

Supplementary Information

Enhancing Butanol Fermentation via Improved Interspecies Electron Transfer in a *Clostridium* Syntrophic Co-culture Using a Biochar-Methyl Viologen Composite

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S1. Media composition

The composition of the seed culture medium is (L^{-1}): 5.0 g fructose or 30.0 g glucose, 5.0 g yeast extract, 2.0 g asparagine, 2.0 g $(NH_4)_2SO_4$, 2.4 g CH_3COONa , 1.0 g K_2HPO_4 , 1.0 g KH_2PO_4 , 1.0 g $NaCl$, 0.01 g $MnSO_4 \cdot H_2O$, 0.75 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.0002 g $Na_2WO_4 \cdot 2H_2O$, 0.004 g 0.004 g terephthalic acid, 0.001 g Resazurin, 0.3 g L-cysteine HCl, 10 mL/L vitamin solution and 10 mL/L trace metal solution,¹ and adjusted to pH 6.50–6.80.

The modified Turbo-CGM medium was prepared as described previously, supplemented with extra $CaCO_3$ (5 g/L).² The P2 medium contained the following components per liter: 2.0 g fructose, 80.0 g glucose, 2.0 g yeast extract, 2.4 g CH_3COONH_4 , 0.5 g K_2HPO_4 , 0.5 g KH_2PO_4 , 0.01 g $NaCl$, 0.01 g $MnSO_4 \cdot H_2O$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.0002 g $Na_2WO_4 \cdot 2H_2O$, 0.004 g terephthalic acid, 0.001 g Resazurin, 0.3 g L-cysteine HCl, 5 g $CaCO_3$. The P2+ medium was supplemented with 10 mL/L vitamin solution and 10 mL/L trace metal solution.

S2. Preparation of Biochar-Methyl Viologen Composite (BM)

BC700 was mixed with MV at varying ratios, followed by the addition of ethanol until the mixture was fully submerged. The mixture was then sonicated for 15 min and dried in an oven at 60 °C. To remove unadsorbed MV, the solid was then soaked with approximately 20 mL of phosphate buffer (pH 8.0) and rinsed under vacuum filtration with an additional portion of the same buffer, totaling 100 mL. After washing, the material was dried again at 60 °C to obtain the biochar-MV composite with varying

MV loadings, designated as BM. The absorbance of the filtrate obtained by vacuum filtration was measured at 260 nm using a UV-visible spectrophotometer. The mass (mg) of the soluble electron mediator fixed on the biochar (BC) was calculated using the following formula:

$$Q_{MV} = (10 \times Q_1 - (A_{260} \times 10.296 + 0.0729)) \times 100 \quad (1)$$

where Q_{MV} is the actual fixed amount of MV (mg); A_{260} is the absorbance at 260 nm; Q_1 is the initial mass of MV mixed with BC (g).

The amount of BM required for fermentation to achieve a target MV concentration was calculated as:

$$Q = \frac{C \times 257.16 \times V}{Q_{MV}} \times Q_2 \quad (2)$$

where Q is the mass of BM to be added per bottle (g); C is the target MV concentration (mM); V is the fermentation volume (mL); Q_2 is the mass of dried BM (g).

BM samples were prepared by mixing 1.0 g BC700 with 0.4, 0.8, 1.2, 1.6, or 2.4 g MV. Approximately 0.225 g of the resulting BM was added to 30 mL of fermentation medium to achieve final MV concentrations of 3, 5, 7, 11, or 15 mM, designated as BM-n mM ($n = 3, 5, 7, 11, 15$).

S3. Electrochemical testing

The testing process is carried out according to the literature description, with slight modifications.³ The electrochemical tests were performed by adding 5 mL buffer solution to the cylinder and continuously purged with N_2 to remove oxygen. The mediator oxidation potential (MEO) was set at +0.40 V, and the mediator reduction

potential (MER) was set at -0.49 V. After the background current stabilized, $40 \mu\text{L}$ of the corresponding mediator was added. After the peaks tended to be stable, 0.1 g of ground sample was added into system. The subsequent electron transfer (oxidation/reduction) of the sample produced a measurable current, yielding the current-time (I-t) curve reflecting the electron-donating or electron-accepting process of the material. Electron accepting capacity (EAC) and electron donating capacity (EDC) can be calculated by the following formulas:

$$\text{EAC} = \frac{\int \frac{I_{\text{MER}}}{F} dt}{m_{\text{biochar}}} \quad (3)$$

$$\text{EDC} = \frac{\int \frac{I_{\text{MEO}}}{F} dt}{m_{\text{biochar}}} \quad (4)$$

Where I_{MEO} and I_{MER} represents the current generated in MEO and MER, respectively, F ($= 96485 \text{ sA/mol } e^-$ m_{biochar}) is the Faraday constant, m_{biochar} (g) is the mass of biochar tested.

