Research Article

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Production of hydroxyectoine from biogas by an engineered strain of *Methylomicrobium alcaliphilum* using a novel Taylor-flow bioreactor

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Abstract

BACKGROUND: The production of compatible solutes, such as ectoine and hydroxyectoine, is of great interest due to their industrial and biotechnological applications. *Methylomicrobium alcaliphilum* was genetically engineered to replace a native gene with a heterologous one, aiming to enhance ectoine production. This study focuses on the optimization of bioreactor conditions to maximize the microbial production of these metabolites from methane.

RESULTS: The engineered strain (*M. alcaliphilum PstEctD*) was cultured in a Taylor flow bioreactor under varying gas recirculation flow rates. Increased flow rates enhanced methane consumption, biomass concentration, and ectoine production. The highest production of ectoine (32 mg/g-VSS) and hydroxyectoine (272 mg/g-VSS) was observed at a flow rate of 0.7 L min^{-1}, while methane removal efficiency improved from 30% to over 60% as flow rates increased.

CONCLUSIONS: Optimizing bioreactor conditions, particularly gas recirculation flow rates, significantly improved both the efficiency of methane consumption and the production of ectoine derivatives. This work provides a scalable approach for the sustainable production of compatible solutes from methane, offering potential applications in biotechnological processes utilizing renewable carbon sources.
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Keywords: ectoine; hydroxyectoine; methanotrophic bacteria; Taylor flow; methane

INTRODUCTION

The surge in European biogas plants, accounting for 19,491 plants with an installed capacity of 179 TWh in 2022 (EBA 2023), reflects a global trend of expanding biogas production. However, the utilization of biogas other than electricity and heat generation remains low, highlighting the untapped potential of this renewable methane. Thus, biogas is primarily used for electricity and heat generation, with cogeneration (64%), heating (27%), and biomethane production (9%) as the main biogas applications. Costly upgrading processes are required to meet the composition standards fixed by European regulations, raising utilization costs. Indeed, physico-chemical methods like membrane-based separation, CO_2 absorption/adsorption and H_2S precipitation can enhance biogas quality at the expense of high consumption of chemicals and energy. Additionally, declining costs in other renewable energies prompt the exploration of new biogas applications, like bioconversion into high added value products.

The use of biogas as a raw material for biorefineries is becoming increasingly important as a more sustainable, environmentally

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friendly, and cheaper raw material compared to those used in current production processes. However, there are still several barriers to overcome regarding the scale-up of these technologies, including limitations in gas–liquid mass transfer due to the low solubility of methane in water, as well as kinetic limitations in the bioconversion of methane due to the low specific growth rate of most methanotrophic bacteria. Methanotrophs are aerobic microorganisms capable of using methane as a source of carbon and energy. These microorganisms are classified into Type I or γ-proteobacteria, which assimilate methane *via* the ribulose monophosphate pathway and exhibit the highest carbon conversion yield; Type II or α-proteobacteria, which use the serine pathway in methane assimilation.

Besides biogas production, over 70 million tons of methane per year are currently being released into the atmosphere,[1] as the result of waste management, mining, farming *etc*. Methane is a gas with high greenhouse potential,[2] thus its utilization as a feedstock for industrial biotechnology, besides reducing feedstock costs, would also contribute to mitigating climate change. In glucose-based fermentations the feedstock accounts for up to 30% of the total production costs and methane has been identified as the most cost-competitive feedstock.[3] In this context, methanotrophs appear to be promising platforms for industrial microbiology. However, the product portfolio which is currently possible to obtain from these organisms is still narrow. Type II methanotrophs accumulate PHB naturally[4-6] and the halotolerant methanotroph *Methylomicrobium alcaliphilum* produces ectoine. To expand the portfolio of chemicals obtained from methane *via* microbial fermentation, it is necessary to develop efficient metabolic engineering approaches in methanotrophs. *Methylomicrobium alcaliphilum* has proved to be a good platform for genetic engineering[7] and there are already two Genome Scale Metabolic Models (GSMMs) available for this organism.[8,9]

Ectoine and hydroxyectoine are cyclic amino acids synthesized intracellularly in response to high salinity environments, as they prevent cellular osmotic dehydration, maintaining osmotic balance. These protective compounds are stored in the cell's cytoplasm and do not alter its natural behavior. They possess stabilizing properties for proteins, nucleic acids, and enzymes. Therefore, their applications are framed in the cosmetic industry, offering excellent protection against UV radiation, dermatology, nutrition, and medicine.[10] Ectoine is one of the products synthesized by microorganisms with the highest added value, with a market price of 900 euros/kg and a consumption of 20 tons per year.[11] Ectoine is currently being produced industrially by *Halomonas elongata*,[12] which uses glucose as a substrate. Hydroxyectoine, a derivative of ectoine, is more common among gram-positive halotolerant bacteria[13] and shows additional protective properties including resistance to heat stress. The market prize of hydroxyectoine is 40% higher than that of ectoine, which makes it an interesting product with high added value. The halotolerant methanotroph *Methylomicrobium alcaliphilum* accumulates intracellular ectoine under high salinity conditions. The Ect operon of *M. alcaliphilum* contains a putative ectoine hydroxylase (*EctD*), which has been shown to lack catalytic activity.[9] Mustakhimov *et al*.[14] engineered *M. alcaliphilum* by adding the enzyme ectoine hydroxylase from *Pseudomonas stutzeri*. One of the engineered strains, when grown in a medium with 6% (w w^{-1}) NaCl, showed an intracellular hydroxyectoine content of 22 mg g-DW^{-1}.

