Unveiling Biomethane Potential: Amino Acids' Influence on Microbial Activity

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Keywords: CO_2 fixation, syntrophy, metabolic modelling, methanogenesis, anaerobic digestion

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Introduction

In our modern world, as our societies grow, so does our need for energy. One of the direct consequences, stemming from unsustainable practices worldwide, is the generation of greenhouse gases (GHG). These gases, particularly carbon dioxide (CO_2), contribute significantly to global warming and climate change. Annually, 54.59 billion tons of GHGs are emitted, with CO_2 accounting near 76% of the total (Friedlingstein et al., 2022). In pursuit of a carbon-neutral economy, innovative methods such as CO_2 methanation and biological biogas upgrading (BBU) have emerged. These approaches aim to reduce GHG emissions by harnessing CO_2 from industrial processes or biogas and converting it into biomethane (CH_4). At the core of these processes is biotechnology, particularly the utilisation of hydrogenotrophic archaea, which play a crucial role in converting CO_2 into CH_4. Providing hydrogen (H_2) derived from renewable sources these microbes can efficiently carry out the methanogenesis in a Power-to-Gas perspective (Angelidaki et al., 2018).

Microbial communities in these processes rely on symbiotic relationships. Anaerobic systems are characterised by syntrophic acetate-oxidizing bacteria (SAOB) working in tandem with methanogens, exchanging H_2 to facilitate methane production. In a system with a constant provision of H_2, this interaction may appear hampered by thermodynamic constraints; however, previous studies have demonstrated the coexistence of these organisms (De Bernardini et al., 2022). Other metabolites, such as amino acids (AA) could be involved in syntrophic interactions between archaea-bacteria. AA play a crucial role in microbial communities' metabolic pathways and their exchanges among community members are essential for sustaining energy-intensive processes like AA biosynthesis and the citric acid cycle (Newsholme et al., 2011).

In this study, we aim to explore syntrophic relationships focusing on the impact of AA on microbial metabolism. Additionally, this work employs antibiotics and inhibitor of methanogenesis to selectively target bacteria in the community, allowing us to study the effects of AA on methane production, microbiome composition, and gene expression.

Material and Methods

A simplified thermophilic pilot-scale reactor community (55°C) fed with H_2 and CO_2 served as the inoculum. Six batch assays, each in triplicate, were conducted in 500 ml anaerobic glass bottles with a working volume of 180 ml. All bottles received 18 ml of the inoculum, along with yeast extract (3.6 mg), Wolin's vitamin solution (1.8 ml at 1X), and $Na_2S{\cdot}7\text{-}9H_2O$ (45 mg) in an anaerobic chamber. Six conditions were studied, two served as control, one providing only BA medium and a second (AA) supplemented with specific AAs (Aspartate, L-Cysteine, Leucine, and Valine). Two conditions were supplemented with antibiotics (Ab and AA+Ab), and another two with 2-Bromoethanesulfonate (BES and AA+BES). Gas feeding was a mix of H_2 and CO_2 (4:1 proportion) at 1.5 bar pressure at the beginning of the experiment and from day 6 onwards a final pressure of 2 bar. Gas composition and Volatile fatty acids (VFA) were monitored by gas chromatography, while microbial growth rates were determined by optical density. Amino acid detection and quantification were performed using UPLC-MS/MS. DNA and RNA were extracted from liquid samples and library preparation was conducted using the Illumina NovaSeq platform.

Metagenome-assembled genomes (MAGs) were reconstructed using Metabat v1,v2, CONCOCT v1.1.0, MaxBin v2.2.7 and Vamb v3.0.9. RNA-seq reads were analysed using Deseq2 v1.40.1. Draft metabolic models were automatically reconstructed based on proteomes of MAGs, refined, and gap-filled using gapseq 1.2. Community-level constraints were set based on feedstock and amino acid concentrations, with net consumption or production rates calculated, and error propagation ensured accuracy with CoCo and MICOM.

Results and Discussion

Both the control and AA conditions exhibited similar growth rate curves and methane production. Conditions with antibiotics and BES (BES, AA+BES, Ab) were inhibited and no growth or CH_4 production was observed. Notably, the addition of amino acids restored community growth hindered by antibiotics, emphasising the pivotal role of these organic compounds in shaping microbial interactions (**Fig 1A-B**). The study also delved into the consumption patterns of specific amino acids, shedding light on their significance in microbial metabolism. Community was dominated by an hydrogenotrophic archeon (*Methanothermobacter thermautotrophicus*), while the remaining organisms belonged to Firmicutes phylum, exhibiting diverse functionalities. *M. thermautotrophicus* highlighted distinct gene expression patterns in response to AAs and antibiotics. Methanogenesis-related hydrogenases, including formylmethanofuran dehydrogenase (fdh),

