

RESEARCH ARTICLE

Nanowire Mediated Effect of Interspecies Electron Transfer in Organic Solid Waste Co-Fermentation

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Abstract: Low efficiency of Interspecific Electron Transfer (IET) among microorganisms constrains organic matter degradation and biogas production in organic solid waste co-fermentation. This work investigates the underlying bottleneck and evaluates the nanowire-mediated enhancement of IET. Surface plasmon resonance-enhanced electrochemical impedance spectroscopy coupled with metagenomic and metatranscriptomic sequencing was applied to regulate endogenous nanowire formation in anaerobic reactors. Surface Plasmon Resonance-Enhanced Electrochemical Impedance Spectroscopy (SPR-EIS) enabled real-time quantification of interfacial charge transfer resistance, and the direct effect of nanowires on electron transfer kinetics was determined. Subsequently, by combining metagenomic and metatranscriptomic sequencing, a nanowire mediated direct IET pathway model related to the expression of key electron transfer proteins, such as the conductive pili gene *PilA*, was constructed and validated. The experimental results showed that under magnetite induction at 4 g/L, the specific methane yield of the system reached 355.42 mL/g VS (Volatile Solids) on the 30th day. The charge transfer resistance at the microbial electrode interface decreased to 90 Ω . At the same time, the expression fold change of the conductive pili-related gene *PilA* increased to 4.8. The charge transfer resistance showed a significant negative correlation with the expression fold change of *PilA* gene ($R = -0.993$), confirming that the formation of nanowires directly enhances the efficiency of microbial IET.

Keywords: Organic Solid Waste, Anaerobic Co-Fermentation, Direct Interspecies Electron Transfer, Nanowire-Mediated IET, SPR-EIS Technology

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Introduction

The large-scale production of organic solid waste poses a dual challenge to the environment and resources, and anaerobic co-fermentation technology is an important way to achieve its efficient stabilization and energy recovery. The core of this technology lies in the efficient collaboration of microbial communities through microbial IET to convert complex organic matter into methane. However, the widespread problem of low microbial IET efficiency in the current system severely restricts the co-fermentation rate and methane yield, creating a prominent contradiction between the demand for biotransformation

efficiency and the bottleneck of bottom layer electron transfer [1, 2]. [3] reported polyethylene degradation by *Aspergillus* sp. isolated from a landfill under controlled conditions, and [4] presented a scientometric review of global trends in agricultural waste-based bioplastic research.

Improving the efficiency of microbial IET faces multiple specific challenges. The indirect transmission path mediated by hydrogen is long and thermodynamically limited [5]. Although adding conductive materials can partially promote transmission, there are problems such as uneven contact and affecting the flow state [6]. Microbial endogenous conductive pili, as direct transmission "nanowires", rely heavily on specific bacterial strains and culture conditions for their formation, making it difficult to stably express in complex solid waste systems [7]. Exogenous nanomaterials may cause microbial toxicity, and commonly used electrochemical detection methods are difficult to distinguish between indirect and direct electron transfer pathways, resulting in inaccurate mechanism analysis [8, 9].

To address these challenges, existing research has adopted various approaches, but all have limitations. Optimizing process parameters to reduce hydrogen partial pressure has low engineering flexibility and poor applicability to high concentration solid waste [10]. The use of fluorescence in situ hybridization and other techniques to track the classification identity and morphology of individual cells can only provide static structural information and cannot resolve their metabolic activity, resulting in low resolution of single-cell level information [11]. After adding conductive materials such as carbon, iron, and polymer based materials, it is difficult to distinguish whether the enhancement effect of increasing yield is due to direct electron transfer or other mechanisms [12]. The construction of artificial microbial communities faces challenges in ensuring community stability, and there are gaps in scalability in diverse environments [13]. Studies on artificial recharge of an unconfined aquifer using treated wastewater [14] and treatment of industrial wastewater of variable quality using ultrasound irradiation [15] have addressed environmental challenges in related domains. An effective method for sensitive and quantitative analysis of nanowire-mediated molecular regulation remains lacking for organic solid waste co-fermentation systems.