One of the main challenges to use methane as a feedstock for industrial biotechnology, is its low solubility in water, which makes difficult its transport to the liquid phase, where it can be used by methanotrophic organisms. Recent advancements in bioreactor technology have introduced capillary bioreactors as a promising avenue for achieving high methane conversion rates while minimizing energy consumption. These innovative bioreactors are characterized by parallel straight microcapillaries, typically ranging in diameter from approximately 1 to 5 mm. Within these capillary bioreactors, gas–liquid hydrodynamics exhibit an alternating sequence of gas bubbles and liquid slugs, forming what is known as Taylor flow. This unique flow pattern offers several advantages, including a substantial increase in gas–liquid interfacial area and reduced thickness of the liquid film across which methane can diffuse. Consequently, these features contribute to an enhanced mass transfer coefficient, facilitating an efficient methane transfer at a minimal energy input.[15] While capillary bioreactors are not yet widely utilized for methane bioconversion, they have shown promising results in preliminary studies, notably achieving a 50% increase in methane removal compared to conventional two-phase partitioning turbulent bioreactors.[16]

Capillary reactors combine efficient mass transfer with relatively low pressure drop, both of which are key factors influencing cost-effectiveness in many industrial applications. Capillary gas–liquid contactors typically consist of parallel straight microchannels (small round or square capillary channels) separated by thin walls. The hydrodynamics of gas–liquid flow in capillary channels have been extensively studied in the context of chemical reaction engineering.[17-19] The solution of Navier–Stokes equations describing the liquid flow around elongated bubbles in capillary channels has shown that the formation of a thin liquid film between the gas bubbles and the capillary walls is the main responsible for the improved mass transfer rates.[20] Areas of study and applications are discussed in Haase *et al*.[21] and Kreutzer *et al*.,[18] covering topics such as chemical processes where back-mixing is undesirable, micro devices (*e.g*., lab-on-a-chip applications) and compact heat exchangers (*e.g*., printed circuit cooling systems). Although capillary gas–liquid bioreactors have proven to be effective platforms for gas treatment,[15,22] they have not yet been systematically explored for dilute methane abatement.

In this work, the gene *ectD* from *P. stutzeri*, coding ectoine hydroxylase, was introduced into *M. alcaliphilum*, resulting in a mutant strain with high intracellular contents of hydroxyectoine. This strategy was previously followed by Mustakhimov and coworkers, who created two mutant strains in which the native *phy* gene, a putative ectoine hydroxylase that was observed to be inactive,[9] was substituted by the *ectD* gene. The native *phy* gene forms part of the *ectABCaskD* operon, which is under the regulation of the salt-inducible Pect promoter, this means that the four members of the operon are co-transcribed. In a first approach Mustakinov *et al*.[14] substituted *phy* by *ectD* keeping the whole operon under the regulation of the same promoter. In a second approach, the native *phy* gene was substituted by a fusion of the Pect promoter and the *ectD* gene, which allows the gene to be transcribed separately from the rest of the operon. In this work the *ectD* gene was added fused to the Pect promoter but, differently from Mustakinov *et al*., the gene was inserted in the opposite sense to the native operon (see Fig. 1), trying to avoid interferences in its transcription.

This study introduces a significant advancement in the bioreactor-based production of ectoine and hydroxyectoine, by optimizing the cultivation conditions for the engineered Methylomicrobium alcaliphilum PstEctD strain. A Taylor flow bioreactor

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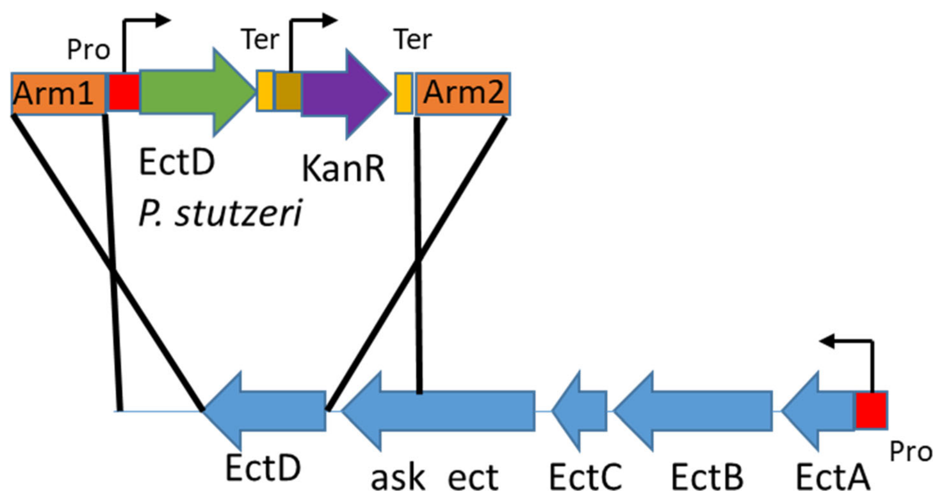

Figure 1. Depiction of the native Ect operon of *Methylomicrobium alcaliphilum* and the insertion of *EctD* from *Pseudomonas stutzeri* by double recombination. The native *EctD* gene, which has been proven to lack catalytic activity, was replaced by the new genetic construction.

was used to efficiently produce these compatible solutes, demonstrating that controlled factors such as gas recirculation flow rate can significantly enhance methane consumption and biomass concentration. The study found that increasing the gas recirculation flow rate from 0.7 to 3.0 L min^{-1} improved methane removal efficiency from 30% to over 60%, which directly correlated with higher biomass and ectoine production. Notably, the strain achieved the highest yields of ectoine (32 mg/g-VSS) and hydroxyectoine (272 mg/g-VSS) at the lowest recirculation flow rate. This innovation in bioreactor operation, coupled with the genetic modifications, not only optimizes the production of valuable metabolites but also provides a scalable model for future bioprocesses that could utilize methane as a carbon source in sustainable industrial applications.

