Application of Stable Isotope Techniques for an In-Depth Understanding of Syngas Biomethanation Conversion Pathways

I. Bonato*, A. Catenacci*, S. Gil-Villalba** and F. Malpei*

* Department of Civil and Environmental Engineering, Politecnico di Milano, Piazza Leonardo da Vinci 32, Milano, 20133, IT
(E-mail: irene.bonato@polimi.it; arianna.catenacci@polimi.it; francesca.malpei@polimi.it)
** Isotope Tracer Technologies S.r.l., Viale Ortles 22/4, Milano, 20139, IT
(E-mail: sergiogilv@it2europe.com)

Abstract
This study investigates the biomethanation of syngas, focusing on microbial interactions that enable the conversion of carbon monoxide into methane. We analyse the effects of environmental factors such as gaseous substrate on microbial consortia, particularly the carboxydotrophic and methanogenic potentials. The proposed experimental plan includes semi-batch tests under mesophilic conditions, with a focus on understanding and optimizing metabolic pathways. The approach integrates microbial population analysis, and the novel stable isotope analysis to provide insights into syngas biomethanation. Future work will address gas-liquid mass transfer limitations using hollow fiber membranes to improve methane production efficiency.

Keywords
Carbon monoxide; microbial interactions; stable isotope analysis; syngas biomethanation

INTRODUCTION

The increasing energy demand and reliance on fossil fuels have significantly boosted greenhouse gas emissions, such as CO_2, CO, and syngas, driving the climate crisis. To tackle this, processes are being developed to capture and recycle C1 gases into valuable products like biofuels. Biomethane, primarily produced through anaerobic digestion of organic waste, shows great promise, although its efficiency is limited with resistant biomasses. Alternative methods, like gasifying biomass into syngas and converting it into methane via biomethanation, offer potential solutions (Grimalt-Alemany et al., 2018). The anaerobic microbial conversion of syngas to methane supports various microbial trophic groups and occurs through different catabolic pathways (Figure 1).

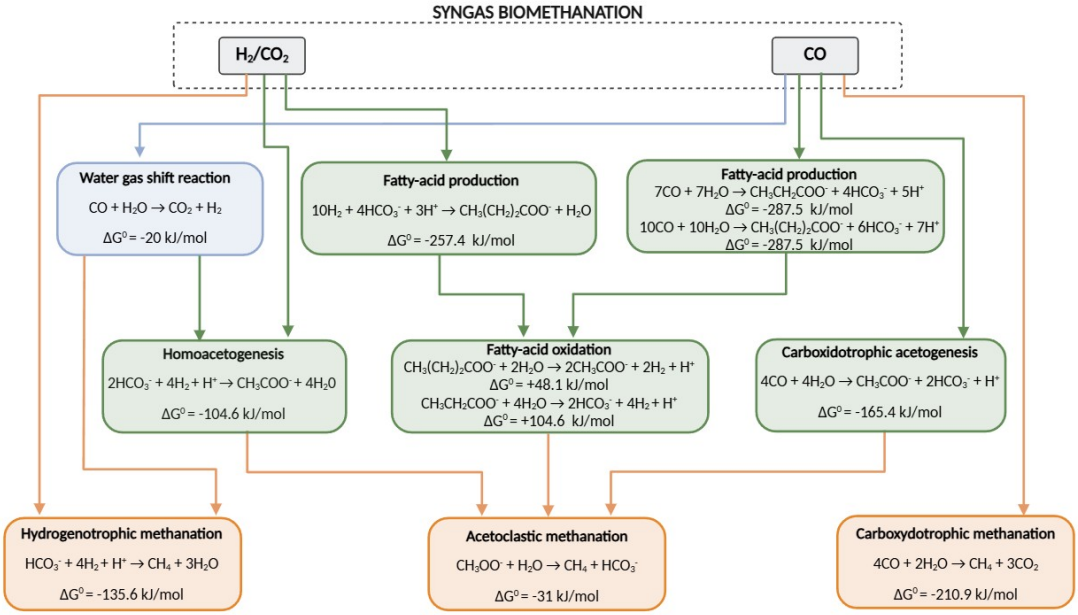

Figure 1. Adapted from Grimalt-Alemany et al., 2018. Pathways leading to the production of methane and their standard Gibbs free energy change (ΔG°). The different colours indicate different bioconversions: ● = carboxydotrophic hydrogenogenesis; ● = fermentation; ● = biomethanation.

