Bacterial carbon monoxide (CO) cycling via the CODH/ACS complex in compost: insights from metagenomic and gene expression analyses

K. Sobieraj[1], A. Urbanek[2], Z. Steczkiewicz[3], T. Strzała[4]

[1]Wrocław University of Environmental and Life Sciences, Department of Applied Bioeconomy, 37a Chełmońskiego Street, 51-630 Wrocław, Poland
[2]Wrocław University of Environmental and Life Sciences, Institute of Biology, Laboratory for Biosustainability, Kożuchowska 5B Street, 51-631 Wrocław, Poland
[3]Wrocław University of Environmental and Life Sciences, Institute of Environmental Biology, Division of Experimental Biology, Norwida 27B Street, 50-375 Wrocław, Poland
[4]Wrocław University of Environmental and Life Sciences, Department of Genetics, Kożuchowska 7 Street, Wrocław, 51-631, Poland
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Presenting author email: karolina.sobieraj@upwr.edu.pl

Bacterial production of carbon monoxide (CO) during biowaste composting may represent a cost-effective and environmentally friendly approach for CO generation, opening a new niche for waste-based biorefineries (Sobieraj et al., 2023). The CO market is growing due to its wide industrial applications, with demand projected to increase at a compound annual growth rate of 4.1% between 2023 and 2032 (Allied Market Research, 2024). Bacteria produce CO through the activity of the bidirectional enzyme CO dehydrogenase (CODH), which catalyzes the reversible reaction: $CO_2 + 2H^+ + 2e^- \rightleftarrows CO + H_2O$ (Jeong et al., 2015). Both aerobic and anaerobic bacteria can produce CODH. However, the structure and function of this enzyme differ between the groups (Jain et al., 2021). In aerobic microorganisms, CODH catalyzes the oxidation of CO to CO_2, whereas in anaerobes it can be either monofunctional (encoded by *cooS*) or bifunctional in complex with acetyl-CoA synthase (CODH/ACS, encoded by *acsAB*). In the latter case, within the Wood–Ljungdahl pathway during growth on H_2 + CO_2 or organic substrates, CO is generated from CO_2 by *acsA* and converted to acetyl-CoA by *acsB* (Jain et al., 2021). However, to date, this process has not been directly investigated in the context of composting.

Therefore, this study aimed to characterize the microbial community and investigate *acsA* and *acsB* gene expression during biowaste composting, providing the first direct evidence of CODH/ACS activity in this process.

Materials and Methods

The mixture of grass cuttings, branches, and food waste (mass ratio 1:1.2:0.2) was composted in four replicates (reactors 1-4). The process was carried out at laboratory scale at 45°C for 14 days in 5-L bioreactors equipped with forced aeration, controlled to maintain O_2 concentration in the headspace above 15%, and placed in a ST3 climate chamber (POL-EKO, Poland). Samples of substrates and composts were collected on days 0, 7, and 14.

Samples were subjected to total DNA extraction using the GeneMATRIX Soil DNA Purification Kit following homogenization with a bead beater. DNA quality and concentration were assessed using a Qubit fluorometer. Sequencing libraries were prepared using the Oxford Nanopore Native Barcoding Kit and sequenced on a MinION platform. Raw reads were quality-filtered, trimmed, and adapter sequences were removed using Porechop and Chopper. Cleaned reads were assembled de novo into contigs using Flye. Taxonomic profiling was performed with Kraken2 and Bracken to estimate microbial community composition. Alpha diversity was evaluated using the Shannon diversity index (Shannon, 1948), calculated at species level based on Bracken abundance tables. This metric was used to characterize temporal changes in both species richness and evenness within each reactor. Beta diversity was assessed using Bray–Curtis dissimilarity (Bray and Curtis, 1957), followed by Principal Coordinates Analysis (PCoA) to visualize global differences in microbial community structure between samples (Gower, 1966).