MATERIALS AND METHODS

Strain and culture conditions

The strain *Methylomicrobium alcaliphilum* 20Z was purchased from DSMZ (Leibniz-Institut). The mineral salt medium (MSM) used for *M. alcaliphilum* 20Z cultivation was prepared according to Akberdin *et al.*[8] with the addition of 0.07 mg L^{-1} of Na_2WO_4, which prevents the accumulation of formic acid from methane oxidation. Avoiding the accumulation of formic acid in the fermentation broth is essential to optimize the process yield and to avoid pH modifications or product inhibition phenomena during the operation of bioreactors. Thus, the *M. alcaliphilum* PstEctD strain was cultured in 1 MSM with 0.07 mg L^{-1} of Na_2WO_4 and 6% NaCl.

To characterize specific growth rates, biomass yields on methane and production of formic acid, sterile gas-tight bottles of 1.2 L with 195 mL of MSM were inoculated with 5 mL of a preculture in exponential growth phase. The headspace contained initial concentrations of 1.9 mmol L^{-1} of methane. The bottles were closed with gas-tight butyl septa and plastic screw caps. The consumption of methane and O_2, as well as the optical density (OD_{650}), were daily monitored as described in the analytical methods. Specific growth rates were obtained by plotting the logarithm of OD_{650} *versus* time and obtaining the slope. Biomass yields were obtained by plotting the accumulated biomass *versus* the methane consumed in the headspace. All the experiments were carried out in triplicate.

Integration of EctD from P. stutzeri by double recombination

The sequence of *EctD* from *Pseudomonas stutzeri*, strain CGMCC 1.1803 was retrieved from GenBank (GeneID:28544874; NC_015740.1). This gene was flanked upstream by 258 base pairs, which correspond to the native promoter of the Ect operon in *M. alcaliphilum*, and a transcriptional terminator 112 bp long. The *EctD* gene is followed by a kanamycin resistance gene (with its corresponding promoter and terminator) and the whole insert was flanked by two regions of homology to the genome of *M. alcaliphilum*, with a length of 800 bp. The homology regions were chosen to replace the native *EctD* gene, which have been shown to code an enzyme that lacks catalytic activity. The described insert (sequence available at supplementary file SF1) was custom synthesized (GenScript) and cloned in the pUC57 plasmid at the cloning site EcoRV/EcoRV. This plasmid cannot be replicated by *M. alcaliphilum* and was used as suicide plasmid. The insertion by double homologous recombination took place as depicted in Fig. 1.

M. alcaliphilum 20Z was cultured in a modified methanol based MSM, which contained only 2 g L^{-1} of NaCl. Lower salinity levels and methanol, instead of methane, were used to speed up the growth of the microorganism for its genetic manipulation. Liquid cultures of 50 mL were grown until a 0.6 OD_{650} was reached. Cells were harvested by centrifugation at 5000*g* and 4 °C for 10 min. To remove salts, cells were resuspended in 50 mL of ice-cold sterilized Milli-Q water and harvested again by centrifugation at 5000*g* and 4 °C for 10 min. After repeating the washing step three times, the resulting pellet was resuspended in 500 μL of ice-cold sterilized Milli-Q water and placed on ice. One hundred microliters of the cell suspension was carefully mixed with 500 ng of plasmid DNA and transferred into an ice-cold 0.1 cm-gap cuvette (Bio-Rad). Electroporation was performed with a MicroPulserTM system (Bio-Rad) set at 1.8 kV. Immediately after the electrical discharge, cells were resuspended in 1 mL of modified MSM (0.1% methanol) and transferred to 120 mL vials containing 19 mL of modified MSM and cultured at 30 °C and 230 rpm for 24 h to

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Table 1. Primers used to test the presence of the recombinant and native ectD genes

Primer	
ectDPs F	*P. stutzeri* ectD (5′-tcaccggcaaggagttctac-3′)
ectDPs R	*P. stutzeri* ectD (5′-cgcagggacttctcgtagtg-3′)
ectDMa F	*M. alcaliphilum* 20Z ectD (5′-gtcgtttgcgaagaggaaac-3′)
ectDMa R	*M. alcaliphilum* 20Z ectD (5′-cgaggccatggagtgatatt-3′)

allow cell recovery. After growth, cultures were transferred into selective plates. To ensure strain purity, 12 single colonies were transferred to new plates and isolated again, this last step was repeated three times.

Successful double recombination was checked by amplifying regions of the native ectD gene (ectDMa) and the ectD gene from *Pseudomonas stutzeri*, using the primers shown in Table 1. The resulting engineered strain was referred to as *M. alcaliphilum* PstEctD.

Taylor flow reactor and experimental setup

The experimental work was performed in a Taylor flow reactor with a total volume of 10 L. The reactor comprised of 25 capillary glass tubes of 3 mm internal diameter and 1.5 m length, a lower polyvinyl chloride (PVC) chamber of 2.5 L and an upper 6.5 L PVC chamber (Fig. S1). The cultivation broth was constantly recirculated from the upper to the lower chamber using a centrifugal pump (Aalborg, GFC17).

