:* In preparation.

Book Chapters

1. **Cantera S, Frutos O.D, López J.C, Lebrero R, Muñoz R (2017).** *Technologies for the bio-conversion of GHGs into high added value products: Current state and future prospects.* Carbon foot print and the Industrial Life cycle.Green Energy and Technology book. Ed. Springer International Publishing AG2017, 359-388.

- In preparation

Lebrero R, Estrada J, Pérez V, Cantera S, Frutos O.D, Muñoz R (2018). *Biological treatment of gas pollutants in partitioning bioreactors.* Advances and Applications of Partitioning Bioreactors book (volume 54). Advances in Chemical Engineering. Ed. El Sevier books. In preparation.

Conference participation

Oral Presentation

Cantera S, Lebrero R, García-Encina P, Muñoz R. *Valorization of methane emissions into the high-added-value products: assessing the effect of Cu^{2+}, temperature, NaCl and CH_4 concentration on ectoine.* 3rd IWA Specialized International Conference Ecotechnologies for Wastewater Treatment 2016 (ecoSTP16). June 27-30th, 2016. Cambridge, UK.

Cantera S, Lebrero R, García-Encina P, Muñoz R. *Evaluation of methane and copper concentration and CH_4 mass transport on the community structure and the*

biodegradation kinetics in methanotrophic cultures. The 6th international conference on biotechniques for air pollution control. September 2-4th, 2015. Ghent, Belgium.

Díez R, De Florio L, Moreno-Ventas X, Herrero M, Pérez P, Cantera S, Tejero I. *Novel anoxic-anaerobic reactor followed by hybrid membrane bioreactor for biological nutrient removal*. *IWA Nutrient Removal and Recovery*. 23-25 September 2012. Harbin, China.

Benítez V, Cantera S, Aguilera Y, Mollá E, Esteban R, Díaz M.F, Martín-Cabrejas M.A. *Evaluación de factores antinutricionales en legumbres no convencionales: Efecto del proceso de germinación.* VII Congreso internacional de nutrición, alimentación y dietética y XV Jornadas Nacionales de nutrición práctica. March 30th to April 1st, 2011. Madrid, Spain.

Martín-Cabrejas M.A, Benítez V, Cantera S, Aguilera Y, Mollá E, Díaz M.F. *Modificaciones de los constituyentes de leguminosas como consecuencia de los procesos Biotecnológicos*. III Congreso de Producción Animal Tropical. 15-19 November 2010. La Habana, Cuba.

Poster participation

Cantera S. *Continuous abatement of methane coupled with ectoine production by Methylomicrobium alcaliphilum20Z: A step further towards GHG biorefineries.*7th congress of European Microbiologists (FEMS 2017). July 9-13th, 2017.Valencia, Spain.

Fellowships

2017 International Mentor Program 2017-18 of IMFAHE Foundation.

2017 Travel and Conference grant for the participation in international conference (UVA fellowship).

2016 Research internship grant to strengthen international cooperation (UVA-Wageningen fellowship).

2015 Travel and Conference grant for the participation in international conference (UVA fellowship).

2014 JCyL-Predoctoral researcher Fellowship. Spanish Research Council (ORDEN EDU/1083/2013).

Research Projects

Participation

2015-18 Biogas Bioconversion to Commodities and HIGH-Added value products: Exploring new strategies for biogas valorization. Spanish Ministry of Economy and Competitiveness (CTM2015-70442-R project). PI: Raúl Muñoz & Raquel Lebrero.

2014-16 Advanced biological processes for the abatement of the greenhouse gases CH_4 and N_2O: targeting the direct gas-cell mass transport and process microbiology. Spanish Ministry of Economy and Competitiveness (CTQ2012-34949 project). P.I: Raúl Muñoz

2012 Red NOVEDAR- CONSOLIDER. Spanish Ministry of Economy and Competitiveness CSD 2007-00055. P.I: Iñaki Tejero Monzón (UNICAN). P.I coordinator: Juan Lema Rodicio.

2010-11 AECID: Incorporación de germinados de *Vigna unguiculata* en piensos para producción animal-A/030613/10. P.I: María Ángeles Martín Cabrejas.

Teaching and student mentoring

2018 Co-supervisor of a Master Thesis in the Master in Environmental Engineering of Valladolid University. Student: Enrique Regidor.

Thesis title: *Production of ectoine from biogas: A new approach*.

2017 Lecturer in the cord course "Atmospheric Pollution Prevention and Control" at the Master in Environmental Engineering of Valladolid University (12 h teaching in the academic year 2017-2018).

2017 Co-supervisor of an international researcher from Cyprus University of Technology (Cyprus) from the laboratory of Ioannis Vyrides: Student Evangelia Panagiotou. Thesis title: *Generation of multiple high performance biomaterials from methane in bio-reactors: Comparative performance evaluation of Methylomicrobium alcaliphilum 20Z and an halotolerant methanotrophic consortia*.

2017 Co-supervisor of a Master Thesis in the Master in Environmental Engineering of Valladolid University. Student: Suní Rodríguez. Thesis title: *Generation of multiple high performance biomaterials from methane in bio-reactors: Comparative performance evaluation of Methylomicrobium alcaliphilum 20Z and an halotolerant methanotrophic consortia*.

2017 Co-supervisor of an undergraduate student Final Degree Project in the Chemical Engineering Degree at Valladolid University. Student: Lidia Sadornil. Title: *Alcalophilic and halophilic methanotrophic bacteria*.

2016 Co-supervisor of an undergraduate student Final Degree Project in the Chemical Engineering Degree at Valladolid University. Student: Suní Rodríguez. Thesis title: ***Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-refineries into high added-value products***.

2016 Co-supervisor of an undergraduate student internship. Student: Lidia Sadornil. Title: *Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement*.

Outreach activities

Workshops and Conferences committee

- **Workshop-** How to Attract Funding from USA to Spain. February 2nd, 2018. Harvard University (IMP-program).
- **Workshop-**How to write an effective Personal statement to be admitted on an Ivy League University. December 12th, 2017. Harvard University (IMP-program).
- **Workshop-** Building worlwide networks to succeed in Science and Education. December 5th, 2017. Harvard University (IMP-program).
- **Member of the Organizing Committee** "Caracterización y Gestión de Olores y gases de efecto invernadero en EDARs". October 5th, 2015. Valladolid, Spain.

Long-term Research Stays

2015 Laboratory of Microbiology at Wageningen University, The Netherlands (12 weeks).

Peer-review in ISI Web of Knowledge journals

Journal of Environmental Management.

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