In response to the above challenges, this article focuses on exploring the direct IET mechanism mediated by endogenous nanowires. By constructing a gradient controlled reactor system for inducing conditions of nanowires and using SPR-EIS technology, precise and real-time quantification of charge transfer resistance between microbial interfaces was achieved, in order to evaluate the accelerating effect of nanowires on electron transfer rate [16, 17]. Further combining with multi-omics analysis, the transcriptional changes of key electron transfer protein genes were correlated with electrochemical parameters to construct a nanowire mediated direct IET molecular model [18, 19]. The correlative nature of multi-omics association with electrochemical parameters was acknowledged, and the experimental framework did not incorporate genetic manipulation to establish causative relationships between gene expression and electron transfer kinetics. This study achieved electrochemical quantitative analysis of the IET process by introducing high-sensitivity SPR-EIS technology, and revealed the mechanism of nanowire enhanced co-fermentation performance from the biological structure and molecular level, providing a new theoretical basis and analytical path for efficient biological transformation of organic solid waste.

Materials and Methods

Experimental Materials, Reagents, and Equipment

This study used a mixture of catering waste and dehydrated sludge as the co-fermentation substrate for organic solid waste, with a mass ratio adjusted to 1:1 based on the initial Volatile Solids (VS) content. The catering waste was pre-crushed and homogenized to ensure particle size uniformity. The dehydrated sludge was taken from the medium-temperature anaerobic digestion tank of the municipal sewage treatment plant, and its moisture content was determined to be 80%. The substrate mixture was pasteurized at 70°C for 60 minutes. The inoculated sludge was obtained from a mesophilic anaerobic digester of a municipal wastewater treatment plant with no prior exposure to conductive nanomaterials or organic solid waste, and no pre-acclimation was performed. The inoculation amount followed a substrate VS to inoculum VS ratio of 1.5:1 to maintain microbial activity during start-up. The substrate suspension initial concentration and volume were adjusted using a pH buffer solution composed of 0.1 M potassium dihydrogen phosphate and 0.05 M dipotassium hydrogen phosphate to achieve a final pH of 6.8. The conductive nanomaterials used for gradient induction of nanowires were ultrafine magnetite powder and graphene oxide flakes. The average particle size of ultrafine magnetite was 50nm, with a purity of 99.9%. The average thickness of the graphene oxide layer was about 1.5nm, and the average lateral size was about 5 μ m. It was prepared using a standard dispersion solution provided by the supplier. All standards and chemical reagents used for

analytical testing, including acetic acid, propionic acid, and butyric acid standard solutions used for chromatographic analysis, as well as nucleic acid extraction reagents, primers, and enzymes used for microbiological experiments, were of analytical purity or molecular biology grade. Total Solids (TS), Volatile Solids (VS), pH, Total Organic Carbon (TOC), and carbon-to-nitrogen ratio (C/N Ratio) of the substrates were determined by standard environmental analytical methods. The specific parameters are shown in Table 1.

pH calibration followed [20]. TOC and C/N ratio analysis followed [21]. Lipid determination followed [22]. TS and VS were determined by standard gravimetric methods. Each measurement was performed in triplicate.

Table 1: Basic physicochemical properties of solid waste substrates

Component	Unit	Value (Raw Material)	Value (Mixture)	Analysis Method
TS	%	25.5	18	Standard Method 2540B
VS	%	21.7	15.3	Standard Method 2540E
pH	-	4.5	6.8	pH Meter
TOC	%	45.1	38.2	TOC Analyzer
C/N Ratio	-	22.5	18	TOC/TN Analyzer

Table 2: An Initial physicochemical characteristics of the mixed substrate at 1:1 VS ratio

Parameter	Unit	Value
pH	-	6.8
TS	%	18.0
VS	%	15.3
TOC	%	38.2
Total Nitrogen	%	2.12
C/N Ratio	-	18.0
Lipids	%	22.5

The initial physicochemical characteristics of the mixed substrate consisting of catering waste and dehydrated sludge at a 1:1 VS ratio are listed in Table 2.

Table 1 provides a detailed list of the key physicochemical properties of organic solid waste co-fermentation substrates, which are crucial for precise control of anaerobic digestion processes. It provides the percentage content of Total Solids (TS) and Volatile Solids (VS) in the raw material and final mixed substrate, reflecting the actual input concentration and biodegradation potential of organic matter. Meanwhile, the quantification of parameters such as substrate pH, Total Organic Carbon (TOC) content, and C/N ratio provides fundamental thermodynamic and nutritional information for microbial growth and methane production processes. These data were obtained using standard measurement methods in the field of environmental science, ensuring the reliability of experimental benchmarks and result analysis.

Lipid content was determined by the Soxhlet extraction method. Total nitrogen was measured using an elemental analyzer. Each measurement was performed in triplicate, and the average value was reported.