Synthetic biogas with a composition of 70%v v^{-1} CH_4 and 30% v v^{-1} CO_2 (Carburos Metálicos, S.A., Barcelona, Spain) was mixed with an air stream in a 0.9 L PVC mixing chamber, resulting in a methane content of 5% v v^{-1}. The resultant biogas/air mixture was passed through a 0.22 μm filter to eliminate solid impurities and microorganisms prior being combined with a gas recirculation stream in a second mixing chamber. Then, the mixture of inlet and recirculation streams was injected into the lower chamber through a custom-made membrane diffuser with a pore size of 0.5 mm to allow the homogeneous sparging of the gas into the liquid media recirculated from the upper chamber. The flow rates of gas and liquid were regulated to ensure that both phases moved through the capillary tubes to the upper chamber in a Taylor-flow hydrodynamic pattern. For this purpose, a flow meter was installed in the liquid recirculation line before it entered the lower chamber of the reactor.

In the upper module, a liquid extraction basket installed in the middle section of the chamber facilitated the separation of liquid from the gas phase, preventing gas bubbles from entering the liquid recirculation stream. The gas outlet positioned at the top of the upper chamber enabled both, the recirculation of a portion of the methane-depleted gas mixture and the discharge of the remaining fraction into the atmosphere. The recirculated gas, controlled by a rotameter (Aalborg, Germany), was directed to a water trap to remove water droplets before being conducted to a gas compressor (H5P3 EAD, Spain), and ultimately mixed with the inlet gas stream. A complete scheme of the Taylor flow reactor is depicted in Fig. 2.

Operational conditions and monitoring of the experimental system

The Taylor flow reactor was initially filled with 9.4 L of modified MSM with a NaCl content of 6% w w^{-1} and inoculated with 600 mL of a culture of *M. alcaliphilum* PstEctD exhibiting an OD_{650} of 0.6, previously grown in methanol. To speed up microbial growth during the first days of operation, and avoid contamination with other microorganisms, methanol (50 g L^{-1}) and kanamycin (50 μg L^{-1}) were added in the initial MSM. The liquid was constantly recirculated at a flow rate of 7.2 L min^{-1}. Gas flow rate in the inlet was set to 100 mL min^{-1} while the gas recirculation was set initially at 0.7 L min^{-1} (R1) and then increased to 1.4 L min^{-1} (day 50, R2) and 3.0 L min^{-1} (day 62, R3). Due to a decrease in biomass concentration from day 14 onwards, 0.5 L of cultivation broth (dilution rate of 0.09 d^{-1}) was exchanged daily by fresh MSM (without methanol and kanamycin), to prevent the potential buildup of inhibitory metabolites.

The experimental system was monitored by analyzing the liquid and gas phases three times a week (for an operation period of 68 days). Liquid samples of 110 mL from the cultivation broth were drawn to measure the total suspended solids (TSS), volatile suspended solids (VSS), OD, dissolved organic carbon (TOC), dissolved inorganic carbon (IC), dissolved total nitrogen (TN), nitrate, nitrite, pH, conductivity, and intra-cellular ectoine and hydroxyectoine. Gas samples of 100 μL were also taken from the inlet and recirculation gas streams to measure the gas composition (CO_2, O_2 and CH_4). Inlet and outlet gas flow rates and the inlet gas pressure were also monitored (the outlet pressure corresponded to atmospheric pressure). All analyses were performed in duplicate.

Analytical methods

Formic acid was measured only in the pre-culture (after filtering the sample with 0.22 μm filters) using a Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation module equipped with a Waters 2414 refractive index detector. A mobile phase of 0.025 M H_2SO_4 was eluted with a flow of 0.6 mL min^{-1} and 50 °C. Detector temperature was 35 °C. OD at 650 nm was measured using SPECTROstar Nano (BMG Labtech).

The concentrations of CO_2, O_2, and CH_4 in the inlet and recirculating gas streams were determined in a Bruker 430 GC-TCD gas chromatograph (Palo Alto, USA) equipped with a CP-PoraBOND Q and a CP-Molsieve 5A columns using helium as a carrier gas.[23] An Ifm differential pressure sensor (Essen, Germany) was used to monitor the inlet gas pressure. The pH was measured with a Basic 20+ pH meter (Düsseldorf, Germany) and the conductivity using an EC-Meter BASIC 30 instrument (Barcelona, Spain). The concentrations of dissolved TOC, IC, and TN were measured using a Shimadzu TOC-VCSH analyzer (Japan) equipped with a TNM-1 chemiluminescence module after sample filtration (0.45 μm pore size). Nitrate and nitrite concentrations were determined after sample filtration by HPLC-IC.[24] TSS and VSS were determined according to standard methods.[25]

Intracellular ectoine and hydroxyectoine were determined using 2 mL of cultivation broth following the extraction procedure as in Rodero *et al.*[26] prior to its analysis *via* HPLC-UV. This system was composed by a pre-column, a Spherisorb Amino (NH2) column (3 μm × 4.6 mm × 150 mm), and a Waters 717 plus automatic sampler (Waters, Bellefonte, USA) coupled with a UV dual wavelength absorbance detector at 210 nm (Waters, Bellefonte, USA). The mobile phase used was a 75–25% v v^{-1} mixture of acetonitrile and MilliQ water at a flow rate of 1 mL min^{-1}. External standards of ectoine and hydroxyectoine (purity ≥95%, Sigma Aldrich, USA) were used for quantification. The specific concentrations were calculated using the biomass concentration in the cultivation broth measured in terms of VSS.