While hydrogenotrophic methanogens commonly convert H_2/CO_2 to CH_4, the direct conversion of CO to CH_4 is rare, with only a few species capable of carboxydotrophic methanogenesis, all exhibiting slow growth (Rother and Metcalf, 2004). In open mixed microbial consortia, carboxydotrophic methanogens are outcompeted by faster-growing groups like acetogens and hydrogenogens, preventing direct biomethanation of CO. Thus, syngas biomethanation relies on syntrophic interactions among microbial groups, involving a complex network of reactions, including the water–gas shift, carboxydotrophic acetogenesis, homoacetogenesis, and hydrogenotrophic and acetoclastic methanogenesis. Understanding microbial community changes under varying conditions is essential for elucidating the population dynamics that determine the success of these processes (Grimalt-Alemany et al., 2018).

Key factors influencing the microbial conversion pathways

The carboxydotrophic and methanogenic potential of a mixed microbial consortium depends on key factors such as pH, temperature, and gas partial pressure, which should be carefully evaluated. The pH is a crucial factor for microbial growth as it influences metabolism and bioenergetics. Acetogenic bacteria tolerate a wide pH range, hydrogenogenic bacteria thrive at neutral pH, and methanogens grow best at neutral to slightly alkaline pH (6.8 to 8.5). Syngas biomethanation typically occurs at pH 7.0-7.6 to favour methanogenesis. However, the effect of pH on syngas biomethanation is not well studied (Grimalt-Alemany et al., 2018). To date, only one study has investigated pH effects, finding that the highest specific CH_4 production occurred at pH 5.8 and 1.0 atm syngas (Pereira et al., 2013). Since pH influences the active transport of substrates and products across cell membranes, as well as cellular bioenergetics, a mechanistic understanding of its predominant effects on shifting metabolic pathways would be beneficial for steering the process toward desired yields.

Temperature significantly impacts syngas biomethanation by influencing microbial interactions and metabolic pathways. At mesophilic conditions, acetate is the main precursor for methanogenesis, while hydrogen (H_2) is more important at thermophilic conditions due to increased hydrogenogenic bacteria and exergonic H_2-producing reactions (Conrad and Wetter, 1990). Higher temperatures lead to increased conversion rates, but also reduce gas solubility, which may cause mass transfer limitations (Grimalt-Alemany et al., 2018). Carbon monoxide (CO), while a substrate for carboxydotrophic microbes, is also an inhibitor for many microbial groups (Rother and Metcalf, 2004). Carboxydotrophic methanogens and sulfate-reducers are particularly sensitive to CO, while acetogens and hydrogenogens exhibit higher tolerance. Increased CO partial pressures generally lead to inhibition of methanogenic activity, reducing CH_4 yield and productivity. Additionally, a shift from acetoclastic to hydrogenotrophic methanogenesis is observed with higher CO concentrations (Sancho Navarro et al., 2016).

Significant progress has been made in understanding how operating conditions affect microbial consortia performance; however, the current literature on the syngas biomethanation process remains limited and, in some cases, contradictory (e.g., the effect of pH), making process optimization and engineering challenging. To bridge this gap, further research and a deeper understanding is needed to explore interactions between factors and their impact on population dynamics. In this context, ongoing tests are being conducted using a combination of approaches, including microbial population analysis via DNA sequencing and stable isotope analysis. Carbon stable isotopes ($\delta^{13}C$)-based calculations have previously been applied to elucidate and quantify methanogenic pathways in the anaerobic digestion of organic waste (Conrad, 2005; Rodrigues Oliveira et al., 2024; Whiticar, 1999). Isotopic analysis ($\delta^{13}C$ and $\delta^{2}H$) is a cutting-edge tool that offers a comprehensive understanding of biomethanation pathways and can provide unique mechanistic insights of syngas biomethanation.