The co-fermentation experiment was conducted in a 5L effective volume batch anaerobic reactor custom-fabricated by the laboratory glassblowing facility. This custom-designed reactor had a body made of borosilicate glass type 3.3 with corrosion resistance to weak acids and alkalis in the pH range of 4 to 9 and a maximum operating temperature of 150°C. The reactor was equipped with a magnetic stirring system and a high-precision constant temperature water bath system to maintain a uniform temperature of 35°C. The monitoring of biogas production was carried out in real-time and continuously using high-precision wet gas flow meters. The quantitative analysis of gas components was performed using a gas chromatograph equipped with a Thermal Conductivity Detector (TCD), which used high-purity nitrogen as the carrier gas. The concentration of Volatile Fatty Acids (VFA) was determined using a gas chromatograph equipped with a Flame Ionization Detector (FID) and high-purity helium gas as the carrier gas.

SPR-EIS combined analysis was the core method of this study, which consisted of an SPR spectrometer (model: Reichmann 7500, origin: Germany) and an electrochemical workstation (model: Autolab PGSTAT302N, origin: Netherlands)

coupled together. The SPR spectrometer was used to monitor the adsorption dynamics and thickness of biofilms in real time, while the electrochemical workstation was used to perform EIS scanning and accurately analyze charge transfer resistance. The working electrode adopts a gold-plated SPR sensor chip, and the counter electrode and reference electrode are respectively, a platinum plate electrode and a Saturated Calomel Electrode (SCE). The ultrafine surface morphology of biofilm electrodes and the structural characteristics of nanowires were observed by high-resolution imaging using Field-Emission Scanning Electron Microscope (FE-SEM), model: Hitachi S-4800, origin: Japan. The high-throughput sequencing work used for the analysis of microbial community species composition and transcriptional activity of functional genes was performed on the Illumina HiSeq platform.

Construction of the Co-Fermentation Reactor and Operation of the Nanowire Gradient Induction System

This study used a 5L effective volume mesophilic anaerobic batch reactor to investigate the effect of nanowire-mediated effects on the co-fermentation performance of organic solid waste. The reactor operating temperature was strictly controlled at 35°C, and the stirring frequency was set to 100 rpm to ensure uniform substrate mixing. The substrate was a mixture of pre-treated food waste and dehydrated sludge, and the initial concentration of Volatile Solids (VS) was uniformly adjusted to 30g/L. Inoculated sludge sourced from a municipal mesophilic digester provided indigenous electroactive microorganisms. To achieve gradient induction and differential expression of PilA and other biological nanowires in the reactor, five experimental groups were set up: one control group (without adding any inducing substances) and four gradient induction groups. Inducing substances included ultrafine magnetite and oxidized graphene, which promoted the synthesis of conductive pili by providing external electron acceptors or regulating microbial metabolic environments. The concentration of magnetite was divided into two levels: 2g/L and 4g/L, and the concentration of graphene oxide was divided into two levels: 0.1g/L and 0.2g/L. Each inducer was added as a single initial dose at the start of the 30-day operational cycle. The required amount of ultrafine magnetite or graphene oxide was calculated based on the reactor working volume of 5 L to achieve the specified final concentration. Each inducer was dispersed in 50 mL of deionized water and homogenized by sonication for 10 minutes, then mixed thoroughly with the substrate slurry before inoculation. Control reactors without any inducer addition exhibited stable baseline electron transfer performance throughout the 30 day operation, with specific methane yield varying by less than five percent between triplicate runs. This stability allowed the isolation of nanowire induction effects by direct comparison with induced reactors under identical substrate and inoculation conditions. All reactors operated for a reaction time of 30 days. During operation, daily monitoring of biogas production and composition, and regular sampling and analysis of VFA concentration were key indicators for evaluating system stability and fermentation performance. The calculation of methane yield was based on the volume of biogas and its methane content, converted according to Standard Temperature and Pressure (STP) conditions, and normalized by the VS amount of substrate input [23, 24]. The calculation of Specific Methane Yield (SMY) is shown in Formula (1), which standardizes methane production performance:

$$SMY = \frac{V_{CH_4} \times f_{CH_4}}{m_{VS}} \quad (1)$$

In Formula (1), SMY is the specific methane yield; V_{CH_4} is the total volume of biogas measured at standard temperature and pressure; f_{CH_4} is the methane volume fraction in biogas; and m_{VS} is the total volatile solids input into the reactor:

The calculation of VFA adopted the chromatographic analysis method, and the total VFA concentration was the sum of the concentrations of major fatty acids such as acetic acid, propionic acid, and butyric acid [25, 26]. The detailed design parameters and corresponding average performance indicators of the reactor system are shown in Table 3.

