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Optimization of CH_4 Removal from Diluted Emissions and Continuous PHB Production by *Methylocystis hirsuta:* Towards GHG Biorefineries

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This work aimed at optimizing the removal of diluted CH_4 emissions (4 % *v/v*) by decoupling the actual gas residence time from the overall empty bed residence time (EBRT) in a continuous bubble column bioreactor (BCB) inoculated with *Methylocystis hirsuta*. Different EBRTs (120, 60, 30 and 15 min) were tested along with six internal gas-recycling ratios (Q_R/Q = 0, 2, 3, 6, 10 and 15, where Q_R is the recycling gas flow rate and Q the inlet gas flow rate treated). The results showed a CH_4 elimination capacity of 35.2 ± 0.4 g m^{-3} h^{-1} (RE=72.9 ± 0.5 %) at an EBRT of 30 min and Q_R/Q of 15, which was identified as the optimum operating conditions in terms of CH_4 abatement. The production of poly-3-hydroxybutyrate (PHB) was initially evaluated batchwise, in experiments limiting micro and macro nutrients such as K, Mn, N, and N with excess of Fe. Nitrogen starvation resulted in the highest PHB accumulation (28.0 ± 1.2 %) batchwise. Based on these results, nine sequential N feast-famine cycles were implemented in the BCB operating at an EBRT of 30 min and a Q_R/Q of 15. N starvation resulted in a gradual increase in the PHB content from 0.4 ± 0.0 % to 25.7 ± 0.1 % in the first cycle and up to 37.2 ± 2.0 % from the fifth cycle onwards. PHB productivities remained roughly constant during operation under N feast-famine cycles at 1.82-2.23 kg m^{-3} d^{-1}, which corresponded to specific PHB productivities ranging from 15.9 to 21.6 mg PHB gX^{-1} h^{-1}.

1. Introduction

Climate change is a global environmental problem prioritized by different governments around the world due to its high impact in areas such as economy, health and environment. This phenomenon is attributed mainly to the emissions of greenhouse gases (GHG) such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and chlorofluorocarbons (CFC), which are mainly emitted by anthropogenic activities and in a minor extent by natural sources (Miphokasap, 2017). In this context, CH_4 emissions are considered one of the major causes of climate change, contributing to 30% of the current net global warming. CH_4 has a global warming potential 25 times greater than CO_2 in a 100 years horizon (Hoo et al. 2017) and its concentration is expected to increase based on the methane leaks associated with the increasing extraction of shale gas. CH_4 is emitted in a wide range of concentrations from many different sources. In this context, CH_4 emissions can be classified in three categories: high purity (>90%), medium purity (5-75%) and diluted (<5%). Diluted methane emissions are of major concern since they are not suitable for energy recovery (Kim et al. 2013). In this regard, the treatment of diluted CH_4 emissions is crucial since they represent > 55 % of the anthropogenic CH_4 emissions released into the atmosphere (Cáceres et al. 2017).

Some technologies such as biofiltration have been developed for abating dilute CH_4 emissions, but low removal efficiencies are typically achieved in this bioreactor configuration (Cáceres et al. 2017). Therefore, new bioreactor configurations have been engineered to effectively eliminate CH_4 in a more cost-effective way (Rahnama et al. 2012). Of them, some configurations allow the synthesis of bioproducts of commercial interest such as proteins, exopolysaccharide and bioplastics (i.e poly-3-hydroxybutyrate) during CH_4 abatement (Pieja et al. 2011). In this context, methanotrophs are a group of bacteria that contribute to CH_4 abatement while producing polymers with similar properties to conventional plastic. In particular, type II methanotrophs such as *Methylocystis hirsuta* have been reported to degrade CH_4 and accumulate bioplastics under nutrient limitation during batch fermentation (Khosravi-Darani et al. 2013). This innovative CH_4 abatement approach has resulted in the concept of CH_4 biorefinery, which has been lately extended to the co-production of ectoine and other added-value bioproducts (Kajaste 2014; Khosravi-Darani et al. 2013). The aim of the present research was to evaluate the effect of internal gas recycling on CH_4 abatement and on the continuous PHB co-production under nutrients limitation in a bubble column bioreactor.

