



Intercellular whole-cell cascade strategy for robust CO-to-formate bioconversion

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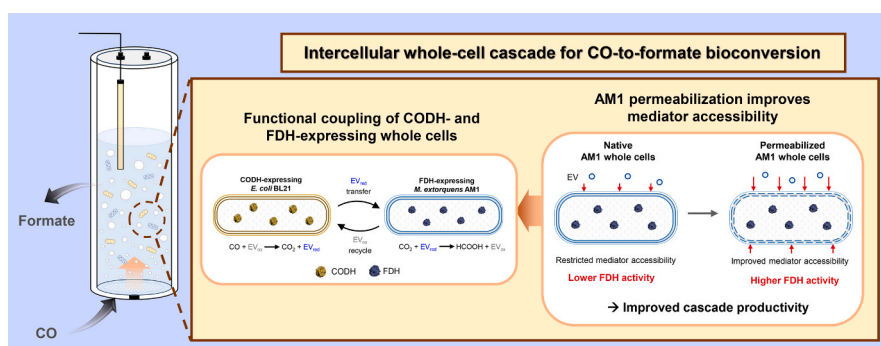
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HIGHLIGHTS

- Intercellular whole-cell cascade converted CO to formate.
- Pre-established enzyme-producing hosts were coupled via ethyl viologen.
- Whole-cell cascade retained productivity during multi-day operation.
- AM1 permeabilization improved FDH activity and cascade productivity.
- Gas-fed reactor validation showed up to 3.5-fold higher productivity.

GRAPHICAL ABSTRACT



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ABSTRACT

Biocatalytic conversion of CO-containing C1 gas to formate provides a route for C1 gas valorization, but purified carbon monoxide dehydrogenase (CODH)–formate dehydrogenase (FDH) enzyme cascades require enzyme purification and immobilization steps. As a practical alternative, this study developed an ethyl viologen (EV)-mediated intercellular whole-cell cascade by coupling CODH-expressing *Escherichia coli* with FDH-expressing *Methylobacterium extorquens* AM1. In this cascade, the two whole-cell biocatalysts performed the oxidative and reductive half-reactions, respectively, and were functionally coupled through EV redox cycling. The native whole-cell cascade enabled CO-to-formate conversion but showed lower initial productivity than the immobilized enzyme benchmark under low-EV conditions, while maintaining activity and productivity more effectively during multi-day operation. Module-level activity analysis, EV-dependent FDH kinetics, and apparent Damköhler number analysis indicated that the initial productivity penalty was mainly associated with restricted mediator accessibility in AM1 whole cells. Mild Triton X-100 permeabilization produced envelope-associated morphological changes, increased FDH activity at 0.1 mM EV by 7.9-fold, and enhanced cascade productivity by approximately 7-fold while maintaining multi-day productivity retention. In a pH-controlled gas-fed bubble-column reactor, the permeabilized cascade outperformed the native cascade under both low- and high-cell-loading conditions and approached the theoretical CO-transfer upper bound when sufficient catalyst loading was supplied. These results demonstrate that pre-established enzyme-producing hosts can be assembled into a

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robust EV-mediated intercellular whole-cell cascade, providing a practical catalyst format for CO-to-formate bioconversion without constructing a single multifunctional strain or relying on purified and immobilized enzyme catalysts.

1. Introduction

The conversion of carbon monoxide (CO)-containing C1 gas streams to formate has attracted increasing attention because it links waste-gas valorization with sustainable chemical production (Bulushev and Ross, 2018; Pacheco et al., 2023). Formate/formic acid is a versatile platform molecule for chemical synthesis and hydrogen storage, and CO-derived formate production provides a route to valorize CO-containing industrial and biomass-derived gas streams. Biocatalytic CO-to-formate processes are particularly attractive because they operate under mild, aqueous conditions compatible with bioprocess engineering (Lee et al., 2024; Shi et al., 2024).

Recent advances in enzymatic cascades employing carbon monoxide dehydrogenase (CODH) and formate dehydrogenase (FDH) have demonstrated that CO can be converted to formate with high molar productivity when suitable redox mediators, such as viologen derivatives, are supplied (Kim et al., 2025; Lee et al., 2024; Shi et al., 2024). In these systems, CODH-catalyzed CO oxidation and FDH-catalyzed CO₂ reduction are coupled through reversible oxidation–reduction of the mediator, establishing CODH–FDH cascades as a well-defined enzyme-level benchmark for CO-to-formate conversion (Lee et al., 2024). From a process-design perspective, however, implementations based on purified and immobilized enzymes generally require additional unit operations for enzyme production, purification, and support coupling, which increase catalyst-preparation complexity and may introduce support-dependent limitations during operation (Maghraby et al., 2023; Sheldon, 2007). These considerations motivate the development of complementary catalyst formats that simplify preparation while maintaining operational robustness in gas-fed aqueous bioprocesses.

In this context, whole-cell biocatalysts provide a complementary catalyst format for CO-to-formate bioconversion. They can reduce the need for enzyme purification and immobilization, while the intracellular environment may help stabilize enzymes during operation (Ladkau et al., 2014; Lin and Tao, 2017). However, constructing a single host that efficiently performs CO oxidation, CO₂ reduction, and mediator or cofactor handling may require extensive strain engineering because enzyme expression, maturation, and redox balance must be simultaneously coordinated in one cellular environment (Pinto et al., 2020; Wu et al., 2016). A conceptually simple and practical way to reduce this burden is to distribute the CODH and FDH functions across two specialized whole-cell modules, thereby allowing established host systems suited for producing each redox enzyme to be used without constructing a single multifunctional strain (Lin and Tao, 2017; Pinto et al., 2020). Nevertheless, mediator-mediated intercellular whole-cell cascades for CO-to-formate conversion remain underdeveloped, and the factors governing their productivity and performance have not been sufficiently clarified.

Distributed whole-cell redox cascades require an electron-transfer route between catalytic modules located in different cells. Soluble redox mediators, including viologen derivatives, provide a simple means of transferring electrons through the bulk liquid phase and have been widely used in cell-free enzyme cascades, bioelectrochemical systems, and single-module whole-cell redox reactions (Lee et al., 2024). However, it remains unclear whether such mediators can productively couple established host systems as distinct whole-cell modules, in which one cell module reduces the mediator and the other consumes the reduced mediator for product formation. In this format, productive coupling requires repeated mediator transfer and recycling between cell modules, as well as mediator access to intracellular redox enzymes

across cell-envelope barriers. Therefore, it is necessary to evaluate established enzyme-producing hosts as practical whole-cell catalyst modules and to clarify whether mediator transfer, recycling, and cellular accessibility can support productive intercellular CO-to-formate conversion under low-mediator conditions.

In this study, an ethyl viologen (EV)-mediated intercellular whole-cell cascade was constructed by functionally coupling CODH-expressing *Escherichia coli* and FDH-expressing *Methylobacterium extorquens* AM1 for CO-to-formate bioconversion using a CO/CO₂/N₂ gas mixture (Fig. 1). A literature-reported EV-compatible *Carboxydotherrus hydrogenoformans* CODH/*M. extorquens* FDH pair was used to focus on whole-cell catalyst-format translation rather than enzyme screening (Lee et al., 2024). By distributing the CODH and FDH functions across two pre-established enzyme-producing hosts, this strategy was designed to reduce the need for single-host strain construction and purified-enzyme preparation and immobilization while preserving modular catalyst preparation. The cascade was then evaluated to confirm CO-driven formate production, benchmarked against an immobilized enzyme format, and diagnosed to identify restricted mediator accessibility in AM1 whole cells as the major initial productivity limitation. Mild AM1 permeabilization was applied to relieve this limitation, and the permeabilized cascade was validated under gas-fed reactor operation.

2. Material and methods

2.1. Microorganisms and biocatalyst preparation

Engineered *Escherichia coli* BL21 expressing CODH from *C. hydrogenoformans* and *Methylobacterium extorquens* AM1 expressing *M. extorquens*-derived FDH were used as whole-cell biocatalysts, respectively. Detailed strain construction and cultivation procedures are provided in the [Supplementary materials](#) (Section S1). Unless otherwise noted, cell loadings are reported on a wet-cell-mass basis. Resting whole-cell biocatalysts were prepared by thawing frozen wet cell pellets, washing them with 200 mM BTP buffer (pH 7.0), and transferring them into an anaerobic chamber for final washing and resuspension in anaerobic BTP buffer. Immobilized CODH and FDH, as well as AM1 lysate used as the membrane-relieved reference, were prepared from the corresponding wet cell pellets as described in the [Supplementary materials](#) (Section S2).