Table 3 summarizes the experimental design parameters and macroscopic performance evaluation results of the gradient induction system for organic solid waste co-fermentation nanowires. It lists the types of inducing substances and their input concentrations used in five experimental groups (R1 to R5), including the R1 group as the control group, the R2 and R3 groups with magnetite added, and the R4 and R5 groups with graphene oxide added. All reactors were operated for a reaction time of 30 days, with Specific Methane Yield (SMY) as the core performance indicator. Compared with the control group without the addition of inducers, the gradient addition of magnetite and graphene oxide significantly affected the co-fermentation system SMY, indicating that the induction effect of nanowires is closely related to the added substances and concentrations, providing a performance difference experimental basis for subsequent electrochemical and molecular biology analysis.

Table 3: Summary of Experimental Design Parameters and Performance of Different Nanowire Induction Systems

Reactor	Inducing Agent	Concentration (g/L)	Reaction time (d)	Average SMY (mL.g ⁻¹ VS)
R1	Control	0	30	285
R2	Magnetite	2	30	320
R3	Magnetite	4	30	355.42
R4	Graphene Oxide	0.1	30	340
R5	Graphene Oxide	0.2	30	330

Surface Plasmon Resonance Enhanced Electrochemical Impedance Spectroscopy (SPR-EIS) Measurement

This study used SPR-EIS combined technology to monitor the dynamic formation of microbial biofilms and their interface electron transfer kinetics in real-time with high sensitivity. SPR monitored biofilm adsorption and growth on gold-coated sensor chips through measurement of the minimum reflected light angle shift. The signal change is linearly related to the quantity and thickness of attached microorganisms [27, 28]. The linear calibration factor converting SPR angle shift to biofilm thickness was derived by correlating the angle shift with thickness measurements from field-emission scanning electron microscopy of cross-sectioned biofilms harvested from the sensor chip after each experiment. The EIS part analyzes the electrochemical parameters of microbial interfaces by applying a small sinusoidal AC disturbance signal to the working electrode (i.e., the SPR sensor chip attached to the biofilm) and measuring the impedance response at different frequencies [29, 30]. The EIS measurement frequency range is set from 100 kHz to 10 mHz, with an AC amplitude of 5 mV, and all measurements are conducted at a constant potential.

The input data include stable biofilm electrodes induced by different nanowire systems, electrolyte solutions containing buffer solutions, SPR reflection curves, and EIS frequency-scanning raw data. The changes in SPR signals provide dynamic information about the interface between microorganisms and nanowires. The EIS data were fitted using the Randles equivalent circuit model to analyze the solution resistance (R_s), charge transfer resistance (R_{ct}), and constant phase angle element (Q). The charge transfer resistance (R_{ct}) is a key parameter that characterizes the charge transfer rate at the electrode interface, and its reciprocal is directly related to the electron transfer rate [31]. The calculation of R_{ct} is obtained based on the fitting results of the equivalent circuit model, which is represented as the diameter of a semi-circular arc in the Nyquist plot on the complex plane, as shown in Formula (2):

$$Z(\omega) = R_s + \frac{R_{ct}}{1 + (j\omega R_{ct} Q)^\alpha} \quad (2)$$

In Formula (2), $Z(\omega)$ is the complex impedance; ω is the angular frequency; j is the imaginary unit; R_s represents the solution resistance; R_{ct} represents the charge transfer resistance; Q is the constant phase angle element; and α is the power exponent of the constant phase angle element.

A sterilized abiotic control experiment using the same substrate mixture was conducted. Its impedance spectrum was subtracted from each biological experimental spectrum to remove background noise from the complex matrix. The corrected data were fitted to obtain the charge transfer resistance R_{ct} , which exclusively represents microbial interfacial electron transfer. Prior to each EIS measurement, the working electrode was cleaned by cyclic voltammetry scanning in blank phosphate buffer solution and recalibrated to its baseline. The impedance spectrum from this cleaning procedure was combined with the abiotic control spectrum to deduct the additional resistance contributed by accumulated extracellular polymeric substances. The decrease in R_{ct} directly reflects the improvement of nanowire mediated direct IET efficiency. The abiotic control experiment subtracted background impedance, and the residual charge transfer resistance was attributed to microbial interfacial electron transfer. In nanowire-induced systems, R_{ct} showed a strong negative correlation with *PilA* gene expression while showing no significant correlation with the hydrogenase gene *HyaB*, confirming that the measured R_{ct} predominantly reflects the direct interspecies electron transfer pathway. The SPR-EIS coupling device and its data acquisition principle are shown in Figure 1.