2. Material and methods

2.1 Mineral medium and inoculum

The mineral salt medium (MSM) composition used to prepare the inoculum was (g L^{-1}): 2.25 $NaNO_3$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.02 $CaCl_2 \cdot 2H_2O$, 0.68 KH_2PO_4, 6.14 $Na_2HPO_4 \cdot 12H_2O$, 1.3 × 10^{-3} $FeSO_4 \cdot 7H_2O$, 3.5× 10^{-3} $MnCl_2 \cdot 4H_2O$, 1.5× 10^{-3} $ZnSO_4 \cdot 7H_2O$, 0.04 × 10^{-3} $Na_2MoO_4 \cdot 2H_2O$, 0.04× 10^{-3} $CuSO_4 \cdot 5H_2O$, 0.32 × 10^{-3} $CoCl_2$, and 0.2 × 10^{-3} H_3BO_3, which was modified from Mokhtari-Hosseini et al. (2009). *M. hirsuta* (DSMZ 18500) inoculum was prepared using a 33%:67% (*v/v*) CH_4:O_2 headspace in sterile 120 mL gas-tight serum bottles incubated at 25 °C and 250 rpm for 48 h, and finally transferred to sterile 1.25 L gas tight serum bottles made-up with sterile MSM in a liquid volume of 200 mL, and incubated at 25º C and 600 rpm to a final total suspended solid concentration (TSS) of 295 ± 16 mg L^{-1}.

2.2 Experimental set up

The experimental system consisted of a PVC bubble column bioreactor (BCB) with 2.5 L of working volume (0.6 m high and 0.08 m of internal diameter) and a 1-L jacketed condenser cooling the internal gas-recycling line (García-Pérez et al. 2018).The CH_4-air stream (4% (*v/v)*) was sparged at the bottom of the BCB using three 0.5 µm-pore size metallic diffusers. The CH_4 stream was supplied via a mass flow controller (Aalborg). The start-up phase was carried out for 13 days at 60 min of EBRT and an initial biomass concentration of 194 ± 4 mg L^{-1}. The optimization of the CH_4 abatement performance in the BCB was performed via investigation of the influence of the empty bed residence time (EBRT = 120, 60, 30 and 15 min) and internal gas-recycling ratios (Q_R/Q = 0, 2, 3, 6, 10, 15, where Q_R is the recycling gas flow rate and Q the gas flowrate fed to the overall system). 500 mL of cultivation broth were drawn every 48 h, centrifuged at 10000 rpm for 7 min, and the biomass pellet was re-suspended in fresh 500 mL MSM and returned to the BCB to ensure an optimum nutrients balance, a stable pH and the absence of microbial activity limitations in the BCB.

2.3. Batch PHB accumulation under micro/macro nutrient limitation and continuous CH_4 abatement and PHB co-production

The influence of limitations in the concentration of N in the presence of low and high Fe^{2+} concentrations, K and Mn on PHB accumulation and CH_4 biodegradation by *M. hirsuta* cultures was evaluated batchwise. The tests started with a growth phase of 15 days in MSM followed by a PHB accumulation phase of 10 days under nutrient limiting conditions. The assays were carried out in duplicate in 2 L gas-tight serum bottles containing 400 mL of MSM inoculated with a biomass concentration of 128 ± 17 mg L^{-1}. CH_4 was then added to the headspace both in the growth and accumulation stages at a concentration of 193 ± 7 g m^{-3} (32.5 ± 1.1 % *v/v*) in a pure O_2 atmosphere. The biomass was centrifuged at the end of the growth phase and re-suspended in the corresponding nutrient-limited MSM prior to the accumulation phase. Control tests with the original MSM were conducted. The CH_4 and CO_2 concentrations in the headspace, and the biomass in the cultivation broth, were periodically monitored throughout the 25 days of experiment while PHB concentrations were monitored throughout the nutrient limitation tests.