2.2. Serum vial CO-to-formate assays with whole-cell cascades

All batch CO-to-formate assays in serum vials were performed in 125 mL crimp-sealed serum bottles with butyl rubber septa and aluminum caps, with a working volume of 20 mL unless otherwise noted. Reactions were carried out at 30 °C and 200 rpm using a temperature-controlled orbital shaker. The standard reaction buffer was 200 mM Bis–Tris propane (BTP, pH 7.0) containing ethyl viologen (EV) at the indicated concentration; the rationale for the EV conditions used in each assay is summarized in the [Supplementary material](#) (Table S1). All solutions and vials were equilibrated in an anaerobic chamber before sealing, and gas mixtures were added as specified for each assay.

2.2.1. Feasibility assays

For CODH-dependent CO oxidation, *E. coli* whole cells were suspended in 200 mM BTP buffer containing 10 mM EV to a final biomass loading of 0.005 g wet cell/mL. The headspace was filled with a CO/CO₂/N₂ gas mixture (50:15:35, v/v/v) to a total pressure of 2.0 bar, and

vials were incubated at 30 °C and 200 rpm. At defined time points, liquid and gas samples were withdrawn; headspace CO and CO₂ were quantified by GC and reported as liquid-volume-equivalent concentrations (mM). Formate was quantified by HPLC as described in Section 2.7.

For formate oxidation assays, AM1 whole cells were suspended in the same buffer (200 mM BTP, 10 mM EV) at 0.05 g/mL. Sodium formate was added from an anaerobic stock to a final concentration of 100 mM. Vials were sealed under an N₂ headspace, incubated at 30 °C and 200 rpm, and sampled over time to quantify formate and CO₂.

For intercellular whole-cell cascade assays at high mediator loading, *E. coli* whole cells and AM1 whole cells were co-suspended in 200 mM BTP buffer containing 10 mM EV at 0.005 g/mL *E. coli* and 0.05 g/mL AM1. The working volume was 40 mL in a 120 mL serum vial. The headspace was charged with a CO/CO₂/N₂ gas mixture (50:15:35) to a total pressure of 2.5 bar, and vials were incubated at 30 °C and 200 rpm. CO, CO₂ and formate concentrations were monitored over time, and initial CO-to-formate volumetric productivities (mM/h) were obtained from the linear region of the formate production profiles.

Control assays were performed under otherwise identical conditions by omitting CO, *E. coli* whole cells or EV. For controls evaluating the effects of H₂ and EV, N₂ was replaced with H₂ to generate a CO/CO₂/H₂ gas mixture (50:15:35), and reactions were compared in the presence and absence of EV. Formate production in these control assays is reported in Fig. S1.

2.2.2. Cascade assays under mediator-limiting conditions

For comparison of the whole-cell cascade with the immobilized CODH–FDH cascade at 0.1 mM EV, serum-vial assays were conducted. A low EV concentration of 0.1 mM was selected because mediator-lean conditions are more relevant to practical process implementation, where excessively high mediator loadings would increase material cost. *E. coli* whole cells and AM1 whole cells were suspended in 200 mM BTP

buffer (pH 7.0) at 0.02 g/mL *E. coli* and 0.04 g/mL AM1 (20 mL working volume). For the immobilized configuration, Ni–NTA resin-bound CODH and FDH prepared as described in Section 2.1 were added at an equivalent wet cell-derived loading. The headspace of each vial was filled with a CO/CO₂/N₂ gas mixture (50:15:35) to a total pressure of 2.5 bar, and vials were incubated at 30 °C and 200 rpm. CO-to-formate volumetric productivity was calculated from the slope of the formate concentration during the initial 1 h of reaction.

2.2.3. Permeabilization screening of AM1 whole cells

To screen for an effective permeabilization condition, AM1 whole cells were first treated with permeabilization reagents (EDTA, CTAB, Triton X-100 or toluene) under the conditions specified in Section 2.4. After treatment and conditioning, the cell suspensions were combined with *E. coli* whole cells in 200 mM BTP buffer (pH 7.0) containing 0.1 mM EV at 0.02 g/mL *E. coli* and 0.04 g/mL AM1. A CO/CO₂/N₂ gas mixture (50:15:35) was supplied to 2.5 bar, and the initial 1 h CO-to-formate productivity was used as the screening readout.

2.2.4. CO-to-formate production assays of native and permeabilized whole-cell cascades

For batch CO-to-formate production assays, native and permeabilized whole-cell cascades were compared under otherwise identical conditions at 0.1 mM EV. *E. coli* whole cells and native or permeabilized AM1 whole cells were suspended at 0.003 g/mL *E. coli* and 0.006 g/mL AM1 in 200 mM BTP buffer (pH 7.0, 20 mL working volume). A single charge of a CO/CO₂/N₂ gas mixture (50:15:35) was added to 2.5 bar, and vials were incubated for 13.5 h at 30 °C and 200 rpm. Formate concentrations were measured over time.

2.2.5. Multi-day productivity-retention assays of the whole-cell cascades

For multi-day productivity-retention assays, 5-day batch

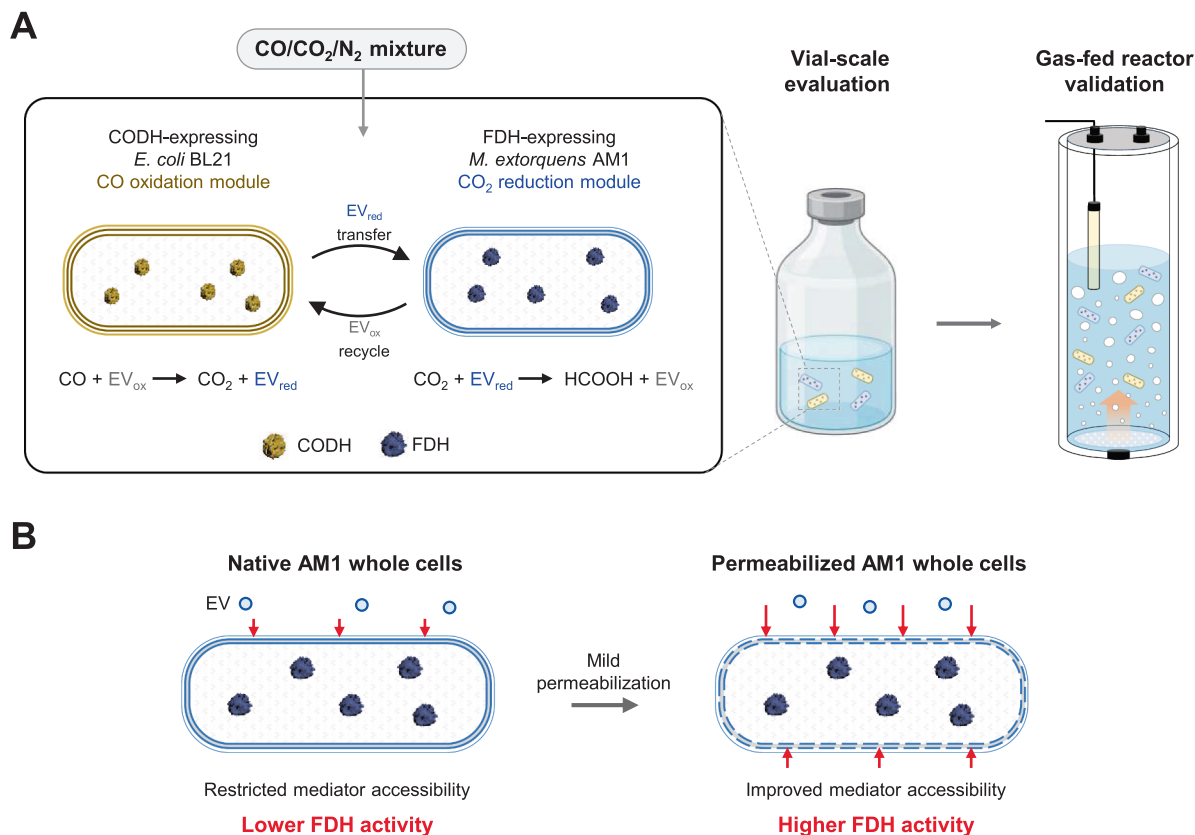


Fig. 1. Ethyl viologen-mediated intercellular whole-cell CO-to-formate cascade. (A) Functional coupling of carbon monoxide dehydrogenase-expressing *Escherichia coli* and formate dehydrogenase-expressing AM1 through ethyl viologen redox cycling. (B) Mediator accessibility in native and permeabilized AM1 whole cells.

measurements were conducted in 125 mL serum vials using 200 mM BTP buffer (pH 7.0) containing 20 mM EV, so that day-to-day changes in productivity primarily reflected catalyst retention rather than low-mediator limitation. The headspace of each vial was filled once with a CO/CO₂/N₂ gas mixture (50:15:35) to a total pressure of 2.5 bar, and the vials were incubated at 30 °C and 200 rpm for 5 days without additional gas supply. Liquid samples were taken once per day to determine formate concentrations. For each day, the average productivity over the corresponding 24 h period was calculated and normalized to the day-1 productivity of the corresponding cascade.