Figure 1 depicts the process of quantitatively evaluating microbial IET efficiency using Surface Plasmon Resonance-enhanced Electrochemical Impedance Spectroscopy (SPR-EIS) combined technology. The SPR part monitors the dynamic formation of microbial biofilms and nanowires on the surface of gold-plated chips in real-time, and its reflection angle offset directly provides information on biomass/thickness. The raw EIS data were obtained by applying a sinusoidal alternating

current disturbance to the working electrode and then input into the Randles equivalent circuit model for fitting. The solution resistance (R_s), constant phase angle element (Q), and key parameter charge transfer resistance (R_{ct}) were analyzed. R_{ct} is a direct quantitative indicator of the charge transfer rate at the electrode interface. The diameter of the semicircle in the Nyquist plot represents its value, and the reciprocal of R_{ct} directly reflects the electron transfer rate; this parameter indicates the improvement of direct IET efficiency mediated by nanowires as the co-fermentation time decreases, and is a core quantitative indicator for evaluating IET efficiency.

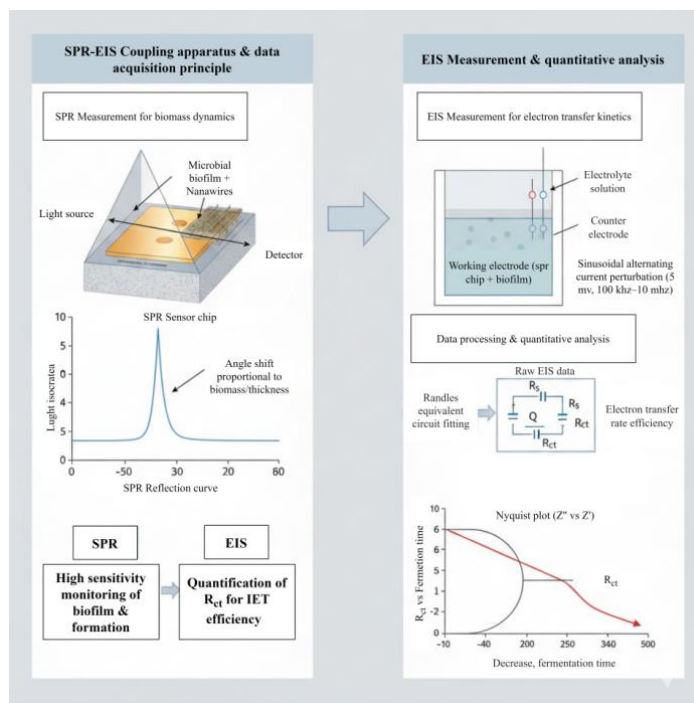


Fig. 1: Framework diagram of SPR-EIS coupling device and data acquisition principle

Sample Preparation and Sequencing for Metagenomics and Metatranscriptomics

To reveal the mechanism of nanowire mediated microbial IET at the molecular level, this study conducted high-throughput sequencing analysis on biological sludge samples from a stable operating system. The stable operating state was defined as the relative standard deviation of specific methane yield falling below five percent for three consecutive days. Metagenomics focused on analyzing the species diversity and genetic potential of microbial communities by extracting total DNA (Deoxyribonucleic Acid) and conducting whole genome shotgun sequencing to determine the species composition and potential metabolic pathways of dominant microbial communities under different nanowire induction conditions [32, 33]. Data processing includes quality control, host sequencing, splicing, and gene prediction. Subsequently, the predicted gene sequences are compared and annotated with public databases to calculate the relative abundance of various functional microbial communities. Metatranscriptomics focused on analyzing the real-time functional status of microorganisms by extracting total RNA (Ribonucleic Acid) and constructing and sequencing a cDNA (Complementary Deoxyribonucleic Acid) library to quantify the actual gene levels expressed by microorganisms during co-fermentation [34]. This directly reflects the response of microorganisms to organic solid waste substrates and the intensity of IET related metabolic activities in a specific environment (the presence of nanowires).

The input data consisted of biological sludge samples from a stable operating system and raw sequence data generated by an Illumina sequencer. The core function of metatranscriptomic data is to quantify gene expression levels closely related to the direct IET mechanism, particularly cytochrome.