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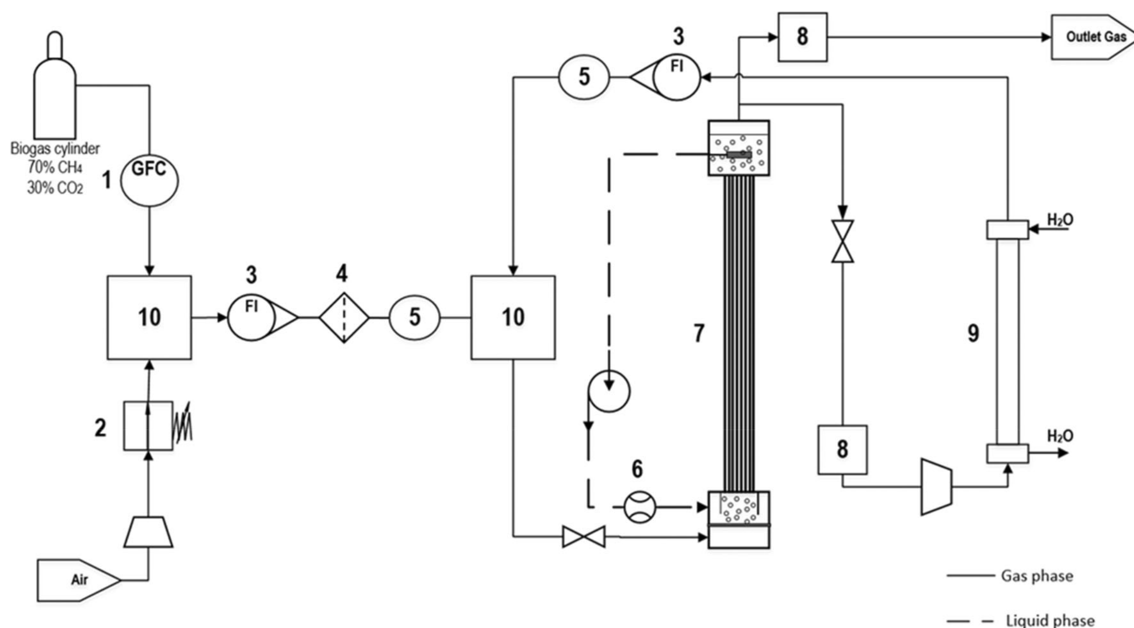

Figure 2. Diagram of the experimental setup. 1: mass flow controller, 2: pressure controller, 3: rotameter, 4: filter, 5: sampling port, 6: flow meter, 7: Taylor reactor, 8: trap, 9: condenser, 10: mixing chamber.

Genome scale metabolic models

Flux Balance Analysis calculations were carried out using the Genome Scale Metabolic model previously published[9] (Bordel *et al.*), which is publicly available at https://github.com/SergioBordel/ModelsMethanotrophs. The Flux Balance Analysis (FBA) calculations were carried out using COBRApy[27] (Ebrahim *et al.*).

RESULTS

Transformation by electroporation and colony selection

Using the method described in Integration of EctD from P. stutzeri by double recombination, 12 colonies were obtained. It was shown by PCR that three of the selected colonies showed successful double recombination (presence of the heterologous gene and absence of the native one), while the rest appeared to be the product of single recombination events (both the native and heterologous genes are present). One of the three colonies was selected for further physiological characterization. Acquisition of resistance to kanamycin, caused by the integration of the *KanR* gene into the bacterial genome after a single or double recombination event. In the cases in which there is a single recombination, the native gene *ectDMa* is still present in the genome. In strains in which a double recombination occurred, the native gene is absent. Therefore, the presence of the heterologous gene *ectDPs* and the absence of the native gene have been used as criteria to confirm that the double recombination was carried out successfully.

Figure 3 shows the PCR results confirming the successful double recombination in the selected mutant (*Methylomicrobium alcaliphilum* PstEctD), in this strain the native gene is absent and the recombinant gene is present. The results are compared to the wild type in which only the native gene is present.

Preliminary physiological characterization of the PstEctD strain

In previous experiments[9] Bordel *et al. M. alcaliphilum* was cultured in the minimal salt medium reported by Kalyuzhnaya *et al.*[28] This resulted in 45% of the consumed methane being transformed into formic acid, which accumulated in the growth medium. This phenomenon occurred independently of the NaCl concentrations of the medium. Akberdin *et al.*[8] reported that formic acid accumulation can be drastically reduced by adding 0.07 mg L^{-1} of Na_2WO_4, which activates a NAD-associated tungsten-dependent formate dehydrogenase.

Specific growth rates and biomass yields on methane were measured. No accumulation of formic acid was detected in the growth medium. The observed growth rate and biomass yield are reported in Table 2. In the absence of Na_2WO_4, the wild type of strain consumed 1.38 mmol h^{-1} g-DW^{-1} of methane and produced 0.62 mmol h^{-1} g-DW^{-1} of formic acid[9] Bordel *et al*.

The strain PstEctD showed molar concentrations of 29.4 mg g-DW^{-1} of hydroxyectoine and 16.8 mmol g-DW^{-1} of ectoine. Compared to the 30.8 mmol g-DW^{-1} of ectoine of the wild type, this amounts to a 40% increase of the total production of ectoine derivatives. The ectoine and hydroxyectoine yields per gram of methane are 10.6 and 18.6 mg g-CH_4 respectively.

