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Multi-production of high added market value metabolites from diluted methane emissions via methanotrophic extremophiles

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ABSTRACT

This study constitutes the first-proof-of-concept of a methane biorefinery based on the multi-production of high profit margin substances (ectoine, hydroxyectoine, polyhydroxyalkanoates (PHAs) and exopolysaccharides (EPS)) using methane as the sole carbon and energy source. Two bubble column bioreactors were operated under different magnesium concentrations (0.2, 0.02 and 0.002 g L^{-1}) to validate and optimize this innovative strategy for valorization of CH_4 emissions. High Mg^{2+} concentrations promoted the accumulation of ectoine (79.7–94.2 mg g biomass^{-1}), together with high hydroxyectoine yields (up to 13 mg g biomass^{-1}) and EPS concentrations (up to 2.6 g L culture broth^{-1}). Unfortunately, PHA synthesis was almost negligible (14.3 mg L^{-1}) and only found at the lowest Mg^{2+} concentration tested. *Halomonas, Marinobacter, Methylophaga* and *Methylomicrobium*, previously described as ectoine producers, were dominant in both bioreactors, *Methylomicrobium* being the only described methanotroph. This study encourages further research on CH_4 biorefineries capable of creating value out of GHG mitigation.

1. Introduction

Methane (CH_4) is currently the second most important greenhouse gas (GHG) as a result of its high global warming potential (85 times higher than that of CO_2 over a 20-y window) and emission rates. CH_4 can be used as an energy vector when its concentration in the gas emission is higher than 30%. However, more than 56% of anthropogenic CH_4 emissions worldwide contain concentrations lower than 4% (European Environment Agency, 2015) which are not suitable for energy recovery. When applied to these diluted gas emissions (such as off-gases from landfills, manure storage tanks, cattle operation and ventilated coal mines), state-of-the-art biological treatment technologies for CH_4 abatement are still not cost-effective (EPA, 2017). The most important and common limitation of biological methane abatement is caused by the low aqueous solubility of this GHG (Dimensionless Henry's law constants (H) = 30 at 25 °C). 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 containing the biomass and therefore, in a reduced GHG biodegradation performance. Hence, this low CH_4 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. 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 Environment Agency, 2015). Of these novel strategies, the bioconversion of CH_4 into high added value products using a bio-refinery approach has emerged as one of the most promising ones (Khmelenina et al., 2015; Strong et al., 2016a,b). In this regard, CH_4-laden emissions can be used by methanotrophs to synthesize essential compounds that cells produce under stress conditions to survive and that have high market value, such as biopolymers, exopolysaccharides or ectoines, turning CH_4 emissions abatement into a sustainable and profitable process. The content of some of these metabolites are governed by the CH_4 concentration in the gas phase. Thus, Cantera et al. (2016b) showed that an increase in the gas CH_4 concentration from 2 to 20% increased ectoine content in *Methylomicrobium alcaliphilum* by a factor of 2.

Ectoine and its hydroxylated derivative (hydroxyectoine) are one of the most profitable bioproducts synthesised by microorganisms. These metabolites are produced by bacteria to resist salinity stress. And they retail in the pharmaceutical industry at approximately US$1000 kg^{-1}, due to their properties as protein DNA-protein complexes and nucleic acids stabilizers (Pastor et al., 2010). Recent studies have demonstrated that *Methylomicrobium alcaliphilum 20Z*, an alkalophilic and halotolerant methanotroph, is able to produce ectoine at 37–70 mg L^{-1} under

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continuous CH_4 fermentation (Cantera et al., 2017a). However, the ectoine productivities obtained in bioreactors by methanotrophs are still low in comparison with those reported for heterotrophic bacteria typically used in the industry (Pastor et al., 2010). Moreover, the halotolerant methanotrophs discovered to date are sensitive to stress by agitation, which hampers the mass load of CH_4 to the microbial community in stirring tanks (Cantera et al., 2017a). Finally, it should be stressed that hydroxyectoine accumulation by methanotrophs has never been reported to date (Reshetnikov et al., 2011). Polyhydroxyalkanoates (PHAs) are intracellular biopolyesters produced by microorganisms under nutrient-limiting conditions (i.e N-, P- or Mg-limitation) as a carbon and energy storage resources (Castilho et al., 2009). Their outstanding mechanical properties, along with their biodegradability and biocompatibility, turns PHAs into an attractive and potential alternative to oil-based plastics (Chen et al., 2015; Strong et al., 2016a,b). Methanotrophic bacteria are able to reach PHAs accumulations ranging from 20 up to 50% (on a dry weight basis) in suspended growth bioreactors operated under batch (Pieja et al., 2012; Zhang et al., 2017) and continuous mode (Rahnama et al., 2012). On the other hand, extracellular polysaccharides (EPS) constitute another potential high profit margin bioproduct resulting from CH_4 biorefineries. These biopolymersre formed by a wide variety of proteins, glycoproteins, glycolipids and polysaccharides (Flemming and Wingender, 2010). EPS are typically excreted by bacteria under stress conditions as a protective barrier or water retainer. These metabolites are taking interest in the food, pharmaceutical and textile industries due to their colloid and adhesive properties, and their effects on liquid rheology (Nwodo et al., 2012). To date, some studies have demonstrated that methanotrophic bacteria are able to synthesize EPS in the range of 0.03–0.43 g $g_{biomass}^{-1}$ (Malashenko et al., 2001).