For the whole-cell versus immobilized cascade comparison, *E. coli* whole cells and AM1 whole cells were suspended at 0.50 mg/mL and 1.0 mg/mL, respectively. For the immobilized configuration, CODH and FDH prepared as described in Section 2.1 were added at immobilized enzyme loadings equivalent to 0.125 mg/mL (CODH, based on the *E. coli* wet-cell origin) and 0.25 mg/mL (FDH, based on the AM1 wet-cell origin).

For the native versus permeabilized whole-cell cascade comparison, *E. coli* whole cells and native AM1 whole cells were suspended at 0.50 mg/mL and 1.0 mg/mL, respectively, whereas *E. coli* whole cells and permeabilized AM1 whole cells were suspended at approximately 0.17 mg/mL and 0.33 mg/mL, respectively.

2.3. Carbon monoxide dehydrogenase and formate dehydrogenase activity assays

CODH and FDH activities were determined spectrophotometrically by monitoring the redox change of EV in quartz cuvettes inside an anaerobic chamber. Unless otherwise noted, all assays were carried out at 30 °C in 200 mM BTP buffer (pH 7.0) under strictly anaerobic conditions.

For CODH activity, reaction mixtures (total volume 2.0 mL) contained 200 mM BTP, EV at the indicated concentration (0.1 mM for process-relevant assays or 20 mM for mediator-saturating assays), and the desired amount of CODH-expressing *E. coli* whole cells or immobilized CODH. Before use, the BTP buffer was purged with 99.95 % CO for at least 1 h to saturate the solution with CO as the substrate. All components were equilibrated in the anaerobic chamber, and reactions were initiated by adding the biocatalyst to the CO-saturated EV/BTP mixture in a quartz cuvette. The increase in absorbance at 578 nm, corresponding to the formation of reduced EV (EV_{red}), was recorded with a UV-Vis spectrophotometer (Biochrom, UK). Initial CODH rates were obtained from the initial linear region of the absorbance–time trace and converted to molar rates using the molar extinction coefficient of reduced EV at 578 nm.

For FDH activity, reaction mixtures contained 200 mM BTP, 100 mM sodium formate as the substrate, EV at the desired concentration, and the appropriate FDH-containing catalyst (immobilized FDH, native AM1 whole cells, permeabilized AM1 whole cells or AM1 lysate). Because the substrate was supplied as dissolved formate, no gas purging was required. FDH activity was assayed in the formate-oxidation direction, and the reaction rate was determined from the initial linear change in EV absorbance at 578 nm under otherwise identical conditions to the CODH assays. The formate-oxidation direction was selected as a reproducible readout of mediator-dependent FDH activity because direct CO₂-reduction assays using reduced viologen can be affected by rapid radical oxidation, leading to uncertainty in initial-rate determination (Lee et al., 2024).

For mediator-saturating measurements (e.g. stability assays), the EV concentration was set to 20 mM. For EV-dependent kinetic analysis, FDH activities were measured over an EV concentration range of 0.05–2 mM for AM1 lysate and 0.1–2 mM for native AM1 whole cells and permeabilized AM1 whole cells, using preparations obtained from the same biomass. Initial rates were converted to $\mu\text{mol}/\text{min}$ using the extinction coefficient of reduced EV and fitted to the Michaelis–Menten equation to obtain apparent K_m and V_{max} values.

An apparent Da analysis for EV in AM1 whole cells was estimated from the ratio of FDH rates in lysate and whole cells under a simple membrane-diffusion model ($\text{Da} \approx v_{\text{lysate}} / v_{\text{wc}} - 1$); the underlying assumptions, parameter values, and detailed calculation procedure are provided in the [Supplementary materials](#) (Section S3). For this analysis, the AM1 lysate was treated as a membrane-relieved reference for the intrinsic catalytic rate.

In all assays, one unit (1 U) of activity was defined as the amount of catalyst that catalyzes the conversion of 1 μmol of substrate per minute under the specified conditions. Specific activities were expressed as U/g cell for whole-cell preparations (based on wet cell mass) and as U/g cell-equivalent for immobilized enzyme preparations (based on the wet cell mass from which the enzyme was obtained). This biomass-origin normalization was chosen to provide a common performance basis between whole-cell and immobilized formats without requiring explicit quantification of purified enzyme yield.

2.4. Permeabilization of *Methylobacterium extorquens* AM1 whole cells

Permeabilization of AM1 whole cells was carried out with four commonly used envelope-perturbing reagents: EDTA, cetyltrimethylammonium bromide (CTAB), Triton X-100 and toluene (all from Sigma–Aldrich). Washed wet AM1 pellets (typically 0.1–1.0 g wet cell mass) were resuspended in 30 mL of 200 mM Bis–Tris propane (BTP, pH 7.0) containing the desired concentration of the permeabilizing reagent. Suspensions were incubated at 25 °C and 100 rpm in conical tubes for 1 h.

After treatment, cells were collected by centrifugation and washed twice with aerobic 200 mM BTP to remove residual reagent. The pellets were then transferred into an anaerobic chamber and washed twice more with anaerobic 200 mM BTP buffer to minimize dissolved oxygen carryover. The final pellets were resuspended in anaerobic 200 mM BTP to the desired AM1 cell loading and used immediately in CO-to-formate cascade assays or EV-dependent FDH activity measurements.

The condition that gave the highest improvement in cascade productivity, 1 % (v/v) Triton X-100, was selected as the standard permeabilization protocol. Throughout the manuscript, AM1 whole cells treated with 1 % (v/v) Triton X-100 and subsequently washed as described are referred to as permeabilized AM1 whole cells, whereas native AM1 whole cells refer to untreated AM1 whole cells subjected to the same washing and handling procedure.

2.5. Transmission electron microscopy

Native and permeabilized AM1 whole cells were prepared for transmission electron microscopy (TEM) to visualize envelope morphology. TEM images were acquired using a JEM-2100F instrument (JEOL, Japan). Detailed TEM sample preparation procedures are provided in the [Supplementary materials](#) (Section S4).

2.6. Bubble column reactor operation

Lab-scale gas–liquid CO-to-formate conversion was performed in a 200 mL bubble column reactor with a working volume of 100 mL. The reactor was filled with 200 mM BTP (pH 7.0), and 99.99 % N₂ was purged through the liquid phase before biocatalyst addition to establish anaerobic conditions. *E. coli* whole cells and AM1 whole cells were added as wet-cell suspensions prepared as described in Section 2.1.