MATERIALS AND METHODS

Experimental Design

Preliminary tests were carried out in batch mode on suspended sludge using CO as the sole substrate, with continuous monitoring of headspace pressure and analysis of gas composition and VFA concentrations at the end. However, this approach proved ineffective for a deep process understating due to the involvement of multiple microbial species in syntrophic relationships. Based on these results, a revised two-phase experimental plan (Figure 2) was designed to improve process control and monitoring. In the first phase, three continuously stirred mesophilic reactors were operated under continuous mode with a hydraulic retention time (HRT) of 14 days, to avoid methanogen washout (Rother & Metcalf, 2004). Each reactor was inoculated with 2L of suspended anaerobic sludge (12 gVS/L) from a wastewater treatment plant, previously sieved and resuspended in 0.05 M phosphate buffer at pH 7 and nutrients. The reactors differed only in the gaseous substrate supplied through the diffuser: B1 is feed with 2 mL/min of pure CO (100% v/v); B2 is feed with 2 mL/min of a H_2/CO mixture (65% H_2, 35% CO); B3 is feed with 2 mL/min of N_2, serving as a blank for monitoring residual CH_4 production. This first phase will be carried out until steady-state conditions are reached and will provide reference values for natural isotope fractionation in the individual tests (B1 and B2). In the second phase, the reactors (B1 and B2) will be operated in fed-batch mode, this time with the addition of ^{13}C-/^{2}H-labelled CO and H_2/CO substrates in the head-space up to an absolute pressure of 2 bars. The objective is to identify and confirm the predominant methanogenic pathway of the enriched cultures.

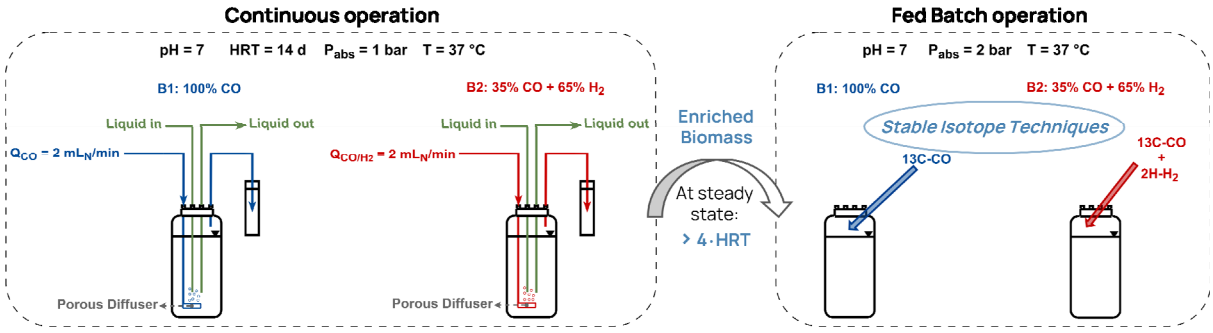

Figure 2. Experimental design overview.

Monitoring plan and analytical methods

The monitoring plan is summarized in Table 1. Volatile fatty acids (VFA) and ethanol concentrations will be measured twice per week, together with soluble chemical oxygen demand (sCOD). In addition, total chemical oxygen demand (tCOD), suspended volatile solids (VSS) and total suspended solids (TSS) will be determined once per week to assess biomass growth. Output flow rate, output gas composition, dissolved gases concentration, pH, and alkalinity will be monitored daily. Microbiological samples will be collected at the start, middle, and end of the first phase. In addition to conventional microbiological and physicochemical analyses, stable isotope analysis will be employed as an innovative technique. This approach enables the quantification of the relative contribution of different methanogenic pathways to overall CH_4 production. Specifically, this can be achieved when the stable carbon isotopic signatures of CO_2, CO, CH_4, and acetate-methyl are measured at the CH_4 production site (B1 and B2), and the isotopic fractionation factors for the conversion of CO_2, CO, and acetate-methyl to CH_4 are known (Conrad, 2005; Rodrigues Oliveira et al., 2024). These fractionation factors will be estimated through the planned isotopic analysis ($\delta^{13}C$ and $\delta^{2}H$) of the gas and of the liquid phase, integrated within the experimental design. All measurements will be validated through mass balance calculations.