PHB co-production was evaluated under continuous CH_4 supply in a BCB operated at an EBRT of 30 min, internal gas-recycling rate of 0.50 m^3_{gas} $m^{-3}_{reactor}$ min^{-1} and N-limitation (identified previously as the optimal operational conditions at an inlet load of 49.8 ± 11.8 g CH_4 m^{-3} h^{-1}). The BCB was inoculated with *M. hirsuta* at an initial biomass concentration of 152 ± 1 mg L^{-1} and operated under nutrient-sufficient condition until reaching a biomass concentration of 4.4 ± 0.6 g TSS L^{-1}. The continuous co-production of PHB during CH_4 abatement was evaluated with nine sequential N-limitation cycles. MSM was added at the BCB with and without nitrogen at a dilution rate (D) of 0.1 d^{-1} during the feast (1 day) and famine (2 days) periods,

respectively. N concentration in the N-supplemented MSM was adjusted to 61 ± 8 mg N L^{-1} during the limitation cycles. The inlet and outlet CH_4 and CO_2 gas concentrations were daily monitored. Likewise, 20 mL liquid samples were daily withdrawn to determine the OD_{600}, pH, TN concentration and PHB content.

2.3 Analytical methods

The concentrations of CH_4 and CO_2 were daily measured in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A column (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q column (25 m × 0.53 µm × 10 µm). The temperatures of the oven, injector and detector were maintained at 45 °C, 150 °C and 200 °C, respectively, using Helium as the carrier gas at 13.7 mL min^{-1}. TSS concentration was determined according to standards methods (APHA, 2005). Culture absorbance was measured at 600 nm using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). Total nitrogen (TN) concentration was quantified following sample filtration (0.45 µm) in a TOC-VCSH analyzer (Shimadzu, Japan) coupled with a chemiluminescence detection TN module (TNM-1) (Shimadzu, Japan). PHB accumulation was quantified in a GC-MS (Agilent Technologies: GC System 7820A MSD 5977E, Santa Clara, USA) equipped with a DB-wax column (30 m × 250 µm × 0.25 µm) according to López et al. (2014). The pH, TSS and TN concentrations were determined every 48 h. The elimination capacity (EC), removal efficiency (RE), CO_2 production rate (PCO_2), PHB content and PHB productivity were calculated according to Zúñiga et al. (2011).