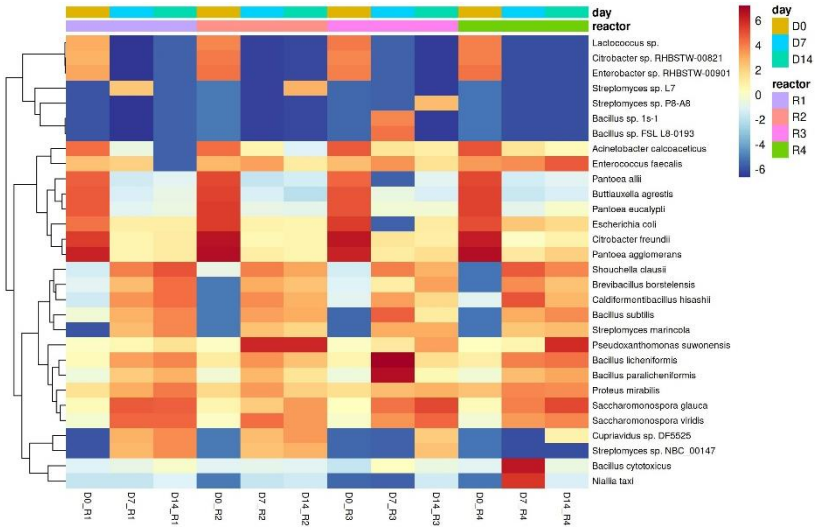

Figure 1. Top 30 microbial species across composting stages (D0–D14)

Functional potential associated with CO metabolism was evaluated through gene-centered analysis of

assembled metagenomes. Open reading frames (ORFs) were predicted from assembled contigs using Prodigal in metagenomic mode (Hyatt et al., 2010), generating translated amino acid sequences for downstream screening. Functional annotation was then performed using HMMER hmmsearch against curated hidden Markov model profiles targeting genes associated with CO transformation pathways, including *acsA* and *acsB* (Eddy, 2011). To account for differences in assembly size and coding sequence recovery between samples, raw HMM hit counts were normalized to the number of predicted ORFs and expressed as hits per 10,000 ORFs. This normalization enabled direct comparison of functional gene representation across reactors and sampling days. Temporal trends in normalized gene abundance were further examined to identify shifts in metabolic potential during compost maturation.

For transcriptomic analysis, RNA was extracted from compost samples using the RNeasy PowerSoil kit under RNase-free conditions. cDNA was synthesized and used for RT-qPCR to quantify the expression of *acsA* and *acsB* genes. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method, normalized to 16S rRNA.

Results

The heatmap showed CLR-transformed (Centered Log-Ratio transformation) relative abundances of the top 30 species across composting time points (D0, D7, D14) in four reactors (R1–R4, Fig. 1). Samples clustered mainly by time, with a clear shift from the initial stage (D0) to later stages (D7 and D14), indicating that composting strongly shaped microbial community composition. At D0, early-stage communities were characterized by higher relative abundances of taxa such as *Pantoea agglomerans*, *Escherichia coli*, and *Citrobacter freundii*. During the process (D7–D14), an increase in *Bacillus* spp. and *Saccharomonospora* spp. was observed, including species previously reported to be capable of CO production, such as *Bacillus subtilis* and *Bacillus licheniformis* (Sobieraj et al., 2024; Zhang et al., 1997).

The Shannon index, calculated at the species level, reflected changes in community diversity by capturing shifts in both richness and evenness across samples. Figure 2 shows the overall patterns of increasing diversity and evenness over time for R1 and R2. In contrast, for R3 and R4, a decrease in the Shannon index was observed on day 7 of composting, followed by an increase by day 14.