Flux balance analysis simulations

As it has been mentioned previously, 45% of the consumed methane gets transformed into formic acid, if a mineral medium without tungsten is used. Akberdin and co-workers argued that, in presence of Na_2WO_4, formic acid is oxidized into CO_2. To test if the observed biomass yield in the presence of Na_2WO_4 is consistent with such phenomenon, a genome scale metabolic model[9] (Bordel *et al.*) was used to predict the theoretical biomass yield. This prediction was carried out using FBA, setting the methane uptake rate to its experimental value, and assuming that 45% of methane is converted to formic acid and further oxidized into CO_2 with NADH production. The predicted values of specific growth rate and biomass yield (Table 2) are equal (within the experimental error intervals) to the observed experimental values. This confirms that the addition of Na_2WO_4 results in the activation of formate dehydrogenase.

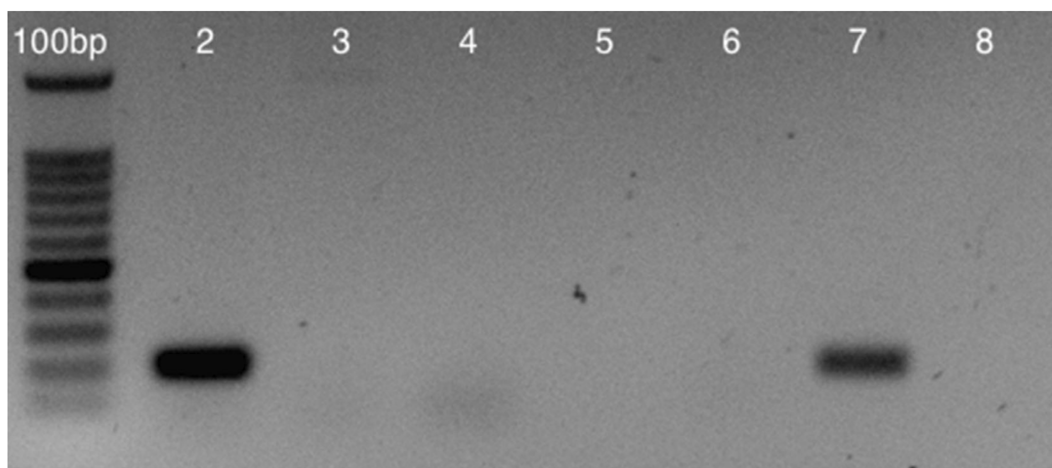

Figure 3. Amplification of *ectD* genes. The DNA in lanes 2, 3 and 4 was amplified with ectDMa primers; lane 2 corresponds to the wild type, 3 to the selected mutant, and 4 is a negative control. The DNA in lanes 6–8 was amplified with ectDPs primers; lane 6 corresponds to the wild type, lane 7 to the selected mutant, and 8 is a negative control.

Table 2. Growth rates and biomass yields, predicted and experimental. Error intervals are standard deviations (n = 3).

	μ (h^{-1})	Yield (g-DW·molCH_4^{-1})	Ectoine (mg·g-DW^{-1})	Hydroxyectoine (mg·g-DW^{-1})
M. alcaliphilum PstEctD with Na_2WO_4	0.015 ± 0.002	10.1 ± 1	16.8 ± 4	29.4 ± 4
Maximal theoretical value with Na_2WO_4	0.0149	10.7	Not modelled	Not modelled

Operation of the Taylor flow bioreactor

The pH and conductivity of the cultivation broth remained constant at 7.99 ± 0.02 and 80.76 ± 0.19 mS cm^{-1}, respectively. Initially, biomass concentration slightly increased over time, but after 8 days, it decreased over the following days (Fig. 4(C)). This decrease was attributed to the generation of inhibitory metabolites resulting from microbial activity. Concentrations of nitrite and nitrate were analyzed to confirm if the reduction of NO_3^- to NO_2^- was the cause of process inhibition[26] (Rodero *et al.*). However, no presence of NO_2^- was detected in the cultivation broth. Consequently, the mineral medium renewal was performed daily throughout the rest of the study. As a result, biomass concentration began to increase, reaching an average value of 0.46 ± 0.07 g L^{-1} under steady state conditions. The increase in the gas recirculation flow rate from 0.7 to 1.4 L min^{-1} from day 44, enhanced the methane gas–liquid mass transfer and, consequently, biomass concentration increased up to 2 g-VSS L^{-1}. Finally, the subsequence increase in gas recirculation flow rate up to 3 L min^{-1} promoted biomass growth until the last day of operation, when it reached a value of 3 g-VSS L^{-1}. TN concentration in the cultivation broth decreased constantly due to bacterial consumption, except between days 10 and 20, when it increased, likely due to cell death. Simultaneously, TOC concentration declined over time due to methanol consumption, reaching low values from day 22 due to the complete depletion of the initial methanol from the cultivation broth (Supplementary material).