3. Results and discussion

3.1 Influence of the EBRT and internal gas-recycling rate in the BCB on CH_4 biodegradation

The influence of the EBRT (120 and 60 minutes) on CH_4 abatement was evaluated. The results showed that at 120 min without internal gas-recycling the EC was 4.7 ± 0.48 g m^{-3} h^{-1}. The EC increased when increasing the Q_R/Q ratio tested (gas-recycling rates of 0.02, 0.03, 0.05, 0.08 and 0.13 m^3 m^{-3} min^{-1}). Similarly, higher gas-recycling rates resulted in concomitant increases in RE and PCO_2. Likewise, process operation at an EBRT of 60 min in the absence of internal gas-recycling resulted in ECs of 8.5 ± 0.3 g m^{-3} h^{-1} and PCO_2 of 12 ± 0.9 g m^{-3} h^{-1}. The increase in the gas-recycling ratio to 2, 3, 6, 10, and 15 resulted in ECs increases up to a maximum EC (EC_{max}) of 18.7 ± 0.2 g m^{-3} h^{-1} (RE = 75 ± 0.6 %) at a Q_R/Q of 15 (table 1 and figure 1). In this context, removal efficiencies of 70 % were reported in a biofilter treating CH_4 at an EBRT of 50 min (du Plessis et al. 2003). The results here obtained were in accordance with those previously reported by Estrada et al. (2014), who recorded a 2.5 increase in CH_4 REs at a Q_R/Q ratio of 18 in a methanotrophic biotrickling filter.
However, the EBRTs above investigated were higher than those reported by other authors for biotrickling filters and would result in costly and prohibitively large reactors (Estrada et al. 2014). Therefore, two more EBRTs of 30 and 15 min were evaluated. The EBRT of 30 min was evaluated at Q_R/Q = 10 and 15, while the EBRT of 15 min was tested at a Q_R/Q = 15. Overall, a decrease in the EBRT mediated an increase in the EC and PCO_2, and resulted in a PCO_2/EC mineralization ratio of 2.2 according to the data linear fit (R^2=0.706) showed in figure 2. An EC_{max} of 35.2 ± 0.4 g m^{-3} h^{-1} and REs of 72.9 ± 0.5 % were achieved at an EBRT of 30 min, while process operation at EBRT of 15 min resulted in ECs of 54.4 ± 0.9 g m^{-3} h^{-1} and REs of 56.6 ± 1.5 %.

Table 1. Influence of the Q_R/Q ratio on elimination capacities at EBRTs of 120 and 60 minutes.

Q_R/Q	EBRT (min)	EC (g m^{-3} h^{-1})	EBRT (min)	EC (g m^{-3} h^{-1})
0		4.7 ± 0.48		8.5 ± 0.30
2		6.6 ± 0.35		12.2 ± 0.36
3	120	7.3 ± 0.12	60	14.0 ± 0.34
6		8.4 ± 0.17		16.5 ± 0.31
10		9.2 ± 0.07		17.48 ± 0.37
15		9.8 ± 0.10		18.73 ± 0.24

In spite of the good results obtained at an EBRT of 15 min and a Q_R/Q ratio of 15, the system stability was only maintained for 3 days. Under these particular conditions, the high shear stress caused by the high turbulence in the cultivation medium caused a severe deterioration in microbial activity. These operational conditions lead to a decrease in the EC from 56.6 ± 1.5 % to 21.1 ± 5.2 g m^{-3} h^{-1} (Inlet load = 99.3 ± 0.7 g m^{-3} h^{-1}). Biomass aggregation and settling at the bottom of the BCB was observed as a result of the high turbulence matching with an increase in the mineralization ratio 1.8 times higher than that recorded at the early stages of process operation. Indeed, *M. hirsuta* is a Gram-negative bacterium sensitive to shear stress limiting the performance of bioreactors devoted to CH_4 treatment (Lindner et al. 2007).

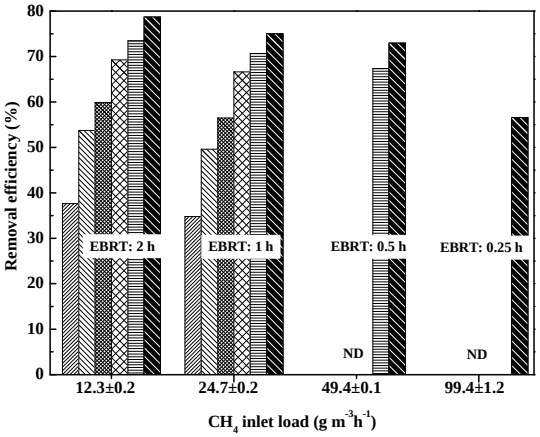

Figure 1: Influence of the EBRT on the RE of the BCB at a Q_R/Q of ( )=0, ( ) 2 , ( ) 3, ( ) 6, ( )10, and ( ) 15 .ND means undetermined.