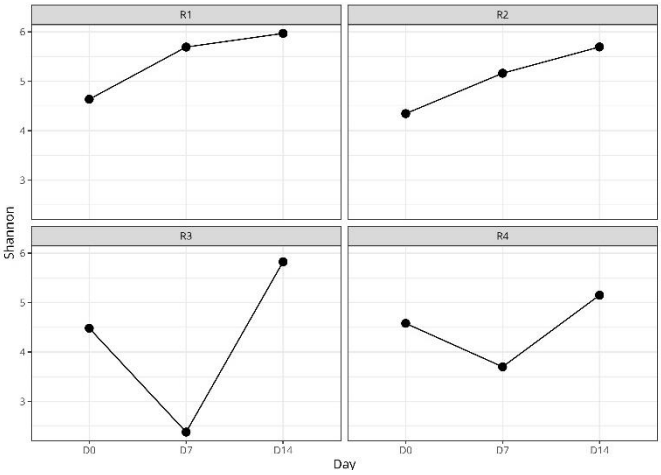

Figure 2. Shannon diversity (Bracken, species) for reactors R1–R4 in days D0/D7/D14

To further explore differences in microbial community composition between samples, beta diversity was assessed using PCoA based on the Bray-Curtis dissimilarity index. The ordinal analysis, shown in Figure 3, revealed clear divergence between samples from day 0 and those collected at later stages along the PC1 axis, which explained 57.8% of the total variance. Samples from days 7 and 14 clustered more closely, suggesting that community structure became more similar over time. Interestingly, on day 7, some temporary differences in community structure were observed in reactors R3 and R4. On day 14, these samples again showed closer clustering, reflecting the convergence of microbial communities across the different reactors.

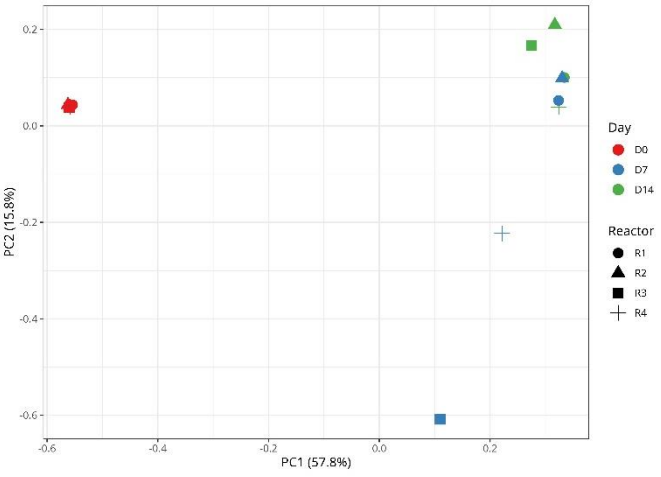

Figure 3. PCoA (Bray–Curtis) of species composition; color = day, shape = reactor

Analysis of temporal dynamics revealed a significant progressive enrichment of *acsA*, which exhibited the most pronounced absolute increase, rising from approximately 10–20 hits at day 0 to nearly 40 hits by day 14 (Kruskal–Wallis, $p = 0.015$, Fig. 4). Conversely, *acsB* abundance did not differ significantly across sampling days ($p = 0.123$ and 0.304).

The expression of the *acsA* gene showed reactor-dependent dynamics, but overall decreased during incubation (Fig. 5). A significant reduction was observed in R1 and R4

after day 7 and persisted until day 14, with relative expression decreasing from 1.0003 to 0.0018 in R1 and from 0.8781 to 0.0026 in R4 between day 0 and day 14. In contrast, R3 showed a transient increase at day 7, while R2 exhibited a different pattern with the highest expression at day 14 (3.5901 vs. 0.5968 at day 0). Analysis of *acsB* expression was limited due to incomplete datasets and revealed inconsistent patterns across reactors. In R1, a transient and statistically significant increase was observed at day 7, whereas R2 showed a non-significant decrease between day 0 and day 7. By day 14, expression decreased in all analyzed reactors (R1, R3, R4) compared to the initial time point, with statistical significance observed only for R3.