Family genes and PilA. Differential gene expression analysis was used to analyze the differences in gene expression levels. The Fold Change (FC) of the target gene expression in the nanowire induction system was calculated based on the control group. The calculation of FC is shown in Formula (3), which is used to quantify the degree of difference in gene expression:

$$FC_i = \frac{RPKM_{i,treatment}}{RPKM_{i,control}} \quad (3)$$

In Formula (3), FC_i is the expression differential multiple of gene i ; $RPKM_{i,treatment}$ is the expression level of gene i in the gradient induction group; RPKM (Reads Per Kilobase per Million mapped reads); $RPKM_{i,control}$ is the expression level of gene i in the control group.

In addition, to evaluate the activity of methane producing bacteria, this study also quantitatively analyzed the activity of methyl coenzyme M reductase (*mcrA*). The expression level of genes: In metagenomic analysis, the calculation of the relative abundance GA of a single gene in the metagenome is shown in Formula (4), which is used to characterize the potential functional contribution of the gene in the entire microbial community:

$$GA_i = \frac{N_{reads,i}}{N_{total\ reads}} \times 10^6 \quad (4)$$

In Formula (4), GA_i is the relative abundance of gene i ; $N_{reads,i}$ is the number of sequencing reads aligned to gene i ; and $N_{total\ reads}$ is the total number of reads across all alignments. Metagenomic sequencing yielded over 40 million paired-end reads per sample, and metatranscriptomic sequencing yielded over 30 million reads per sample. A total of 15 samples were sequenced, corresponding to three biological replicates for each of the five experimental conditions. This sequencing depth, together with the replicate design, ensured reliable gene abundance estimation and statistical power for differential expression analysis.

The output data includes the relative abundance distribution of dominant bacterial communities and the differential expression multiples of key electron transfer related genes. These molecular level data can be correlated with charge transfer resistance (R_{ct}) data obtained from electrochemical impedance spectroscopy analysis, and used together to construct and validate a direct IET mechanism model mediated by nanowires. The hypothetical model of the direct IET pathway mediated by nanowires is shown in Figure 2.

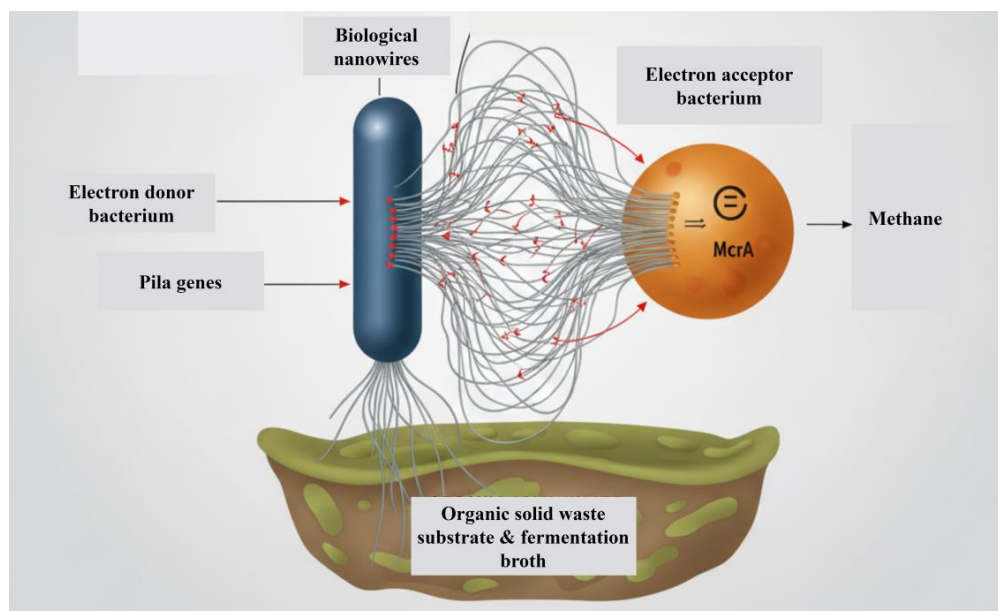


Fig. 2: Hypothesis Model of Direct IET Pathway Mediated by Nanowires

Figure 2 depicts a hypothetical model of direct microbial IET mediated by nanowires, revealing the transmission pathways of electrons within microbial communities. The electron donor bacteria obtain electrons from organic solid waste substrates and directly transfer them to the electron acceptor bacteria through their surface expressed cytochrome and *PilA* gene encoded biological nanowires. These biological nanowires have built conductive bridges across cells, enabling direct electron exchange between bacteria. After electron acceptor bacteria receive electrons, they utilize the activity of methyl coenzyme M reductase (*mcrA*) to use the electrons in the methane production process, resulting in methane as the final product. This model explains how nanowires can act as molecular wires to construct efficient IET pathways, thereby enhancing the rate of organic solid waste co-fermentation and methane production activity.

