

Supplementary Data

Enhanced methane production during long-term UASB operation at high organic loads as enabled by the immobilized Fungi

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Text S1 Fungus culture conditions

Fungal strains were maintained on fungal culture medium (potato dextrose agar medium (PDAM)) at 4°C in the laboratory. Several agar plugs were taken from the periphery of fungal colonies on PDAM plates, and then cultivated for 5–7 days at 30°C, 150 rpm in PDM (PDAM without agar). Glucose and agar were then added to give final concentrations of 15–20 g/L, respectively. Mycelia were collected from cultures in clean bench (AIRTECH, Suzhou, China) and then followed by centrifugation (12,000 × *g*, 10 min). 7.5 ± 1.5 g (wet weight) inoculum was suspended in the 100 mL sterilized deionized water to make a fungus suspension. All media and experiment materials related to fungus were autoclaved at 121°C for 30 min before use.

Text S2 Preparation of AEBs

Firstly, an aqueous mixture containing the above fungus suspension, 3–4% (w/v) sodium alginate solution, and 6–9% (w/v) polyvinyl alcohol (PVA) was prepared. Secondly, the mixture was added dropwise by micro syringe pump to the saturated solution of CaCl₂–boric acid under slow magnetic stirring to form AEBs (about 3 mm in size). Afterwards, AEBs were kept in the CaCl₂–boric acid for 2 h and immersed in the 1.5% (w/v) glutaraldehyde solution for 20 min. Finally, AEBs were rinsed with deionized water and stored in sterile environment for use.

Text S3 Optimization of the sludge-to-AEBs ratio

The effects of different ratios (100:5, 100:10, 100:20, and only AEBs included) of sludge and AEBs on methane production were investigated, and the modified Gompertz equation was used to produce the optimal proportion.

$$M = P \times \exp \left\{ - \exp \left[\frac{Rm \times e}{P} (\lambda - t) + 1 \right] \right\} \quad (1)$$

P, the maximum methane yield (mL g VSS⁻¹); *Rm*, the methane production rate (mL/(g VSS d)); *e*,

Euler's constant; λ , the methane generation lag phase (day). The fitting parameters are listed in Table S1.

Text S4 Composition of synthetic wastewater

Beef extract 0.60 g/L, yeast extract 0.60 g/L and peptone 1.80 g/L are used to provide the nutrient. The mineral elements concentrated solution consisted of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.10 g/L and $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g/L. Trace elements contained of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.50 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.10 g/L; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.10 g/L; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g/L; H_3BO_4 , 0.01 g/L; $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.01 g/L; ZnCl_2 , 0.13 g/L; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.15 g/L; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.03 g/L (Morita et al., 2011). The buffer was served by NaHCO_3 .

Text S5 PCR procedure

The PCR reactions were conducted using the following program: an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s; an annealing step at 50 C for 45 s and an elongation step at 72°C for 10 min. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of $5 \times$ FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The resultant PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™ -ST (Promega, USA). Purified amplicons were pooled in equimolar ratio and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Analysis of data was performed with the Majorbio online analysis (<http://www.majorbio.com/>).

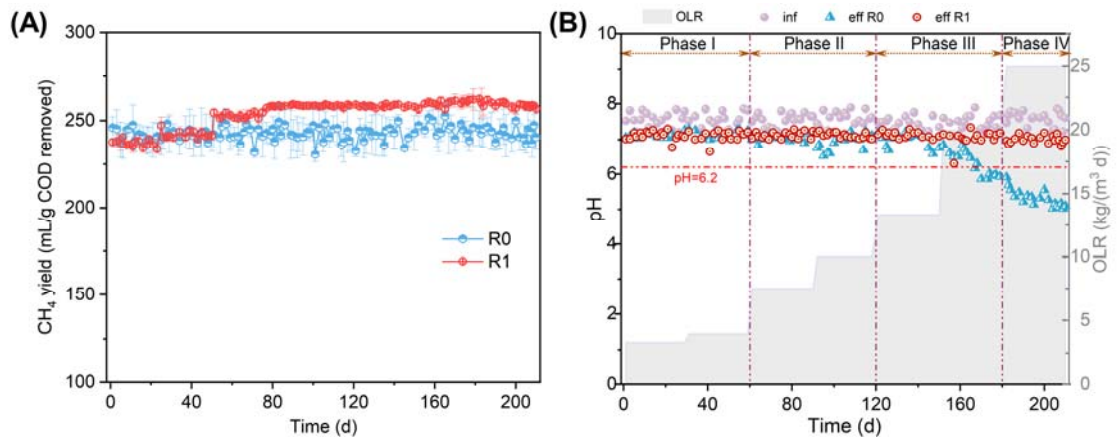


Fig. S1. (A) CH₄ yield and (B) pH variation of influent (inf) and effluent (eff). (The red line represents pH=6.2.)