Two catalyst-loading conditions were used for reactor operation. Under the low-cell-loading condition, *E. coli* whole cells and AM1 whole cells were supplied at 0.004 g/mL and 0.04 g/mL, respectively (0.4 g and 4.0 g wet cells in 100 mL). Under the high-cell-loading condition, the total whole-cell loading was increased 5-fold, and *E. coli* whole cells and AM1 whole cells were supplied at 0.020 g/mL and 0.20 g/mL, respectively (2.0 g and 20 g wet cells in 100 mL). Native and permeabilized AM1 whole cells (1 % v/v, 1 h; Section 2.4) were compared

under both loading conditions. The reactor was operated at 30 °C, and pH was controlled between 6.95 and 7.05 using a pH-stat with 5 M NaOH as titrant. The gas substrate was supplied as a CO/CO₂/N₂ (50:15:35, v/v/v) at a total flow rate of 300 mL/min via a gas sparger at the bottom of the column, and the gas phase was vented through a condenser and gas outlet fitted with sterile filters. EV was present at 1 mM in all bubble-column operations to ensure sufficient mediator availability during gas-sparged reactor operation. The liquid phase was operated in batch mode for 12 h without liquid feed or withdrawal, except for periodic sampling. Liquid samples were withdrawn with syringes, immediately filtered (0.2 µm), and analyzed for formate by HPLC as described in Section 2.7. Volumetric productivities (mM/h) were obtained from the initial linear region of the formate concentration versus time profiles. The theoretical CO-transfer upper bound used for comparison was estimated from independent mass-transfer analysis based on the measured volumetric mass-transfer coefficient (k_La); the calculation procedure is provided in the Supplementary materials (Section S5).

2.7. Analytical methods

Formate was quantified by HPLC using a Hi-Plex H column with 0.005 N H₂SO₄ as the mobile phase at 0.6 mL/min. Gas-phase CO and CO₂ were quantified by GC-TCD using argon as the carrier gas and calibrated with standard gas mixtures. EV redox changes in activity assays were monitored at 578 nm by UV-Vis spectroscopy, and rates were calculated using $\epsilon_{578} = 10 \text{ mM}^{-1}\text{cm}^{-1}$. Enzyme activities were then expressed as U/g cell or U/g cell-equivalent as defined in Section 2.3. All experiments were performed at least in duplicate unless otherwise noted, and reported values represent the mean $\pm$ standard

deviation. Statistical analyses were performed according to the experimental design. Pairwise comparisons between whole-cell and immobilized-enzyme groups were analyzed using an unpaired two-tailed Welch's *t*-test. For permeabilizing-agent screening, one-way ANOVA followed by Holm–Bonferroni correction was used for multiple comparisons against the untreated control. For 5-day productivity-retention analysis of permeabilized whole cells, repeated-measures one-way ANOVA was performed across time intervals, and Greenhouse–Geisser-corrected *p*-values were used when the sphericity assumption was not met. Statistical significance is denoted as ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; and *****p* < 0.0001.

3. Results and discussion

3.1. Ethyl viologen-mediated intercellular whole-cell cascade for CO-to-formate bioconversion

To examine whether established enzyme-producing hosts could be functionally coupled through ethyl viologen (EV), CODH-expressing *E. coli* whole cells and FDH-expressing AM1 whole cells were first evaluated separately and then combined as a two-cell cascade (Fig. 2). An EV concentration of 10 mM was used in these feasibility assays to ensure clear quantification of EV-mediated substrate conversion and product formation. The resulting module- and cascade-level assays were used to determine whether EV could mediate the individual redox reactions and support CO-driven formate production in the combined whole-cell system.

The CO oxidation step was first evaluated using *E. coli* whole cells in the presence of EV under a CO/CO₂/N₂ atmosphere (50:15:35, 2.0 bar) (Fig. 2A). Cumulative CO consumption and CO₂ formation reached

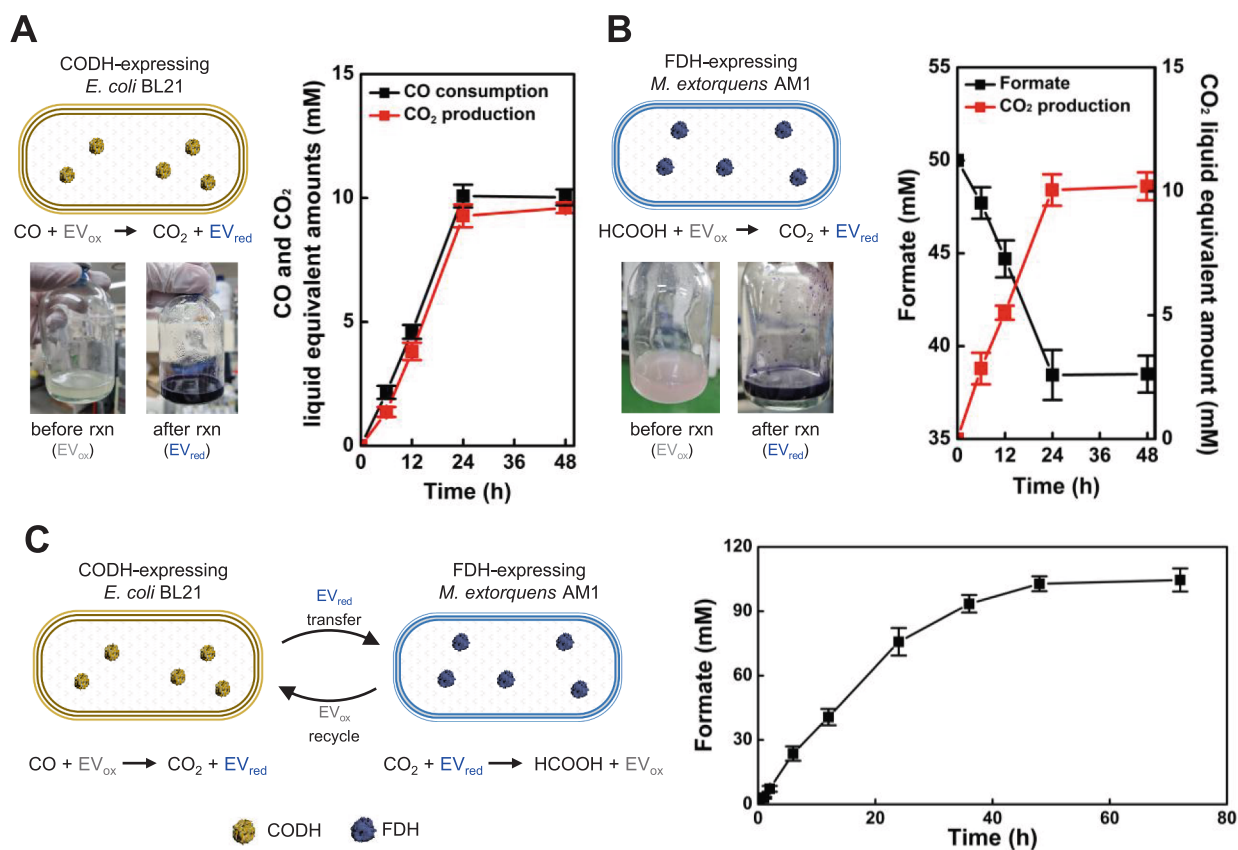


Fig. 2. Feasibility of ethyl viologen-mediated whole-cell reactions. (A) CO oxidation to CO₂ by carbon monoxide dehydrogenase-expressing *Escherichia coli* whole cells. (B) Formate oxidation to CO₂ by formate dehydrogenase-expressing AM1 whole cells. (C) Intercellular CO-to-formate conversion by the combined *Escherichia coli*–*Methylobacterium extorquens* AM1 cascade.

10.03 $\pm$ 0.32 and 9.60 $\pm$ 0.21 mM, respectively, when gas-phase amounts were expressed on a liquid-volume-equivalent basis; these values corresponded to an approximately 1:1 stoichiometric ratio between CO oxidation and CO₂ production. The extent of CO conversion was close to the initial EV loading of 10 mM, indicating that EV reduction by the *E. coli* whole-cell module proceeded nearly to the mediator-limited level. The concomitant color change of the EV solution from the oxidized to the reduced state further supported EV-mediated redox activity in the *E. coli* whole-cell module.

The FDH-expressing AM1 whole cells were then evaluated for EV-dependent formate oxidation (Fig. 2B). In the presence of 10 mM EV and 100 mM sodium formate, AM1 whole cells consumed 11.50 $\pm$ 0.99 mM formate and produced 10.20 $\pm$ 0.57 mM CO₂ on a liquid-volume-equivalent basis. These values corresponded to a near 1:1 stoichiometric ratio between formate oxidation and CO₂ formation, and the extent of conversion was comparable to the initial EV loading. The accompanying color change of the EV solution supported the reduction of EV during formate oxidation, indicating that FDH activity in AM1 whole cells could be functionally coupled to the EV redox pair under the tested conditions.