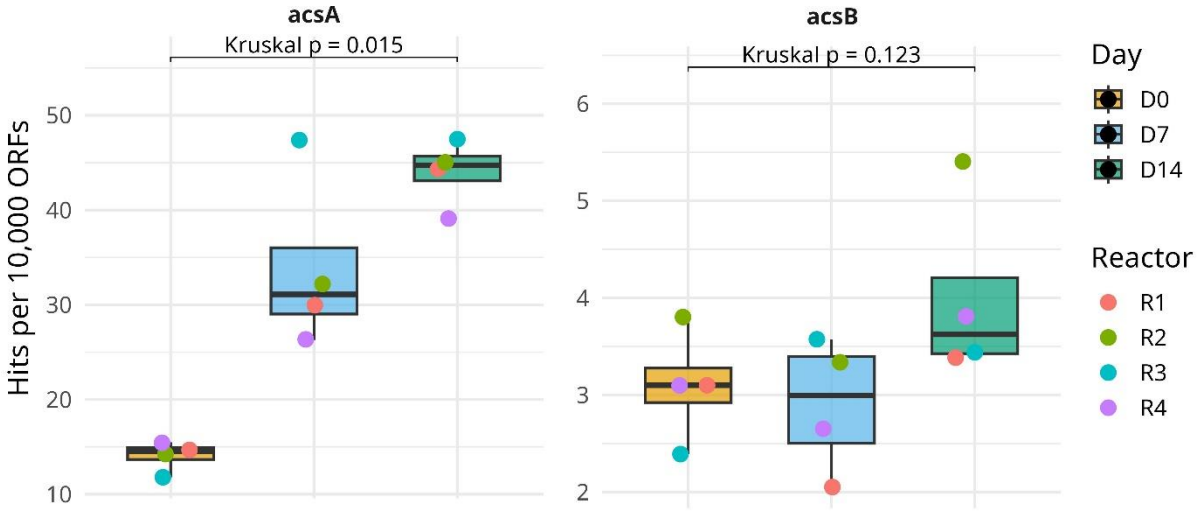

Figure 4. Temporal changes in normalized CO-related gene abundance across composting days. Values represent HMM hits normalized to 10,000 predicted ORFs. Boxplots show the distribution across reactors, while individual points correspond to reactor-specific values. Global p-values from the Kruskal–Wallis test are shown separately for each gene.

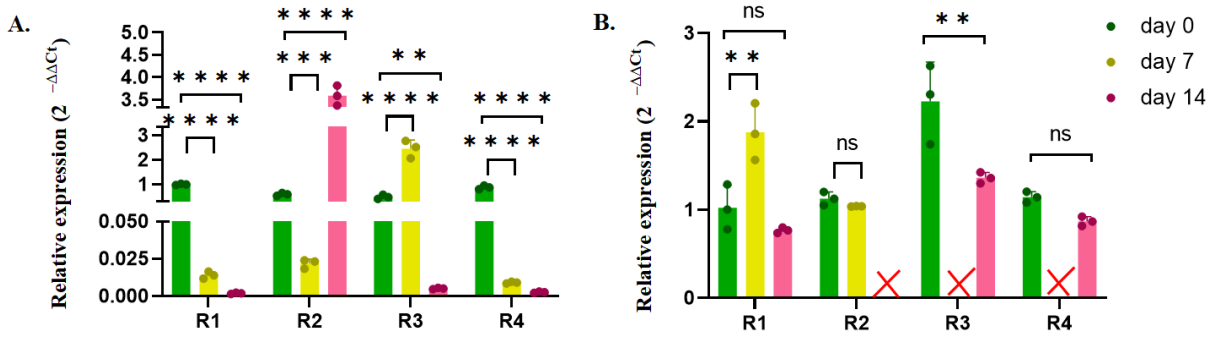

Figure 5. Temporal changes in relative expression of *acsA* (A) and *acsB* (B) genes during composting in reactors R1–R4. Statistical significance was indicated in the graphs using asterisks as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

The results demonstrate that the CODH/ACS pathway is active during biowaste composting, indicating that microbial communities are capable of CO-related metabolism in this environment. This is supported by changes in gene expression, including a general decrease in *acsA* and variable *acsB* expression, suggesting dynamic regulation of the pathway. Additionally, composting led to clear temporal shifts in microbial community structure, with an increased abundance of taxa potentially involved in CO metabolism, although the activity of this pathway appears to depend on reactor-specific conditions.

Acknowledgements

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