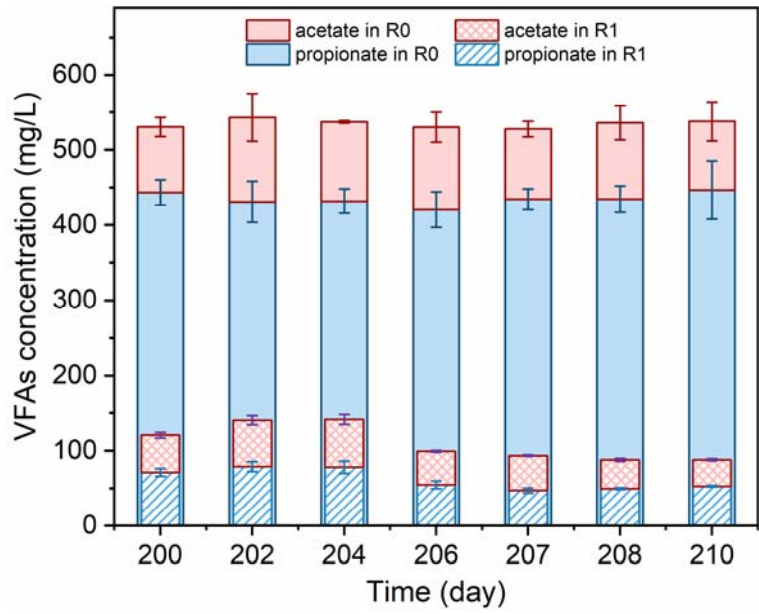


Fig. S2. Variation in the composition of the VFAs in Phase IV. (Other VFAs concentration is not shown in order to more clearly show the main components.)

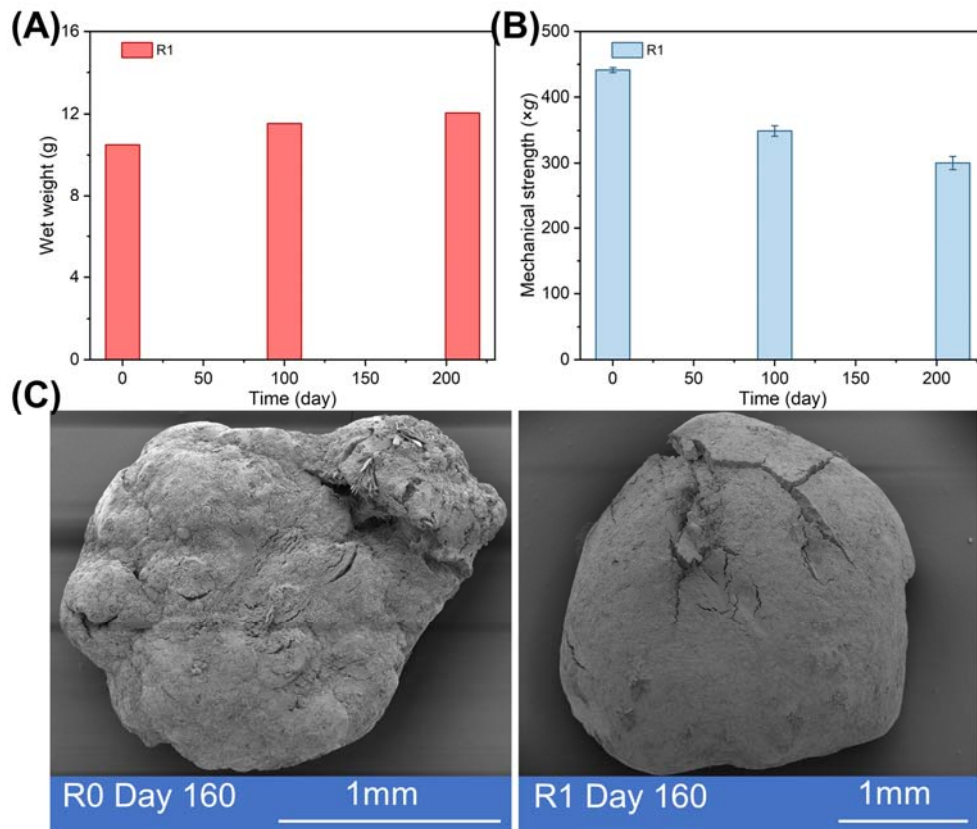


Fig. S3. (A) Average wet weight and (B) mechanical strength of AEBs in R1 during different phases of operation,

$n = 5$. (C) SEM image of granular sludge in R0 and R1 on Day 160 with an OLR of $20.0 \text{ kg}/(\text{m}^3 \text{ d})$.