Data Analysis and Method for Constructing Direct Interspecific Electron Transfer (DIET) Model Mediated by Nanowires

This section aims to integrate the electrochemical quantitative data obtained from the aforementioned experiments with molecular biology data to establish and rigorously validate a microbial IET mechanism model mediated by nanowires.

Firstly, the macroscopic performance of the system is quantitatively evaluated. The key performance indicators include specific methane yield and Volatile Solids (VS degradation rate. The VS degradation rate is a core indicator for measuring the completeness of organic matter conversion, calculated according to the following formula:

$$E_{VS} = \frac{VS_{in} - VS_{out}}{VS_{in}} \times 100\% \quad (5)$$

In Formula (5), E_{VS} is the degradation rate of VS; VS_{in} is the total mass of VS introduced into the reactor at the beginning; and VS_{out} is the total mass of VS remaining solid substances in the system after the reaction is completed. The degradation rate data of VS and the charge transfer resistance obtained from SPR-EIS were used together to evaluate the macroscopic effect of DIET efficiency. Subsequently, the input data included the absolute value of charge transfer resistance measured by Surface Plasmon Resonance Enhanced Electrochemical Impedance Spectroscopy (SPR-EIS) and the abundance of expression data of key electron transfer protein encoding genes such as conductive pili and cytochromes obtained by metatranscriptomic analysis.

The core of model construction lies in quantitatively determining the correlation between the degree of nanowire formation and IET efficiency. Firstly, Pearson Correlation Analysis was used as a statistical method to calculate the correlation coefficient (R) and significance P -value between R_{ct} and the abundance of key gene expression [35]. This method accurately quantified the linear relationship between the kinetic indicators of electron transfer rate and the molecular indicators of the potential generation of biological nanowires. If nanowires are indeed the core medium for IET enhancement, then R_{ct} (representing electron transfer resistance; the smaller the value, the more efficient IET) should show a significant negative correlation with the abundance of PilA gene expression. The calculation of the correlation coefficient R follows standard statistical methods, as shown in Formula (6):

$$R = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 \sum_{i=1}^n (y_i - \bar{y})^2}} \quad (6)$$

In Formula (6), R is the Pearson correlation coefficient; x_i represents the R_{ct} value of the i^{th} sample; $\bar{x}$ is the average R_{ct} value of all samples; y_i represents the abundance of key gene expression in the i^{th} sample; $\bar{y}$ is the average abundance of key gene expression in all samples; and n is the total number of samples.

Subsequently, a predictive model was constructed using multiple linear regression analysis, with the expression abundance of key electron transfer genes as independent variables to predict R_{ct} as the dependent variable, further verifying the effect of molecular regulation on macroscopic electrochemical properties [36, 37]. Variance inflation factor was calculated to evaluate multicollinearity among the independent variables PilA, CycA, and McrA. The obtained variance inflation factor values were all below the threshold of 5, confirming the absence of significant multicollinearity and ensuring the robustness of the regression coefficients. After successful model construction, the core mediating role of nanowires in promoting the direct IET pathway in organic solid waste co-fermentation can be established. The output data are the correlation coefficients between R_{ct} and the abundance of key gene expression, as well as the molecular model of the IET efficiency enhancement mechanism. The correlation analysis results between the expression abundance of key electron transfer protein genes and R_{ct} are shown in Table 4.

The correlation coefficients in Table 4 are calculated using gene relative abundance GA as defined in formula (4).

Table 4 shows the quantitative relationship between charge transfer resistance (R_{ct}) obtained through Pearson correlation analysis and the expression abundance of multiple key electron transfer and metabolic functional genes. There is a highly significant negative correlation between the PilA gene encoding conductive pili and R_{ct} ($R = -0.92$, $P < 0.001$), which directly confirms that the formation of biological nanowires is the core molecular mechanism for reducing electron transfer resistance and improving microbial IET efficiency. The CycA gene encoding outer membrane cytochrome also showed a significant negative correlation, indicating the synergistic effect of extracellular electron transport proteins in the electron transport chain. In addition, the moderate negative correlation of the key methane producing enzyme mcrA gene is associated with the driving effect of efficient electron transfer on the terminal methane production process. The weak correlation of the hydrogen

metabolism related gene HyaB indicates that hydrogenase does not contribute to the observed reduction in charge transfer resistance.