S4. Calculation Methods

Carbon recovery rate refers to the proportion of carbon elements converted into products during the fermentation process using sugar and acetate as carbon sources.

The calculation formula is:

$$\text{Carbon recovery rate}(\%) = \frac{n_1 * N_1 + n_2 * N_2 + \dots + n_6 * N_6 + n_7 * N_7}{n_{0h1} * N_{0h1} + n_{0h2} * N_{0h2}} \times 100\% \quad (5)$$

where n_i represents the molar amount (mol) of products (ethanol, acetate, isopropanol,

butanol, butyrate, 2,3-butanediol, and glucose) at the end of fermentation; n_{0h_1} and n_{0h_2} denote the molar amounts (mol) of glucose and acetate at time 0 h, respectively; N_i denotes the number of carbon atoms in each substance. It is worth noting that the carbon recovery reported here is defined on the basis of quantifiable aqueous-phase metabolites and does not account for carbon incorporated into biomass, consistent with the convention adopted in comparable sugar-based fermentation studies.

Butanol yield refers to the proportion of consumed glucose converted into butanol.

The calculation formula is as follows:

$$\text{Butanol yield}(\%) = \frac{c_{\text{butanol}}}{c_{\text{glucose}}} \times 100\% \quad (6)$$

where c_{butanol} represents the butanol concentration in the product (g/L), and c_{glucose} denotes the amount of glucose consumed (g/L).

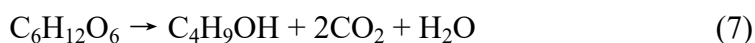
S5. The Stoichiometric Equation

System Boundary Definition:

Single strain: *C. acetobutylicum* fermentation only

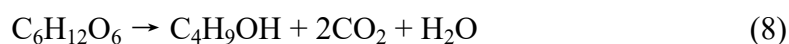
Co-culture: *C. acetobutylicum* fermentation + *C. ljungdahlii* CO₂ re-assimilation

(1) *C. acetobutylicum* single strain:



(Maximum butanol yield: 0.41 g/g glucose; Carbon recovery: 66.7%)

(2) *C. acetobutylicum* and *C. ljungdahlii* co-culture system (without external reducing power):

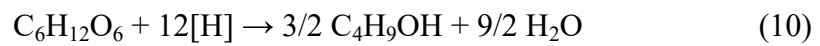


(Maximum butanol yield: 0.41 g/g glucose)



(Maximum carbon recovery: 100%)

(3) *C. acetobutylicum* and *C. ljungdahlii* co-culture system (with external reducing power ([H])):



(Maximum butanol yield: 0.617 g/g glucose; Maximum Carbon recovery: ~100%)

Table S1. Comparison of the BET surface area, pore volume, average pore size, and CO₂ adsorption capability for different BC/BM

Sample	BET (m ² /g)	Pore volume (cm ³ /g)	Average pore size (nm)	CO ₂ adsorption capability (mmol/g)
BC400	5.53	0.019	13.70	0.79
BC550	7.52	0.022	11.66	1.23
BC700	143.13	0.083	2.32	1.77
BC800	104.64	0.066	2.52	1.00
BM700	13.01	0.021	6.38	1.27

Table S2. Pearson correlation analysis of the characteristic properties of BC400, BC550, BC700, and BC850 with the corresponding butanol titer and butanol yield

	I _D /I _G	Electrical conductivity	EDC	EAC	Butanol titer	Butanol yield
BC400	1.908	0.906	0.195	0.009	12.42	0.31
BC550	1.412	5.761	0.122	0.015	16.48	0.37
BC700	1.457	12.012	0.107	0.024	17.16	0.39
BC850	1.170	12.469	0.086	0.037	14.63	0.37
r ₁	-0.58	0.63	-0.70	0.29	-	-
r ₂	-0.81	0.87	-0.91	0.62	-	-

r₁: Pearson correlation coefficient between BC properties and butanol titer; r₂: Pearson correlation coefficient between BC properties and butanol yield.