Methane consumption started to increase when there was no longer any presence of methanol in the cultivation broth, and the microorganisms began to utilize methane as a carbon and energy source (Fig. 4(A)–(B)). From this point onwards, the increase in biomass concentration led to a higher consumption of methane and, consequently, an increase in CO_2 production. Thus, methane depletion efficiencies of 30.0 ± 3.1% and elimination capacities of 10.3 ± 1.6 g-CH_4 h^{-1} m^{-3} were obtained under steady state at gas recirculation flow rates of 0.7 L min^{-1} (R1). Methane removal efficiencies increased with the gas recirculation flow rate reaching maximum values of 50.1% (R2) and 61.3% (R3), which corresponded to elimination capacities of 18.1 g-CH_4 h^{-1} m^{-3} and 22.9 g-CH_4 h^{-1} m^{-3} at recirculation rates of 1.4 L min^{-1} and 3.0 L min^{-1}, respectively. CO_2 production was observed from the early days of operation since initially, the microorganisms transformed methanol into CO_2, producing up to 14.1 g-CO_2 h^{-1} m^{-3}. From day 28 onwards, when the microorganisms began to consume methane, CO_2 production values of 31.9 g h^{-1} m^{-3} were reached (Supplementary material).

No analysis of ectoine and hydroxyectoine concentrations was carried out before day 22 due to the low biomass growth and the presence of methanol in the cultivation broth. Maximum ectoine and hydroxyectoine concentrations of 32 mg-EC g-VSS^{-1} and 272 mg-HE g-VSS^{-1} were achieved under a gas recirculation flow rate of 0.7 L min^{-1}. Osmolytes accumulation was lower with the increase in gas recirculation flow rate (Fig. 4(D)). In this context, specific ectoine contents of up to 17 mg-EC g-VSS^{-1} and 16 mg-EC g-VSS^{-1} and hydroxyectoine contents of 136 mg-HE g-VSS^{-1} and 122 mg-HE g-VSS^{-1} were reached at recirculation flow rates of 1.4 L min^{-1} and 3.0 L min^{-1}, respectively.

DISCUSSION

Ectoine and hydroxyectoine are compatible solutes (also known as osmoregulatory solutes), primarily produced by certain microorganisms as part of their stress response mechanisms. These compounds help protect cells from environmental stresses such as high salinity, desiccation, and temperature extremes.

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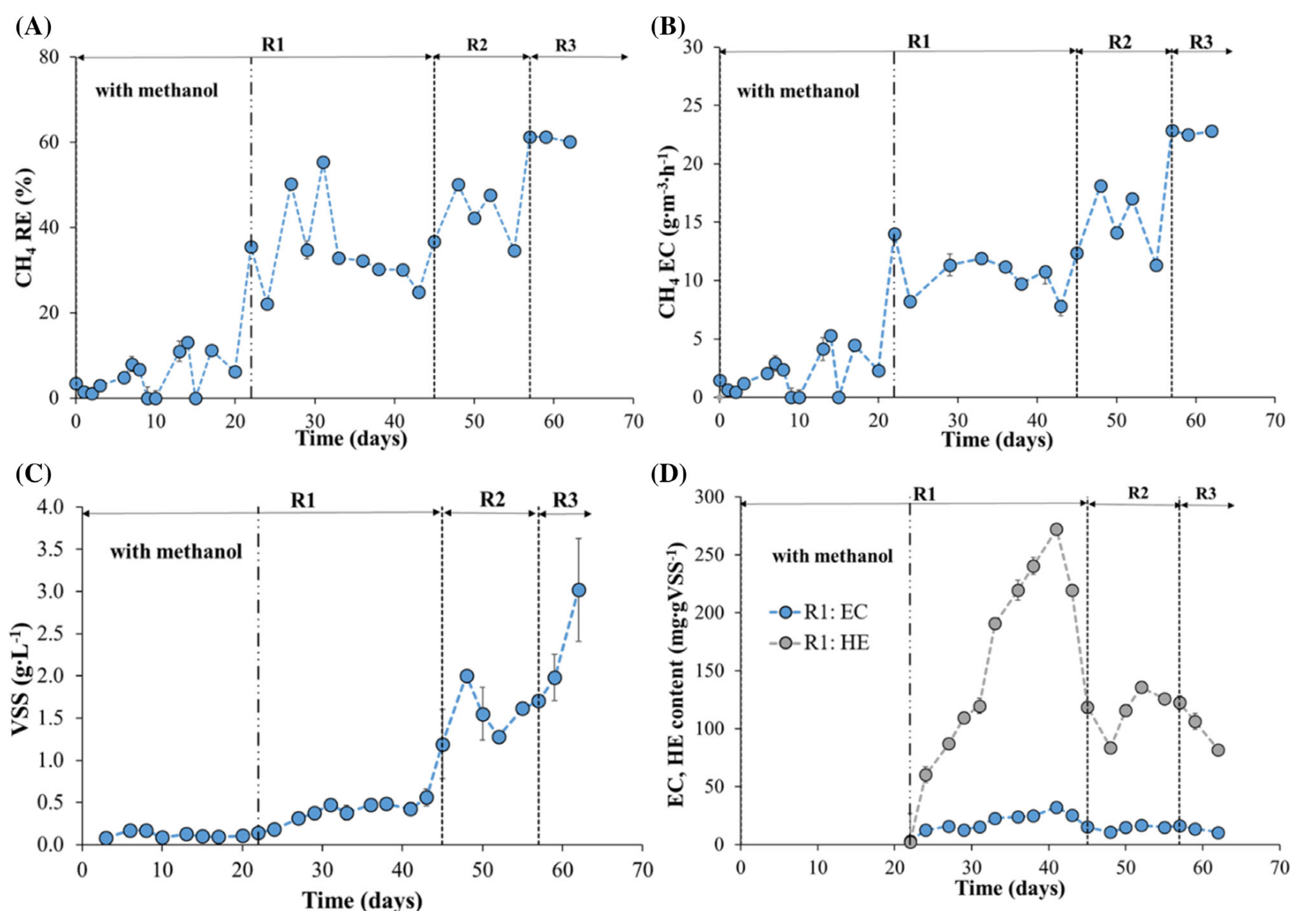

Figure 4. Time course of (A) CH_4 removal efficiency (RE), (B) CH_4 elimination capacity (EC), (C) volatile suspended solids (VSS), (D) ectoine (EC), and (b) hydroxyectoine (HE) yields in the cultivation broth. The error bars represent the standard deviation between duplicate measurements.