Finally, the complete intercellular whole-cell cascade was implemented by combining *E. coli* whole cells and AM1 whole cells in a single serum vial containing 10 mM EV under a CO/CO₂/N₂ atmosphere (50:15:35, 2.5 bar) (Fig. 2C). Under these conditions, formate accumulated continuously during the initial reaction period, with an average volumetric productivity of 3.44 $\pm$ 0.31 mM/h over 0–24 h. The final formate concentration approached 104.60 $\pm$ 5.37 mM, which was consistent with near-complete conversion of the initially supplied CO on a liquid-volume-equivalent basis. The gradual decrease in volumetric productivity at later reaction times was consistent with depletion of the initially supplied CO in the closed gas–liquid system.

Taken together, the module- and cascade-level assays demonstrated that EV could functionally couple CODH-expressing *E. coli* whole cells and FDH-expressing AM1 whole cells for CO-driven formate production. Source-dependency controls further showed only trace formate signals in the absence of CO, *E. coli* whole cells or EV, supporting that formate accumulation required CO-driven EV reduction by the *E. coli* whole-cell component (Fig. S1A). Additional H₂ $\times$ EV control assays showed that replacing N₂ with H₂ did not measurably alter formate accumulation in the EV-containing whole-cell cascade, whereas EV-free conditions resulted in only trace formate signals regardless of H₂ presence (Fig. S1B). These results established the feasibility of the EV-mediated intercellular whole-cell cascade and provided the basis for subsequent evaluation of the catalyst-format-dependent productivity and retention

behavior of the native whole-cell cascade.

3.2. Catalyst-format-dependent productivity and retention of the native whole-cell cascade

After confirming cascade feasibility, the native whole-cell cascade was compared with the immobilized CODH–FDH cascade under a low-EV condition (0.1 mM EV), which was selected as the process-relevant mediator loading for evaluating catalyst-format-dependent productivity (Fig. 3A). The immobilized CODH–FDH cascade served as a previously established enzyme-format benchmark for mediator-dependent CO-to-formate conversion (Kim et al., 2025; Lee et al., 2024) (Fig. 3A). At 0.1 mM EV, the immobilized CODH–FDH cascade produced formate with an initial volumetric productivity of 15.3 $\pm$ 0.5 mM/h, whereas the native whole-cell cascade reached 1.7 $\pm$ 0.1 mM/h under the same mediator loading. Thus, although EV-mediated intercellular whole-cell conversion was feasible, the native whole-cell format showed an approximately nine-fold lower initial productivity than the immobilized enzyme benchmark under low-EV conditions. This result indicated an activity limitation in the native whole-cell catalyst format; therefore, productivity retention during repeated operation was next evaluated.

For activity- and productivity-retention assays, excess EV (20 mM) was used to minimize mediator-related limitations and focus the comparison on catalyst-format-dependent retention behavior. The two catalyst formats were first compared in terms of activity retention under anaerobic aqueous storage condition (Fig. 3B). *E. coli* whole cells and AM1 whole cells maintained CODH and FDH activities close to their respective initial values over 5 days. In contrast, the immobilized enzyme format showed a gradual decrease in FDH activity, whereas immobilized CODH remained comparatively stable; the residual FDH activity of the immobilized system decreased to 63.2 $\pm$ 2.1 % of its initial value after 5 days. These results indicated that the whole-cell format retained activity more effectively under the tested storage conditions.

The productivity-retention behavior of the two catalyst formats was then evaluated during the 5-day batch accumulation assay under the same excess-EV condition to minimize mediator-related limitations during the retention assay. The productivity measured on each day was expressed relative to the day-1 value (Fig. 3C). The native whole-cell cascade maintained productivity close to its day-1 value throughout the 5-day assay, whereas the immobilized CODH–FDH cascade showed a progressive decrease in retained productivity. The retained productivity of the immobilized CODH–FDH cascade decreased to 21.2 $\pm$ 12.1 % on day 5 relative to the day-1 value. This relatively large final standard

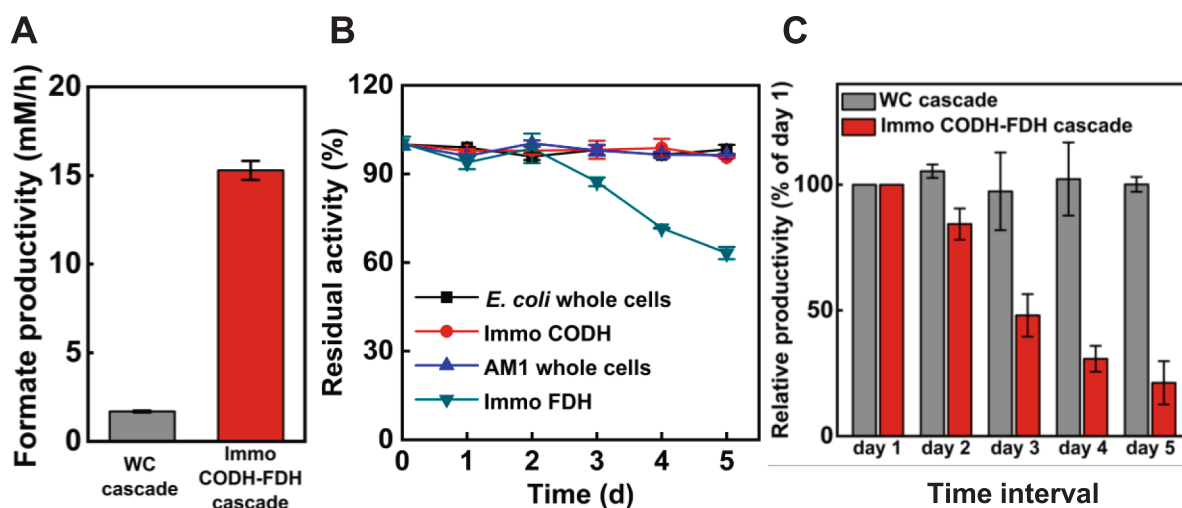


Fig. 3. Catalyst-format-dependent productivity and retention. (A) Initial productivity at 0.1 mM ethyl viologen. (B) Activity retention during anaerobic storage. (C) Productivity retention during 5-day cascade operation.

deviation was mainly associated with the low retained productivity of the immobilized cascade during the day 4–5 interval; because the additional formate accumulation during this interval was only approximately 0.8 mM, small absolute differences among replicates had a greater relative effect on the calculated interval productivity, as supported by the primary formate accumulation profile (Fig. S2). Because the immobilized CODH–FDH cascade was prepared using a Ni-NTA affinity support, the possible contribution of passive enzyme leakage was also evaluated. The Ni-NTA-immobilized cascade was used as a previously reported immobilized enzyme benchmark with high initial retained activity, rather than as an optimized long-term immobilization format. In a leakage control assay, immobilized CODH and FDH were incubated in serum vials at 30 °C and 200 rpm under an N₂ atmosphere without supplying the CO/CO₂/N₂ reaction gas mixture. Although this control assay did not fully reproduce the reactive cascade condition, cumulative enzyme leakage remained below 10 % over 5 days for both immobilized enzymes (Fig. S3), indicating that the decrease in retained productivity was unlikely to be explained solely by passive enzyme leakage from the Ni-NTA support. Enzyme deactivation or stability loss during repeated operation may also have contributed. These results showed that the higher activity retention of the whole-cell format was also reflected in multi-day batch cascade operation.

Taken together, the results in Fig. 3 revealed distinct catalyst-format-dependent performance characteristics. The immobilized CODH–FDH cascade provided higher initial productivity under low-EV conditions, whereas the native whole-cell cascade showed higher activity and productivity retention during multi-day operation. Thus, the native whole-cell cascade was not simply a lower-performing version of the immobilized enzyme benchmark, but a catalyst format with distinct strengths and limitations, showing improved retention behavior despite lower initial productivity. The source of this initial productivity penalty was therefore examined by module-level analysis in the following section.

3.3. Module-level analysis of the initial productivity penalty in the native whole-cell cascade

The source of the lower initial productivity of the native whole-cell

cascade was next examined at the module level. First, CODH- and FDH-associated activities were compared between the immobilized enzyme benchmark and the corresponding whole-cell biocatalysts under the same low-EV condition used for catalyst-format productivity comparison (0.1 mM EV) (Fig. 4A). EV-dependent FDH kinetics and apparent Damköhler number (Da) analysis were then performed using AM1 whole cells and AM1 lysate as a membrane-relieved reference to evaluate whether the AM1-associated activity penalty was consistent with restricted mediator accessibility (Fig. 4B, C).