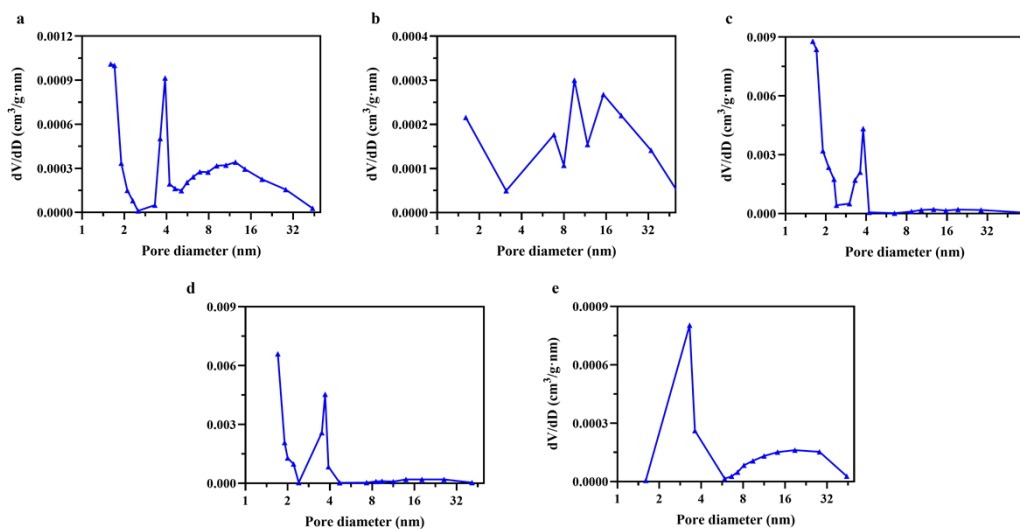


Fig.S1 The BJH pore size distribution curves of BC400(a), BC550 (b), BC700 (c), BC800 (d), and BM700 (e).

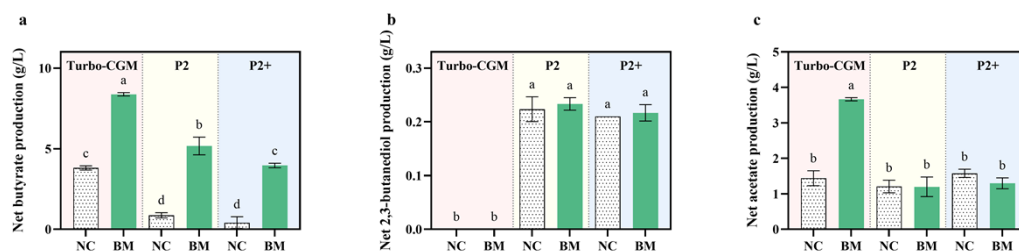


Fig.S2 The net production of butyrate (a), 2,3-butanediol (b), and acetate (c) in NC/BM systems at different medium composition.

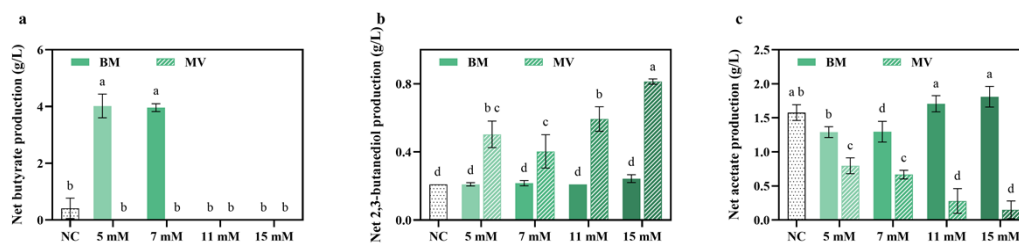


Fig.S3 The net production of butyrate (a), 2,3-butanediol (b), and acetate (c) in BM systems at different MV concentration.