Ectoine and hydroxyectoine have a range of valuable applications, which include:

Cosmetics, skincare, pharmaceuticals, and agriculture. Both ectoine and hydroxyectoine are used in skincare products for their ability to protect cells from dehydration and oxidative stress. They have anti-inflammatory properties and are employed to maintain skin hydration, especially in dry or harsh environments. These compounds have potential in the pharmaceutical industry for developing treatments for inflammatory skin conditions, as well as for improving cell preservation in medical applications. The osmoregulatory properties of ectoine and hydroxyectoine may be beneficial in agricultural applications to protect plants under drought or salt stress.

Mustakhimov *et al.*[14] have already shown the possibility of transforming *M. alcaliphilum* into a hydroxyectoine producer. However, this work herein obtained the engineered strain *M. alcaliphilum* PstEctD by integrating in the chromosome the gene EctD form from *Pseudomonas stutzeri* under the control of a separate promoter.

The ability of *M. alcaliphilum* PstEctD to grow in high-mass transfer reactors, specifically in Taylor flow reactors, using biogas as the only carbon and energy source has been demonstrated in this study. The maximum methane elimination capacities hereby obtained, 22.9 g-CH_4 h^{-1} m^{-3}, were significantly higher than those reported for *M. alcaliphilum* 20Z in bubble column reactors (6–8 g-CH_4 h^{-1} m^{-3}) Cantera *et al.*[23] The elimination capacities were similar to those previously obtained for mixed cultures in bubble column reactors engineered with internal gas recirculations and in Taylor flow reactors under optimized conditions (20–26 g-CH_4 h^{-1} m^{-3}) Rodero *et al.*; Herrero-Lobo *et al.*[29,30]. However, methane removal efficiencies in this study were lower compared with the maximum obtained for mixed cultures under optimized Taylor flow conditions (66% *vs* 84%)[30] Herrero-Lobo *et al*. Nevertheless, the operational conditions in this study were optimized yet, with the gas recirculation flow rate and liquid to gas ratio exerting a significant impact on methane bioconversion.

Maximum specific hydroxyectoine concentrations of 272 mg g-VSS^{-1} have been reached using the engineered *M. alcaliphilum*, which are far from those typically found in mixed cultures (up to 24 mg-HE g-VSS^{-1})[31] Carmona-Martínez *et al*. In addition, this concentration was higher than that reported in *Hansenula polymorpha* modified to produce hydroxyectoine (58 mg-HE g-VSS^{-1})[32] Eilert *et al*. Otherwise, ectoine concentrations were lower due to its conversion into hydroxyectoine. However, the maximum values hereby achieved were in the range of those reported under steady state by mixed cultures (≈30 mg-EC g-VSS^{-1})[29] Rodero *et al*. Unfortunately, those maximum values of specific production of hydroxyectoine and ectoine were not stable, being necessary to optimize the process to achieve high hydroxyectoine and ectoine production along the time. In this

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regard, the increase in gas recirculation flow rate, which led to higher biomass concentration in the reactor, exerted a negative impact on the accumulation of these osmolytes. Similarly, Rodero *et al.*[26] observed a decrease in specific ectoine accumulation at higher biomass concentrations, which was accompanied by a lower specific methane uptake rate. The results obtained confirm that using the engineered *M. alcaliphilum* PstEctD to produce hydroxyectoine from biogas in Taylor flow reactors is feasible under continuous operation and the high shear forces expected under this flow regime do not negatively impact hydroxyectoine production. Despite these promising results, process optimization is still needed to enhance both methane bioconversion yields and hydroxyectoine productivity.

The economic viability of converting methane into ectoine and hydroxyectoine depends on several factors, including the cost of methane as a feedstock, the efficiency of the microbial production process, and the market value of the final products. While the yields of 10.6 mg of ectoine and 18.6 mg of hydroxyectoine per gram of methane are useful, further optimization is likely required for this process to be economically competitive with other industrial production methods for these compounds.

Scaling up the production process will require addressing issues such as reactor design, methane supply infrastructure, and downstream processing to isolate and purify ectoine and hydroxyectoine. However, as methane becomes an increasingly abundant and underutilized resource, and as demand for biochemicals like ectoine grows, the feasibility of this approach is likely to improve.

AUTHOR CONTRIBUTIONS

Raquel Herrero-Lobo carried out the experiments with a Taylor flow reactor, María del Rosario Rodero supervised her and contributed to writing the manuscript, while Nuria Fernández-González and Eva Marcos constructed the mutant strain. Raúl Muñoz and Pedro García-Encina obtained the funding and designed the project, and Sergio Bordel designed the project and wrote the manuscript. Alejandra Martínez contributid to the design of the cloning strategy used to obtain the strain.

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DATA AVAILABILITY STATEMENT

Data are available upon request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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