In this context, the present study represents an assessment of the potential of the continuous bioconversion of CH_4 into multiple added-value products (i.e ectoine, hydroxyectoine, PHAs and EPS) as an innovative strategy for the valorization of diluted CH_4 emissions and therefore to overcome the lack of cost-effectiveness this process. For this purpose, a systematic comparison of the performance of two bubble column bioreactors inoculated with haloalkaliphilic methanotrophs, a pure strain *M. alcaliphilum* 20Z and an enriched haloalkaliphilic consortium, was performed. The influence of magnesium (Mg^{2+}) concentration on the synthesis of the target bioproducts and on the structure of the bacterial communities was evaluated.

2. Materials and methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used for the cultivation of the haloalkaliphilic methanotrophs in this study was a high pH (9.0) and high-salt content (6% NaCl) medium recommended for the enrichment of methane oxidizing bacteria from soda lakes (Kalyuzhnaya et al., 2008). Magnesium was supplemented to the MSM in the form of $MgSO_4$ at the 3 concentrations tested (0.2 (C1), 0.02 (C2), 0.002 (C3) g L^{-1}). The limitation of Mg^{2+} in C2 and C3 was carried out with the aim of increasing PHAs production according to Khanna and Srivastava (2005). Nitrogen limitation was not tested due to the negative effect on ectoine and hydroxyectoine production. 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 inocula preparation

Reactor 1 (R1) was inoculated with a pure strain of *M. alcaliphilum 20Z* acquired from DSMZ (Leibniz-Institut). A 10× dilution of the *M. alcaliphilum 20Z* stock culture from DSMZ was grown at 25 °C in 120 mL sterile glass bottles containing 40 mL of MSM at 0.2 g $MgSO_4$ L^{-1}. The bottles were closed with gas-tight butyl septa and aluminum caps, and 50% (v/v) of the air headspace was replaced by CH_4. The inoculum, which was grown up to a biomass concentration of 0.1 ± 0.06 g, was transferred to two sterile gas-tight glass bottles (1.2 L) closed with butyl septa and plastic screw caps, and containing 180 mL of MSM at 0.2 g $MgSO_4$ L^{-1} (20 mL per bottle) prior reactor inoculation. CH_4 was injected to obtain a CH_4 headspace concentration of 55.0 ± 6.2 g CH_4 m^{-3}. The agitation was set at 600 rpm and the temperature at 25 °C.

Reactor 2 (R2) was inoculated with an enrichment of haloalkaliphilic bacteria able to grow using methane as the only external carbon and energy source. Fresh activated sludge from a wastewater treatment plant with seawater intrusion (Cantabria, Spain) and fresh cow manure and soil from a dairy farm on the coastline of Cantabria (Spain) were used as inoculum for the enrichment. Culture enrichment was performed in two sterile gas-tight glass bottles (1.2 L) containing 190 mL of medium at 0.2 g $MgSO_4$ L^{-1} inoculated with 10 mL of the cow manure-soil mixture and 10 mL of the activated raw sludge. The bottles were closed with butyl septa and plastic screw caps, and CH_4 was injected to obtain a CH_4 headspace concentration of 55.1 ± 2.7 g CH_4 m^{-3}. The enrichments were transferred 7 times to fresh medium bottles upon CH_4 depletion using 10% inoculum aliquots. The agitation was set at 600 rpm and the temperature at 25 °C.

2.3. Experimental set-up and operating conditions

Two 2.0 L bubble column reactors (Afora S.A., Spain) with ultra-fine bubble diffusers were used for continuous CH_4 abatement combined with the co-production of ectoines, EPS and PHAs. These bioreactors can enhance the mass transfer of methane to the aqueous phase without compromising the cell growth due to mechanical stress (Cantera et al., 2017a). The influence of three different Mg^{2+} concentrations (0.2 g L^{-1}, 0.02 g L^{-1} and 0.002 g L^{-1}) on the production of the above mentioned bioproducts was assessed in both reactors during stages C1, C2 and C3, respectively. R1 was inoculated with *M. alcaliphilum 20Z* at an initial concentration of 520 mg L^{-1}, while R2 was inoculated with the haloalkaliphilic bacteria enrichment at 500 mg L^{-1}. A 0.060 L min^{-1} CH_4-air emission containing 25.6 ± 2.1 g CH_4 m^{-3} (methane load of 46.1 ± 2.2 g m^{-3} h^{-1} and aqueous concentration of 0.8 ± 0.1 g m^{-3}) was fed into R1 and R2 via three diffusers (10 µm porous) situated 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 in the reactors. Both reactors were operated at 25 °C and a pH of 9.0 ± 0.3, which was maintained via daily replacement of 50 mL of MSM to wash out toxic compounds and avoid nitrogen limitation. These cultivation broth aliquots were used for the determination of the concentrations of biomass (measured as total suspended solids (TSS)), ectoine, hydroxyectoine, EPS and PHAs. Gas samples to determine the concentrations of CH_4 and CO_2 were also daily measured with gas-tight syringes (HAMILTON, USA) from the inlet and outlet of the bioreactors. The elimination capacity (EC, g m^{-3} h^{-1}) and removal efficiency (RE, %) of the column reactors were calculated using the following equations:

$$EC = \frac{([CH_4]_{IL} - [CH_4]_{out}) \cdot Q}{V}$$

$$RE = \frac{([CH_4]_{IL} - [CH_4]_{out})}{[CH_4]_{IL}} \cdot 100$$

A steady state operation was considered when the EC and RE deviated < 10% from the mean.

A mass transfer test was developed under steady state at the end of C1, C2 and C3 in order to elucidate the limiting step during CH_4 biodegradation under the experimental conditions evaluated. For this purpose, the inlet CH_4 concentration was increased from 25.6 ± 2.1 g m^{-3} to 52.1 ± 3.6 g m^{-3} for a period of 4 h, and the