Table 1. Summary of the analytical methods adopted. *CSIA = Compound specific isotope analysis

Analysis type	B1 reactor	B2 reactor	B3 reactor
VFA and other metabolites	Gas chromatography; bulk analysis of ^{13}C + ^{2}H and CSIA* of VOCs	Gas chromatography; bulk analysis of ^{13}C + ^{2}H and CSIA* of VOCs	Gas chromatography
TSS and VSS	Standard Method	Standard Method	Standard Method
Soluble/Total COD	Hack Lange kit tests	Hack Lange kit tests	Hack Lange kit tests
Dissolve gases (H_2; CO; CO_2; CH_4)	Mass spectrometry	Mass spectrometry	Mass spectrometry
Gas composition	Gas chromatography; analysis of ^{13}C + ^{2}H CSIA*	Gas chromatography; analysis of ^{13}C + ^{2}H CSIA*	Gas chromatography
Microbial composition	NGS sequencing and qPCR	NGS sequencing and qPCR	NGS sequencing and qPCR

EXPECTED RESULTS, CONCLUSIONS, AND FUTURE DEVELOPMENTS

The experiments are still ongoing, with first results expected in the coming months. However, we are confident that this innovative and integrated experimental approach will provide valuable insights into the complex processes involved in syngas biomethanation. By applying stable isotope analysis, we aim to enhance our understanding of the metabolic pathways driving the conversion of syngas into methane. Once optimized, the focus will shift to overcoming gas-liquid mass transfer challenges, which we plan to address by incorporating hollow fiber membranes in the reactors to enhance mass transfer and methane production efficiency. These efforts aim to engineer the biomethanation process and contribute to more efficient renewable energy systems.

ACKNOWLEDGEMENTS

This study was conducted within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (Piano Nazionale di Ripresa e Resilienza (PNRR) - Missione 4 Componente 2, Investimento 1.4 - D.D. 1032 17/06/2022, CN00000022).

REFERENCES

Conrad, R., 2005. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. Organic Geochemistry 36, 739–752.

Conrad, R., Wetter, B., 1990. Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. Archives of Microbiology 155, 94–98.

Grimalt-Alemany, A., Skiadas, I.V., Gavala, H.N., 2018. Syngas biomethanation: state-of-the-art review and perspectives. Biofuels Bioprod Bioref 12, 139–158.

Pereira, F.M., Alves, M.M., Sousa, D.Z., n.d. Effect of pH and pressure on syngas fermentation by anaerobic mixed cultures.

Rodrigues Oliveira, H., Mendes Anacleto, T., Carraro, G., Abreu, F., Enrich-Prast, A., 2024. A novel approach to estimate methanogenic pathways in biogas reactors via stable carbon isotope analysis. Biomass and Bioenergy 183, 107167.

Rother, M., Metcalf, W.W., 2004. Anaerobic growth of Methanosarcina acetivorans C2A on carbon monoxide: An unusual way of life for a methanogenic archaeon. Proceedings of the National Academy of Sciences 101, 16929–16934.

Sancho Navarro, S., Cimpoia, R., Bruant, G., Guiot, S.R., 2016. Biomethanation of Syngas Using Anaerobic Sludge: Shift in the Catabolic Routes with the CO Partial Pressure Increase. Frontiers in Microbiology 7.

Whiticar, M.J., 1999. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chemical Geology 161, 291–314.