For the CODH side, the specific activities of immobilized CODH and *E. coli* whole cells were 539.50 ± 21.92 and 307.50 ± 10.61 U/g cell, respectively, corresponding to a 1.7-fold difference. This relatively small activity gap indicated that *E. coli* whole cells retained substantial EV-dependent CODH activity. In contrast, the FDH activities of immobilized FDH and AM1 whole cells at the same EV concentration were 19.43 ± 0.22 and 0.58 ± 0.03 U/g cell, respectively, corresponding to an approximately 33.5-fold difference. Thus, the apparent activity penalty was much more pronounced on the AM1/FDH side than on the *E. coli*/CODH side. These results suggested that the low initial productivity of the native whole-cell cascade was primarily associated with the AM1 whole-cell component, motivating further analysis of EV-dependent FDH activity in AM1 whole cells.

To further examine the AM1-associated activity limitation, EV-dependent FDH kinetics were compared between AM1 whole cells and AM1 lysate, which was used as a membrane-relieved reference prepared from the same biomass (Fig. 4B). FDH activities were measured in the formate-oxidation direction over an EV concentration range of 0.05–2 mM for AM1 lysate and 0.1–2 mM for AM1 whole cells. The initial rates were fitted to the Michaelis–Menten equation to obtain apparent kinetic parameters. AM1 lysate exhibited an apparent K_m of 0.54 ± 0.03 mM and an apparent V_{max} of 143.6 ± 6.9 U/g cell, whereas AM1 whole cells showed a higher apparent K_m of 1.2 ± 0.1 mM and a markedly lower apparent V_{max} of 7.6 ± 0.4 U/g cell. Thus, compared with the membrane-relieved lysate reference, AM1 whole cells exhibited an approximately two-fold increase in apparent K_m and an approximately 19-fold decrease in apparent V_{max} . This kinetic pattern was consistent with restricted mediator accessibility in intact AM1 whole cells,

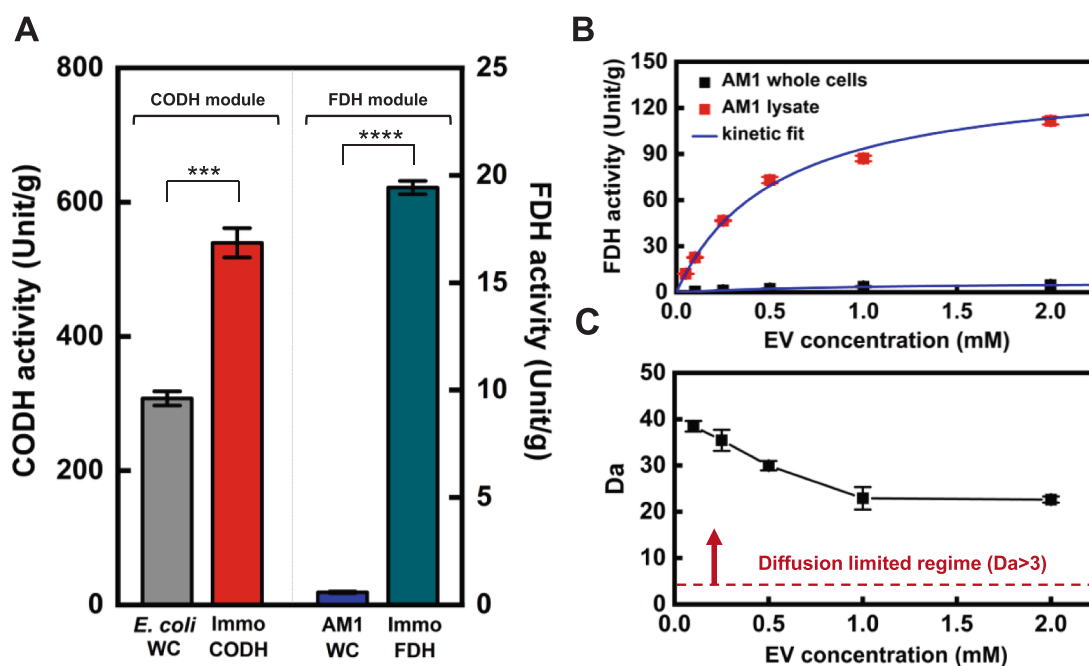


Fig. 4. Module-level analysis of the initial productivity penalty. (A) Carbon monoxide dehydrogenase- and formate dehydrogenase-side activities at 0.1 mM ethyl viologen. (B) Ethyl viologen-dependent formate dehydrogenase activity of AM1 whole cells and lysate. (C) Apparent Damköhler number analysis. Statistics in (A): unpaired two-tailed Welch's *t*-test.

although contributions from the intracellular microenvironment cannot be fully excluded.

An apparent Damköhler number (Da) analysis was then performed to further evaluate the contribution of mediator accessibility to the observed AM1 whole-cell FDH rate (Fig. 4C). Using AM1 lysate as the membrane-relieved reference for the catalytic rate, Da was approximated as $Da \approx v_{\text{lysate}}/v_{\text{wc}} - 1$, where v_{lysate} and v_{wc} denote the FDH rates measured in the lysate and AM1 whole cells, respectively. Across the EV concentration range where both lysate and whole-cell rates were available (0.1–2 mM), the calculated Da values remained well above 1, ranging from 22.6 ± 0.7 at 2 mM EV to 38.5 ± 1.2 at 0.1 mM EV. These values further supported that the apparent FDH activity of AM1 whole cells was strongly constrained by mediator accessibility under the tested low-EV conditions, rather than by intrinsic FDH catalytic capacity alone.

Taken together, the module-specific activity comparison, EV-dependent FDH kinetics, and Da analysis indicated that the initial productivity penalty of the native whole-cell cascade was mainly associated

with the AM1 side. Because the AM1 lysate served as a membrane-relieved reference, the lysate-to-whole-cell activity gap, elevated apparent K_m , reduced apparent V_{max} , and Da values well above unity provided functional evidence that restricted EV access to intracellular FDH, rather than loss of intrinsic FDH catalytic capacity, was the dominant AM1-side limitation (Kasche, 1983; Ni and Chen, 2004; Riley et al., 1997). Based on this interpretation, mild permeabilization of AM1 whole cells was next evaluated as a practical intervention to improve EV-dependent FDH activity and cascade productivity.

3.4. Mild permeabilization improves mediator-dependent activity and cascade productivity

Based on the AM1-associated mediator-accessibility limitation identified in Section 3.3, mild permeabilization was evaluated as a practical strategy to improve EV-dependent FDH activity while retaining the whole-cell catalyst format (Fig. 5A). The effect of permeabilization