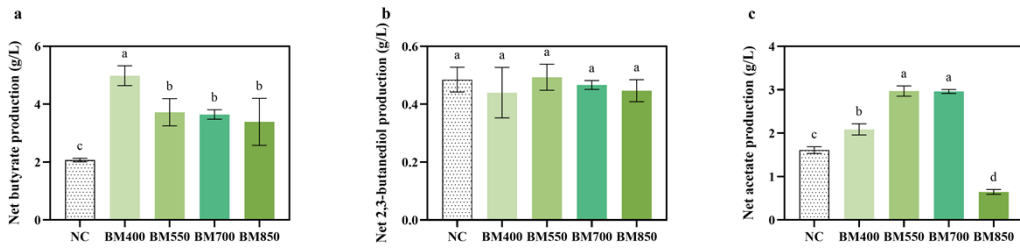


Fig.S4 The net production of butyrate (a), 2,3-butanediol (b), and acetate (c) in BM systems prepared from corn stover at different pyrolysis temperatures.

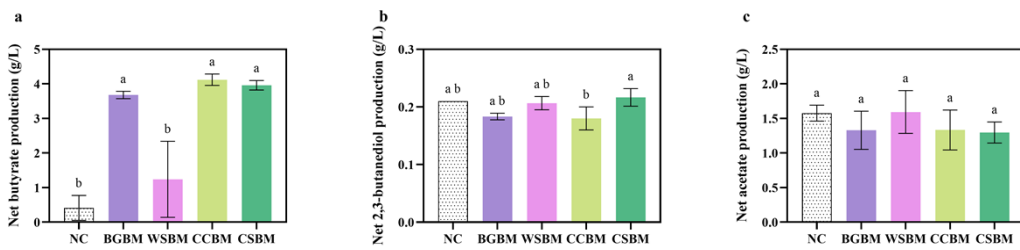


Fig.S5 The net production of butyrate (a), 2,3-butanediol (b), and acetate (c) for BM700 derived from corn stover (CSBM), wheat straw (WSBM), barley grass (BGBM), and corn cob (CCBM).

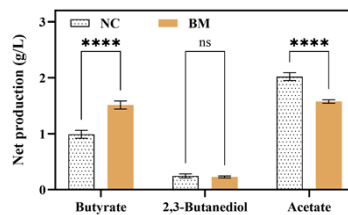


Fig.S6 The net production of butyrate, 2,3-butanediol, and acetate under optimized conditions.

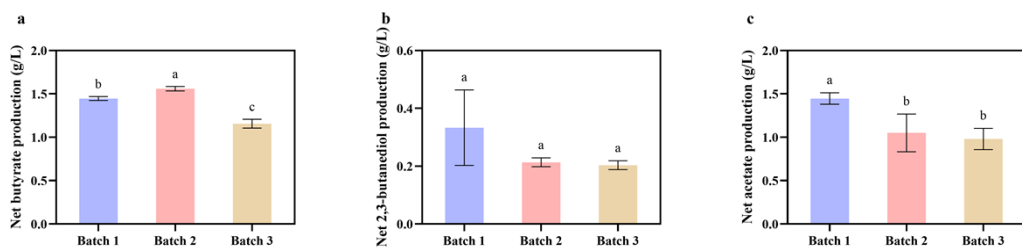


Fig.S7 The net production of butyrate (a), 2,3-butanediol (b), and acetate (c) under repeated-batch fermentation.

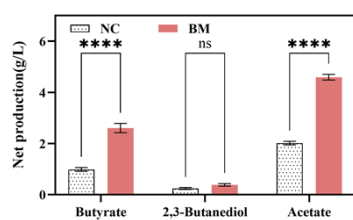


Fig.S8 The net production of butyrate, 2,3-butanediol, and acetate under low-temperature conditions.

References

- 1 W. Li, C. Cheng, J. Zhao, Q. Li, M. Liu, C. Wang and C. Xue, *ACS Sustain. Chem. Eng.*, 2024, **12**, 13244–13252.
- 2 K. Charubin and E. T. Papoutsakis, *Metab. Eng.*, 2019, **52**, 9–19.
- 3 F. Lü, X. Lu, S. Li, H. Zhang, L. Shao and P. He, *Chem. Eng. J.*, 2022, **429**, 132203.