H_2-dependent methylene-H_4MPT dehydrogenase (hmd), hydrogenase expression/formation protein HypE showcased overexpression in the conditions where AAs were supplemented. On the contrary, two hydrogenases, coenzyme F420 oxidoreductase and anaerobic carbon-monoxide dehydrogenase (cdh) were overexpressed in absence of AAs. Transporters for aspartate, leucine/isoleucine/valine, and arginine/lysine/histidine/glutamine demonstrated condition-specific expression patterns. Moreover, genes involved in the interconversion of pyruvate to acetolactate and CO_2 in the archeon showed on averpression in the condition with AAs with more than 1.9 $\log_2$ fold change (**Fig 1C**). Moreover, our study identified six organisms putative as potential homoacetogens through the Wood-Ljungdahl pathway (2 species of *Tepidanaerobacteraceae* sp., *Moorellia* sp., *Acetivibrionales* sp. and *Moorella thermoacetica*). Metatranscriptomics-guided genome-scale metabolic modelling of microbial communities revealed the importance not only of Leucine Valine and aspartate but also two aminoacids in the incorporation of *M. thermautotrophicus*: L-Alanine and L-Glutamate. The first one, provided mainly by two bacteria: *Symbiobacterium* sp, and Limnochordia sp, meanwhile the second one produced mainly by *C. subterraneus* (**Fig 1D**).

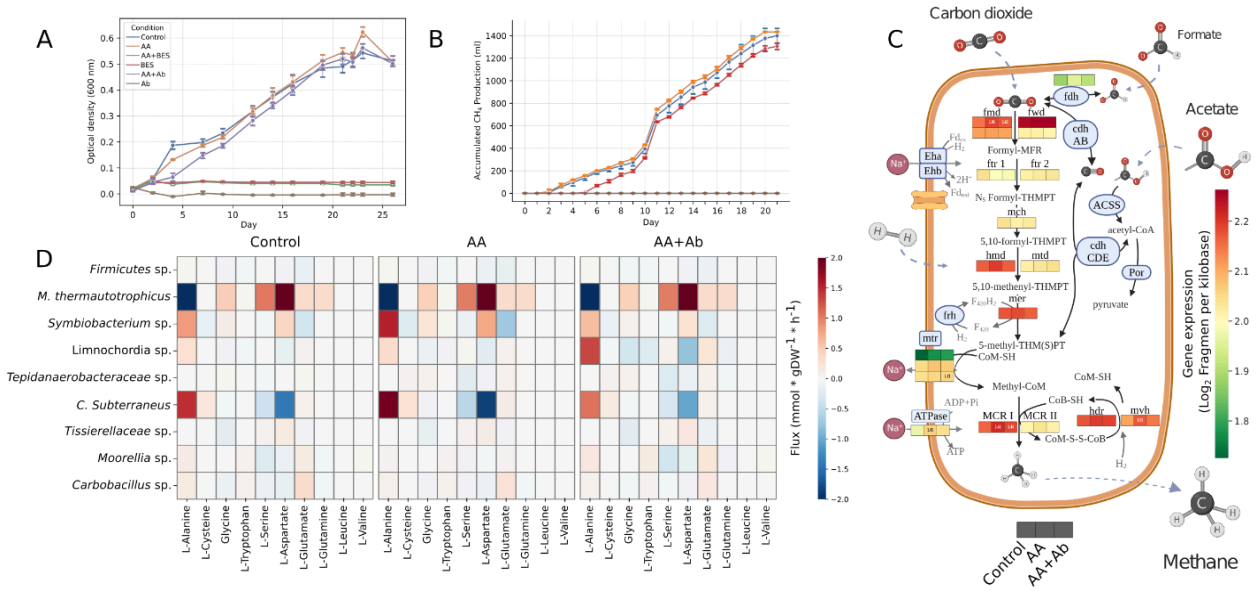

Figure 1. A. Community's growth curve (Optical density at 600 nm). **B.** Methane production (accumulated production in ml). **C.** Gene expression of methanogenesis pathway of the main archeon *M. thermautotrophicus*. Numbers inside boxes represent differential expression in ($\log_2$ fold change) compared with control. **D.** Amino acids fluxes of main bacteria and archeon.

Conclusions

Overall, our research delves into the intricate relationships within microbial communities involved in BBU processes, shedding light on their importance in achieving a more sustainable and circular economy. Through this understanding, we aim to contribute to the development of more efficient and environmentally friendly technologies for mitigating GHG emissions.

Acknowledgements

This work was supported by LIFE+ financial instrument of the European Union under the project LIFE20 CCM/GR/0001642-LIFE CO_2 to CH_4 and EMBO Postdoctoral fellowship (to E.O. - ALT 739-2022)

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