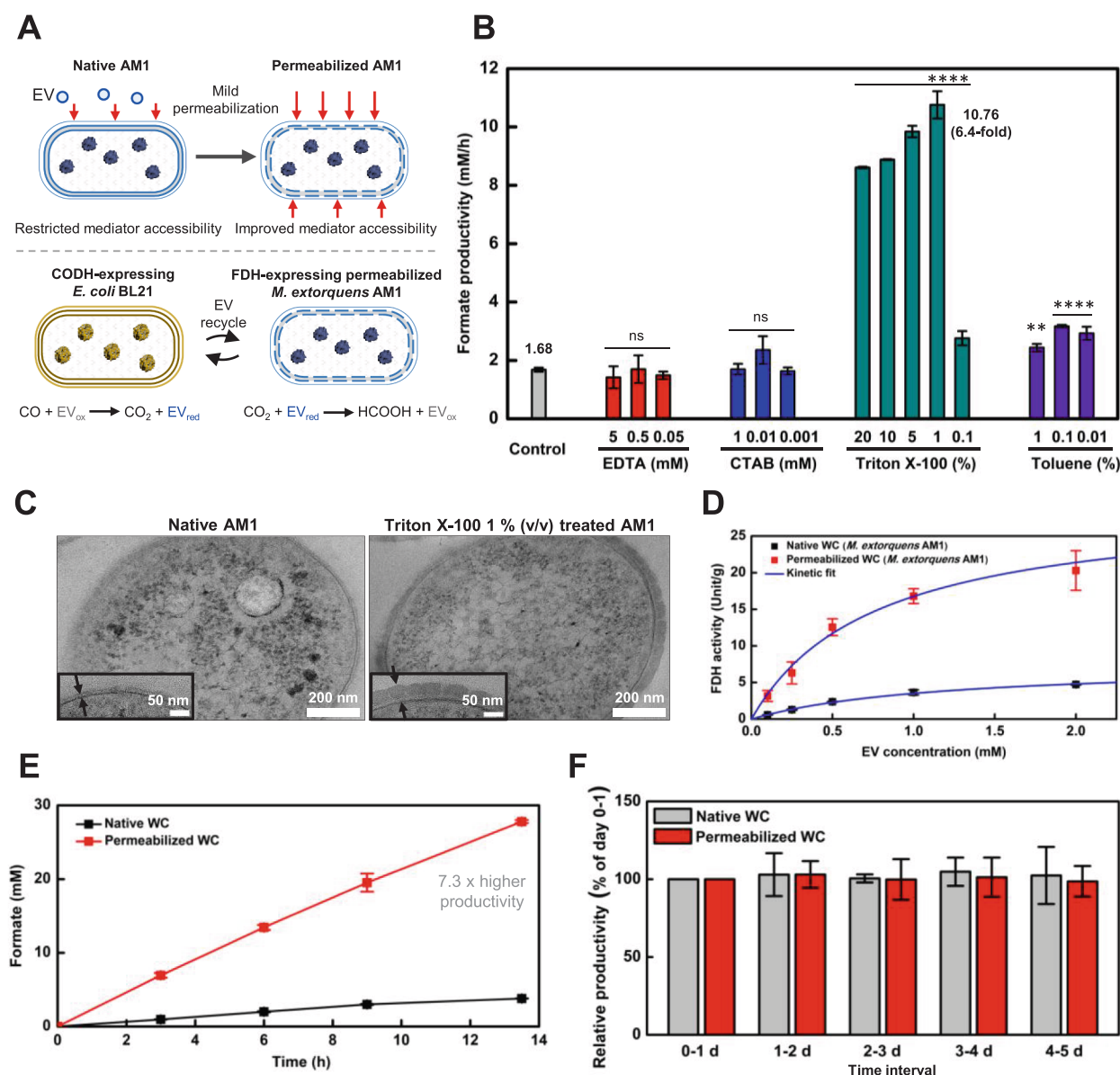


Fig. 5. Effect of AM1 permeabilization on cascade productivity and retention. (A) Permeabilization concept. (B) Reagent screening based on 1 h cascade productivity. (C) Transmission electron microscopy images of native and Triton X-100-treated AM1 whole cells. (D) Ethyl viologen-dependent formate dehydrogenase activity. (E) Batch CO-to-formate production. (F) Multi-day productivity retention. Statistics: analysis of variance with Holm–Bonferroni correction was used in (B), and repeated-measures analysis of variance was used in (F), where no significant time-interval effect was observed (Greenhouse–Geisser-corrected $p = 0.640$).

was assessed through a stepwise evaluation consisting of reagent screening, morphological observation, EV-dependent AM1 FDH activity measurement, cascade productivity testing, and multi-day productivity-retention analysis (Fig. 5B–F).

To identify an effective treatment, AM1 whole cells were treated with representative, commonly used permeabilization reagents that perturb Gram-negative cell envelopes through different mechanisms, including EDTA, CTAB, Triton X-100, and toluene (De Smet et al., 1978; Patil et al., 2017), and the treated cells were evaluated in the EV-mediated intercellular whole-cell cascade using the initial 1 h formate productivity at 0.1 mM EV as an integrated screening metric (Fig. 5B). Among the treatments tested, EDTA, CTAB, and toluene did not substantially improve cascade productivity over the tested concentration ranges. In contrast, Triton X-100 treatment increased the CO-to-formate rate, with the highest productivity observed at 1 % (v/v). Under otherwise identical conditions, the volumetric productivity increased from 1.68 ± 0.07 mM/h for native AM1 whole cells to 10.76 ± 0.47 mM/h after treatment with 1 % Triton X-100, corresponding to a 6.4-fold increase. Lower Triton X-100 concentrations produced smaller improvements, whereas concentrations above 1 % did not provide additional gains. These results identified 1 % Triton X-100 treatment as the most effective condition for improving cascade productivity under the tested low-EV condition.

Before morphological observation, short-term integrity checks were performed after the selected Triton X-100 treatment. Cell turbidity remained unchanged after Triton X-100 treatment and during short-term incubation, and no detectable FDH leakage into the supernatant was observed during the same period. Transmission electron microscopy was then used to examine whether Triton X-100 treatment produced envelope-associated morphological changes in AM1 whole cells (Fig. 5C). Native AM1 whole cells showed a compact envelope region, as also observed in the magnified envelope insets. In contrast, AM1 whole cells treated with 1 % (v/v) Triton X-100 retained their overall cell morphology but showed localized expansion and partial loosening of the envelope region, consistent with membrane-associated alterations reported for detergent-treated Gram-negative bacteria (Duché and Houot, 2019; González-Mañas et al., 1990). These observations indicated that the selected Triton X-100 condition induced detectable envelope-associated morphological changes while largely retaining the whole-cell morphology.

The module-level effect of permeabilization was first evaluated by comparing EV-dependent FDH activity between native and permeabilized AM1 whole cells over the tested EV concentration range (Fig. 5D). Under otherwise identical assay conditions, permeabilized AM1 whole cells displayed higher FDH activity than native AM1 whole cells across the EV range examined. This improvement was especially evident at 0.1 mM EV, the mediator loading used for low-EV cascade experiments, where Triton X-100 treatment increased FDH activity from 0.58 ± 0.04 to 4.57 ± 0.08 U/g cell, corresponding to a 7.9-fold enhancement. Fitting of the EV-dependent activity data further showed that permeabilization reduced the apparent K_m from 1.2 ± 0.1 to 0.76 ± 0.16 mM and increased the apparent V_{max} from 7.6 ± 0.4 to 29.6 ± 3.1 U/g cell. These changes indicated that mild AM1 permeabilization improved EV-dependent FDH performance, and the recovery of FDH activity after envelope perturbation further supported the interpretation that mediator accessibility was a major constraint in native AM1 whole cells.

Having confirmed that Triton X-100 treatment improved EV-dependent FDH activity in AM1 whole cells, the effect of AM1 permeabilization was next evaluated at the full-cascade level (Fig. 5E). Native and permeabilized whole-cell cascades were constructed by combining native *E. coli* whole cells with either native or permeabilized AM1 whole cells, respectively, and operated under otherwise identical low-EV conditions. After 13.5 h, the native whole-cell cascade produced 3.80 ± 0.03 mM formate, whereas the permeabilized whole-cell cascade produced 27.80 ± 0.21 mM formate. This corresponded to an

approximately 7-fold increase in cascade-level volumetric productivity, indicating that the improvement in EV-dependent AM1 FDH activity was translated into enhanced CO-to-formate productivity in the combined whole-cell cascade; the corresponding apparent mediator-based turnover metrics are summarized in Table S2.

The multi-day productivity retention of the native and permeabilized whole-cell cascades was then compared under the same operating conditions, with the productivity measured on each day expressed relative to the day-1 value (Fig. 5F). In both cases, the relative productivity remained close to the day-1 level throughout the 5-day assay, and no progressive decrease was observed for the permeabilized whole-cell cascade. The residual FDH activity of permeabilized AM1 whole cells was also maintained over 5 days under the tested storage conditions (Fig. S4). These results indicated that AM1 permeabilization increased cascade productivity without causing a detectable loss of productivity retention under the tested conditions. Taken together, the results in Fig. 5 demonstrated that mild Triton X-100 treatment improved EV-dependent FDH activity in AM1 whole cells, increased the productivity of the EV-mediated intercellular whole-cell cascade, and maintained multi-day productivity retention under the tested conditions.

3.5. Gas-fed reactor validation of the permeabilized whole-cell cascade

To examine whether the productivity improvement achieved by AM1 permeabilization was retained under gas-fed reactor operation, native and permeabilized whole-cell cascades were compared in a pH-controlled bubble-column reactor operated with continuous CO/CO₂/N₂ gas sparging (Fig. 6A). Reactor experiments were conducted at 1 mM EV to ensure sufficient mediator availability during gas-sparged operation, and low- and high-cell-loading conditions were tested to examine the effect of AM1 permeabilization across different catalyst loadings. The resulting formate production profiles were compared with the theoretical CO-transfer upper bound estimated from independent mass-transfer analysis (Section S5).