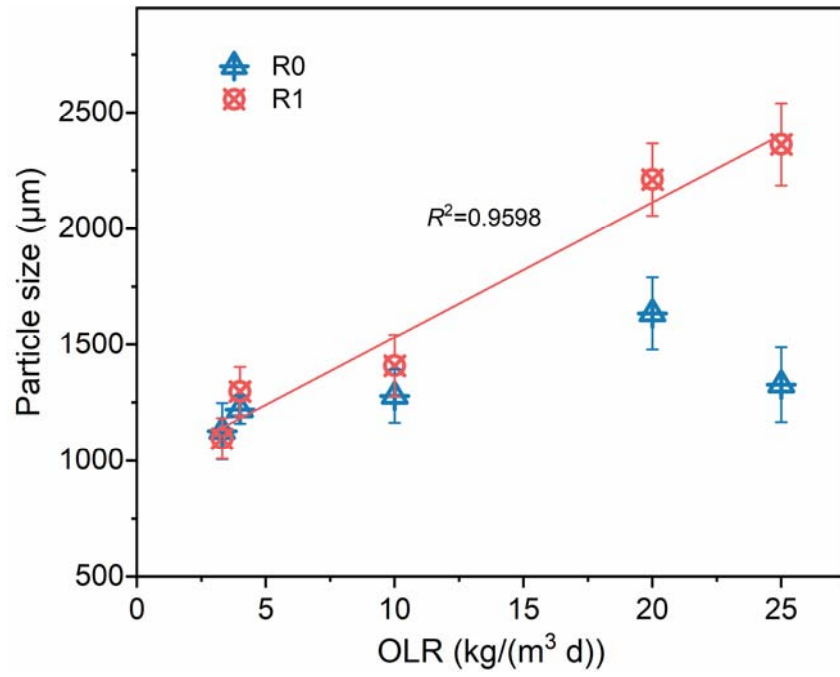


Fig. S4. Correlation analysis between mean particle size (diameter) and OLR. (Samples were randomly collected on Day 20, 50, 100, 160 and 200. $50 < n < 60$, error bar means standard deviation.)