In addition, the results confirmed that the dilution rate (D) of 0.1 d^{-1} tested to prevent nutrient limitations in the BCB was effective. Previous studies demonstrated that N-depletion severely deteriorated the performance of the methanotrophic bioreactor when gas-recycling is implemented (Estrada et al. 2014). In this study, the lowest TN concentrations were 146 ± 2 mg N L^{-1}, which were recorded at an EBRT of 15 min and a Q_R/Q ratio of 15 as a result of an enhanced nitrogen assimilation by *M. hirsuta*. These concentrations were sufficient to maintain an optimal performance during the study of the influence of different gas-recycling ratios on CH4 biodegradation.

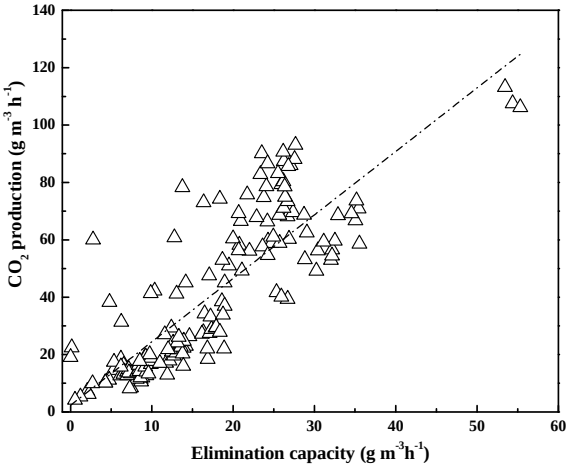

Figure 2: Carbon dioxide production versus elimination capacity. The linear fit showed a R^2 value of 0.706.

3.2 Influence of micro/macro nutrient limitation on PHB accumulation

The growth stage during the nutrients-sufficient conditions showed a biomass yield (Y_x) of 0.63 ± 0.04 gX gCH_4^{-1} and a PHB content of 7.8 ± 1.0 % (*w/w*). Under nutrient deficient conditions, a rapid accumulation of PHB was observed within the first 24 h concomitant with the biodegradation of CH_4. Mn limitation (PHB = 8.1 ± 1.1 %) and K limitation (PHB= 12.5 ± 1.1 %) induced only a slightly higher PHB content compared with the control test. In this context, the PHB content here recorded under K-limitation was 2-times lower than that recorded in a type II *Methylocystis* sp. consortium, likely due to the different MSM composition and to the presence to other methanotrophic species (Helm et al. 2008). N limitation induced the highest PHB accumulation (28.0 ± 1.2 %), which was in agreement with the results obtained in other methanotrophic species (Pieja et al. 2012) belonging to the genera *Methylocystis* and *Methylosinus* (Helm et al. 2008)**.** Compared to the test conducted exclusively under N limitation, a poor CH_4 consumption and a low PHB accumulation (19.2 ± 1.8 %) was recorded under excess of Fe^{2+} and N limitation, which suggested the occurrence of microbial inhibition in *M. hirsuta* at high Fe^{2+} concentrations (60 µM). On the contrary, previous

studies indicated that Fe^{2+} concentrations of 40-80 µM are required for an effective methane monooxigenasa activity and that both Fe^{2+} and Cu^{2+} are important co-factors in the metabolism of methanotrophs (Karthikeyan et al. 2015). In this context, it can be hypothesized that the presence of high concentrations of Cu^{2+} could promote the formation of hydrogen peroxide, which can react with Fe^{2+} at such high concentrations and produce inhibitory free hydroxyl radicals (Sun et al. 2011).