Under the low-cell-loading condition, both native and permeabilized whole-cell cascades produced formate at rates below the theoretical CO-transfer upper bound, indicating that catalyst-side activity still contributed strongly to the observed productivity (Fig. 6B). The permeabilized whole-cell cascade showed faster formate accumulation throughout the 12 h reactor operation, reaching 93.18 ± 9.02 mM formate compared with 26.28 ± 3.50 mM for the native whole-cell cascade. The corresponding average volumetric productivities were 7.76 ± 0.75 and 2.19 ± 0.29 mM/h, respectively, corresponding to an approximately 3.5-fold increase. This result demonstrated that the productivity improvement achieved by AM1 permeabilization was retained after transition from serum-vial assays to gas-fed reactor operation. The smaller fold increase compared with the 0.1 mM vial assay was consistent with the higher EV concentration used in the reactor experiment, which was expected to partially alleviate mediator limitation in native AM1 whole cells.

When the total whole-cell loading was increased 5-fold, the permeabilized whole-cell cascade still showed higher formate productivity than the native whole-cell cascade, although the productivity gap became smaller than that observed under the low-cell-loading condition (Fig. 6C). During the first 12 h of reactor operation, the native whole-cell cascade reached 120.75 ± 12.09 mM formate, whereas the permeabilized whole-cell cascade reached 330.65 ± 24.25 mM formate, corresponding to average volumetric productivities of 10.06 ± 1.01 and 27.55 ± 2.02 mM/h, respectively. Thus, AM1 permeabilization increased reactor volumetric productivity by approximately 2.7-fold under the high-cell-loading condition. Notably, the productivity of the permeabilized whole-cell cascade slightly exceeded the theoretical CO-transfer upper bound of 25.1 mM/h estimated from independent mass-transfer analysis (Section S5), likely due to the enhanced gas-liquid mass transfer in the high-density cell suspension, whereas the native whole-cell cascade remained substantially below this limit. These results

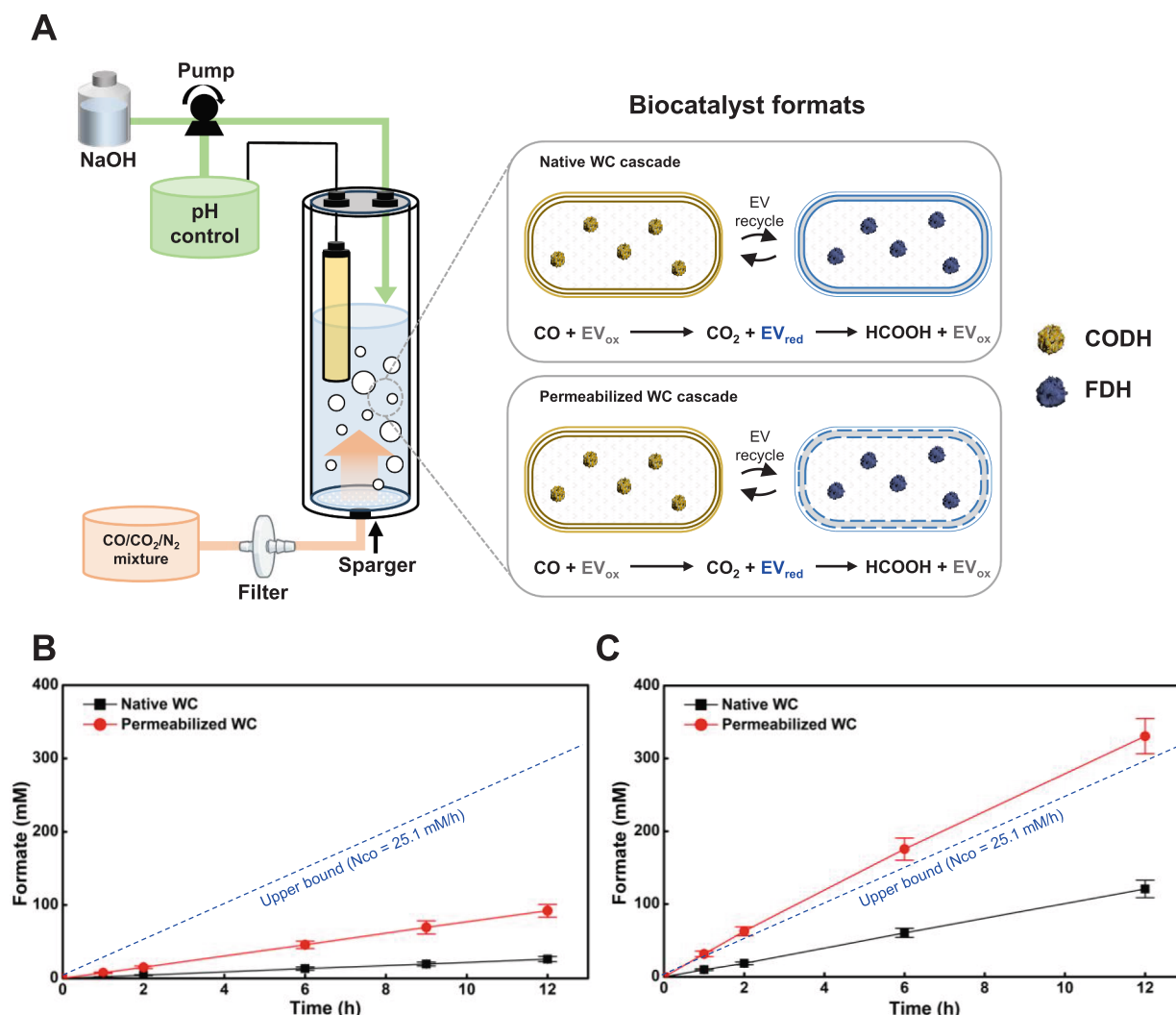


Fig. 6. Gas-fed reactor operation of native and permeabilized whole-cell cascades. (A) Reactor schematic. (B) Low-cell-loading operation. (C) 5-fold higher-cell-loading operation. Data in (B) and (C) are presented as mean $\pm$ standard deviation.

indicated that AM1 permeabilization reduced the catalyst-side limitation sufficiently for CO transfer capacity to become a more prominent constraint under high-cell-loading reactor operation.

Taken together, the reactor data in Fig. 6 demonstrated that the productivity enhancement achieved by AM1 permeabilization was retained under pH-controlled gas-fed reactor operation. The permeabilized whole-cell cascade outperformed the native whole-cell cascade under both low- and high-cell-loading conditions, and its productivity approached the theoretical CO-transfer upper bound when sufficient catalyst loading was supplied. These findings indicate that AM1 permeabilization can improve EV-mediated intercellular whole-cell CO-to-formate conversion in gas-fed reactor systems.

4. Conclusion

An ethyl viologen-mediated intercellular whole-cell cascade composed of carbon monoxide dehydrogenase-expressing *Escherichia coli* and formate dehydrogenase-expressing *Methylobacterium extorquens* AM1 was established for CO-to-formate bioconversion. The native cascade confirmed functional coupling between two specialized whole-cell biocatalysts through mediator redox cycling, but showed lower initial productivity than the immobilized enzyme benchmark under low-mediator conditions. Module-level activity analysis, mediator-dependent formate dehydrogenase kinetics, and apparent Damköhler

number analysis indicated that this productivity penalty was mainly associated with restricted mediator accessibility in AM1 whole cells. Mild Triton X-100 permeabilization improved mediator-dependent formate dehydrogenase activity and increased cascade productivity while maintaining multi-day productivity retention. Under pH-controlled gas-fed reactor operation, the permeabilized cascade outperformed the native cascade and approached the theoretical CO-transfer upper bound. Overall, this format couples pre-established enzyme-producing hosts without single-host construction or purified-enzyme immobilization; greener mediators remain targets after enzyme-level compatibility validation.

CRedit authorship contribution statement

Tae-Hwan Kim: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Jang-Seob Lee:** Investigation. **Jinwon Lee:** Resources. **Yong Hwan Kim:** Resources. **Jeong-Geol Na:** Writing – review & editing, Supervision, Funding acquisition. **Byung-Keun Oh:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2026.135310>.

Data availability

Data will be made available on request.

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