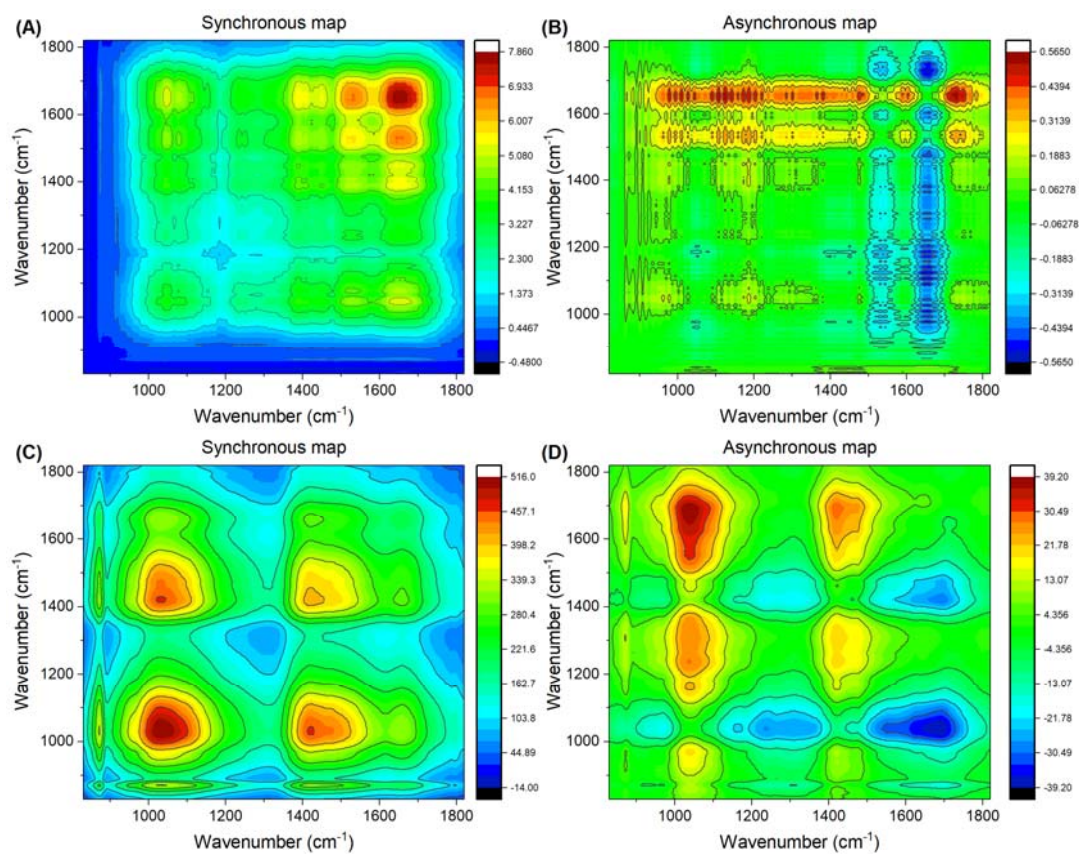


Fig. S5. (A, C) Synchronous and (B, D) asynchronous 2D correlation maps generated from 800–1850 cm^{-1} region of the FT-IR spectra of anaerobic sludge in R0 (A, B) and R1 (C, D) along with the increasing OLR. (The seed sludges and other sludge samples were collected on Day 0, 50, 100, 160 and 200. Positive and negative values represent positive and negative correlations, respectively. Higher color intensity indicates a stronger positive or negative correlation.)

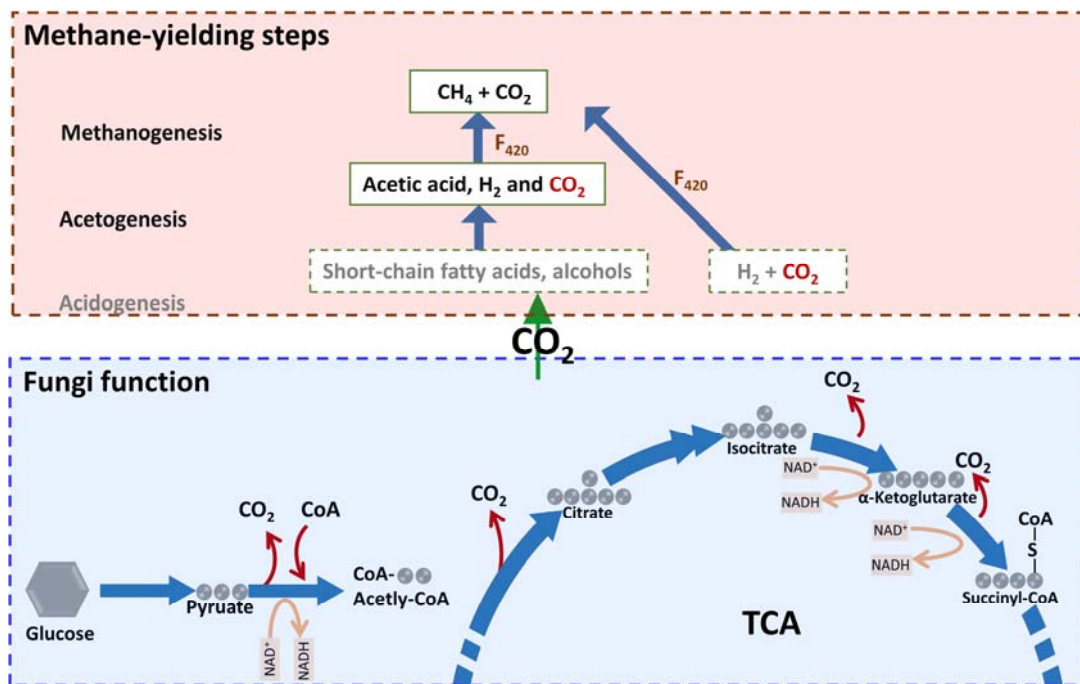


Fig. S6. Possible routes of fungi in promoting methanogenesis.

Table S1 Methanogenic parameters based on the modified Gompertz equation

Group	The maximum methane yield (mL/g VSS)	The methane rate (mL/(g VSS d))	The methane generation lag phase (d)	r^2
Sludge	295.2 ± 16.0	44.3 ± 4.1	1.0 ± 0.3	0.98855
Sludge + 5% AEBs	264.8 ± 7.6	64.0 ± 5.9	2.0 ± 0.2	0.99305
Sludge + 10% AEBs	350.1 ± 33.3	56.8 ± 7.5	2.5 ± 0.4	0.98027
Sludge + 15% AEBs	442.8 ± 13.7	68.6 ± 3.8	1.7 ± 0.2	0.99653
Sludge + 20% AEBs	299.9 ± 21.4	37.2 ± 3.2	0.7 ± 0.3	0.98803
15% AEBs	NA	NA	NA	NA