3.3 Continuous CH_4 abatement and PHB co-production

The continuous bioreactor was operated under the optimal mass transfer conditions determined in the previous test (EBRT of 30 min, Q_R/Q ratio of 15, N limitations). The reactor was operated under a nitrogen feast-famine regime in order to support a stable and efficient CH_4 abatement coupled to PHB production. The ECs in the BCB remained at steady values of ~ 27.9 ± 2.1 g m^{-3} h^{-1} (REs of 57.8 ± 4.5 %) by day 10 with biomass concentrations of 4.5 ± 0.6 g L^{-1} from day 20 onwards. Additionally, PCO_2 and biomass productivities of 79.9 ± 8.4 g CO_2 m^{-3} h^{-1} and 26.4 ± 18.5 gX m^{-3} h^{-1}, respectively, were recorded. The implementation of nine N feast-famine cycles induced a gradual increase in the PHB content from 0.4 ± 0.0 % to 25.7 ± 0.1 % during the first N limitation cycle, and up to 37.2 ± 2.0 % from the fifth cycle onwards (with a maximum accumulation of 40 % in the fifth and eighth cycles). Process operation under a nitrogen feast-famine 1d:2d regime was enough to improve significantly the EC and to maintain PHB accumulation steady (figure 3). Interestingly, a slight decrease in the PHB content (1.1 %-6.8 %) was consistently observed after N addition, which can be explained by the fact that PHB is used as a readily available energy source by type II methanotrophs following N supply to the cultivation broth (Pieja et al. 2011). CO_2 production decreased with EC during the N starvation cycles, which can be attributed both to the reduced CH_4 uptake and the CO_2 requirements for PHB production in type II methanotrophs (Pieja et al. 2012). PHB productivities remained roughly constant during operation under N feast-famine cycles at 1.82-2.23 kg m^{-3} d^{-1} (specific PHB productivities ranging from 15.9 to 21.6 mg PHB gX^{-1} h^{-1}). These productivities ranked among the highest reported in methanotrophic cultures in continuous CH_4 abatement bioreactors (Khosravi-Darani et al. 2013). Finally, it is important to stress that the high PHB productivities reached in this study were obtained with CH_4 diluted streams (4 % *v/v*), compared to studies conducted at higher CH_4 concentrations and supporting slightly higher accumulations of PHB (Rahnama et al. 2012).

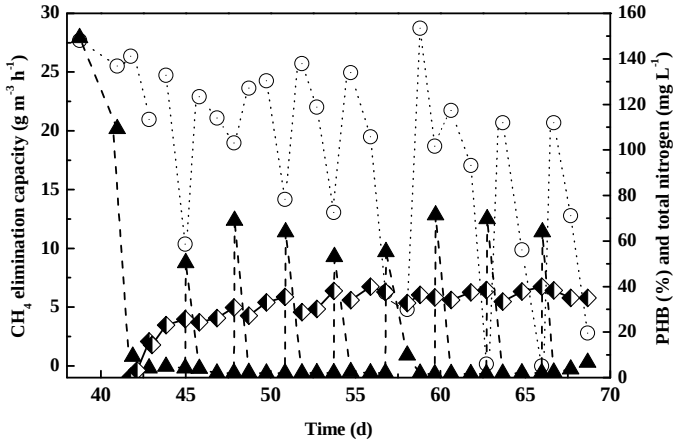

Figure 3: Time course of the PHB cell content (◆), total nitrogen (▲) and elimination capacity (○) during continuous elimination of CH_4 and PHB production.

3. Conclusion

The implementation of gas-recycling strategies in a BCB allowed improving the abatement of diluted CH_4 emissions. The results also demonstrated that N limitation was the most effective approach to induce PHB synthesis in *M. hirsuta* and it implementation under optimum mass transfer conditions (*i.e.*, Q_R/Q= 15 and an EBRT of 30 min) resulted in ECs of 16.2 ± 9.5 g m^{-3} h^{-1}, PHB contents of 34.6 ± 2.5 % and PHB productivities of 1.4 ± 0.4 kg m^{-3} d^{-1}. Therefore, this study demonstrated for the first time the potential of internal gas-recycling BCBs for the continuous bioconversion of diluted CH_4 emissions into PHB at high productivities and under long-term operation. This technological approach paves the way towards the development of CH_4 biorefineries implemented in landfills, coal mines, wastewater treatment plants and even liquefied natural gas carriers.

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