Table S2 Operating characteristics of continuous operation

Reactor & Inoculum	Inoculum total suspended sludge (g/L)	Phase I (Day 0–60)		Phase II (Day 61–120)		Phase III (Day 121–180)		Phase IV (Day 181–210)				
		COD (mg/L)	HRT (h)		COD (mg/L)	HRT (h)		COD (mg/L)	HRT (h)			
			Day	Day		Day	Day		Day	Day		
			0–30	31–60		61–90	91–120		121–150	151–180	181–210	
R0 (Seed sludge without AEBs)	16.1 ± 0.7	5,000	36	30	7,500	24	18	10,000	18	12	12,500	12
R1 (Seed sludge with 15% AEBs)	16.4 ± 0.8	5,000	36	30	7,500	24	18	10,000	18	12	12,500	12

Table S3 Comparison of the methanogenic treatment of high OLR wastewater using UASB.

Wastewater (carbon source of synthetic wastewater) ^a	COD _{inf} (mg/L)	HRT (d)	OLR _{max} (kg/(m ³ d))	COD removal (%)	Maxmium COD removal improved (%) ^b	Maxmium CH ₄ production improved (%) ^b	Strategy	Ref.
Fresh leachate	40,000	2.0	20	48.0	~ 26.3	N.A.	Combined UASB and aerated lagoon	(Govahi et al., 2012)
SW (ethanol)	4,110	~ 0.3	~ 12	93.0	~ 22.9	~ 45.0	Conductive materials addition (improved DIET)	(Zhao et al., 2015)
SW (sucrose)	10,000	0.5	20	~ 97.0	~ 47.0	~ 109.1	Magnetite addition (improved DIET)	(Wang et al., 2019)
SW (ethanol, ethyl acetate and 1-ethoxy-2 propanol)	7,600	~ 0.4	20	~ 96.0	~ 3.8	~ 10.6	Chitosan addition (improved granulation)	(Torres et al., 2018)
SW (glucose)	12,500	0.5	25	85.9	~ 305.7	~ 333.6	Immobilized fungus addition	This study

Note: OLR, organic loading rate. **a**, SW means synthetic wastewater. **b**, compared to the control group over the same period. N.A., data not available.

Table S4 Band assignments for FT-IR spectral features (cm^{-1}) of sludge (Maquelin et al., 2002; Badireddy et al., 2010; Larkin, 2011; El Fels et al., 2014).

Wavenumber (cm^{-1})	Assignments
3376	broad band, hydrogen bonded N-H and O-H stretching vibrations
3050–2810	weak shoulder, C-H stretching vibrations of alkyl groups; intense and sharp peaks, methyl-metal stretching vibration
2520	-SH stretching vibration
1800	M-O stretching vibration of the metal-binding organic molecule
1643–1633, 1639	the C=O stretching vibration, C=O stretch (amide I) associated with proteins; NH ₂ scissors of primary amines
1639	broad band, C=O stretching vibration of ionized form of the carboxyl group
1600–1500	the N-H deformation in -CO-NH
1470–1430	symmetrical deformation of -CH ₂ group
1250–1000	the S=O, C-C stretching vibration and CH ₂ wagging vibration of -S-CH ₂
862	ring “breathing” associated with C-C and C-OH stretching vibration
775–420	small sharp peaks, metal-alkyl stretching vibration

Table S5 FT-IR 2DCOS results on the assignment and sign of tech cross-peak in synchronous and asynchronous (in the brackets) maps of R0 and R1.

Reactor	Position (cm ⁻¹)	Vibration	Functional group or component	Sign	1056	1180	1528	1650
R0	1056	C-O stretching	Polysaccharides polysaccharide-like substances	or +	+	+(-)	+(+)	+(+)
	1180	C-OH, C-O	Amino acids polysaccharides	and/or	+	+(+)	+(+)	
	1528	N-H in - plane bending vibration	Benzene ring				+	+(+)
	1650	C=O, C-N	Proteins or lipids					+
R1	872	=C-H bending	Unsaturated hydrocarbons	+	+	+(-)	+(+)	+(-)
	1040	C-O and C-C stretching, (C-O-H) bending	Cellulose, polysaccharides or polysaccharide-like substances		+	+(+)	+(+)	+(+)
	1300	C=N stretching	Aromatic primary and secondary amines				+	+(-)
	1420	COO ⁻ and C=N stretching	Carbonate, amide Aromatic primary	III,				+

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