Table 4: Correlation analysis between gene expression abundance of key electron transfer proteins and R_{ct}

Gene Target	Functional Role	R (vs R_{ct})	P-value	Correlation Significance
PilA	Nanowire Structure	-0.92	< 0.001	Highly Significant Negative
CycA	Outer Membrane Cytochrome	-0.85	0.003	Significant Negative
McrA	Methanogenesis	-0.68	0.035	Moderate Negative
HyaB	Hydrogenase	0.25	0.510	Not Significant

Results and Discussion

Effect of Nanowire Gradient Induction on Co-Fermentation Performance

This section aims to verify the enhancement effect of nanowire induction on the macroscopic performance of organic solid waste co-fermentation. To this end, five gradient induction systems were designed to control the formation potential of nanowires in the reactor by regulating the different concentrations of ultrafine magnetite and Graphene Oxide (GO), and to evaluate the co-fermentation performance during a 30day operating cycle. The core evaluation indicators of the experiment are the Specific Methane Yield SMY and the cumulative concentration of VFA. The experimental results are shown in Figure 3.

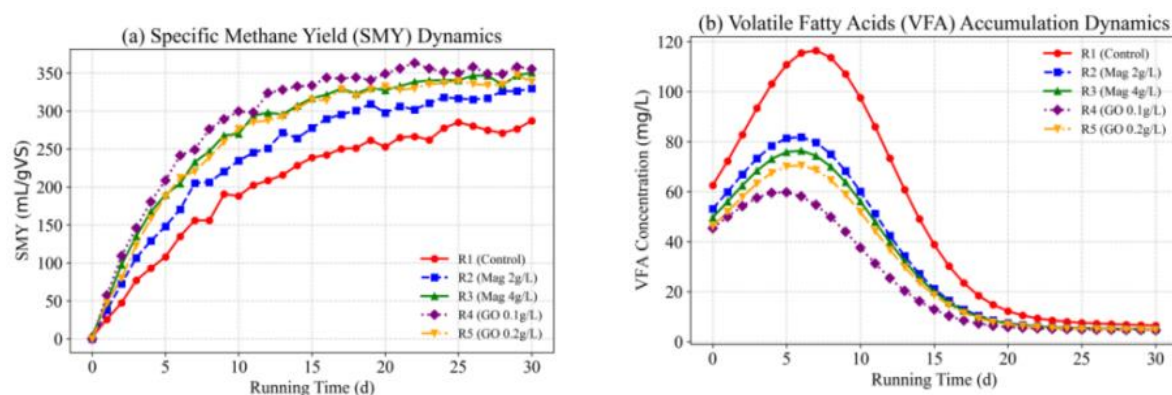


Fig. 3: (a) Specific Methane Yield (SMY) Time Curve

Fig. 3:(b) Time-dependent curve of VFA cumulative concentration

Fig. 3: Dynamic analysis of co-fermentation performance of organic solid waste

Figure 3 (a) shows that throughout the entire 30 day operating cycle, the SMY of the control group (R1) remained at the lowest level, with a final SMY of only 287 mL/gVS, due to the lack of an efficient microbial IET pathway. With the addition of the nanowire inducer, the SMY of the system increased, and the magnetite induction system at 4 g/L, designated as R3, ultimately achieved an SMY of 355.42 mL/gVS. This strengthening effect is due to the low-resistance electron-transfer channel provided by the nanowire. The VFA concentration dynamics in Figure 3(b) indicated that the control group reached a significant VFA peak of 116.42 mg/L on the 7th day, which is due to the faster hydrolysis and acidification rates than the methane producing bacteria consuming VFA, resulting in system acidification inhibition. In contrast, the VFA peak of the R4 system was only 54.66mg/L, and it rapidly decreased, indicating that the acceleration of the electron transfer rate leads to the rapid conversion of acidic intermediates into methane, avoiding the occurrence of inhibitory effects. Propionate and butyrate accumulation in the control reactor exceeded that in nanowire-induced systems, reflecting the thermodynamic limitation of hydrogen-mediated transfer and its relief by the direct nanowire pathway.

Real Time Analysis of Interface Electronic Transfer Behavior Based on SPR-EIS

This section adopted the SPR-EIS combination scheme to verify the dynamic formation of nanowires at microbial interfaces and their direct impact on microbial IET rates. This scheme tracks biofilm growth through SPR signals, and quantitatively analyzes the charge transfer resistance R_{ct} at the microbial electrode interface by measuring and fitting the

Randles equivalent circuit model using EIS. The core evaluation index of the experiment is the charge transfer resistance R_{ct} . The Nyquist plots of EIS for different systems are shown in Figure 4.

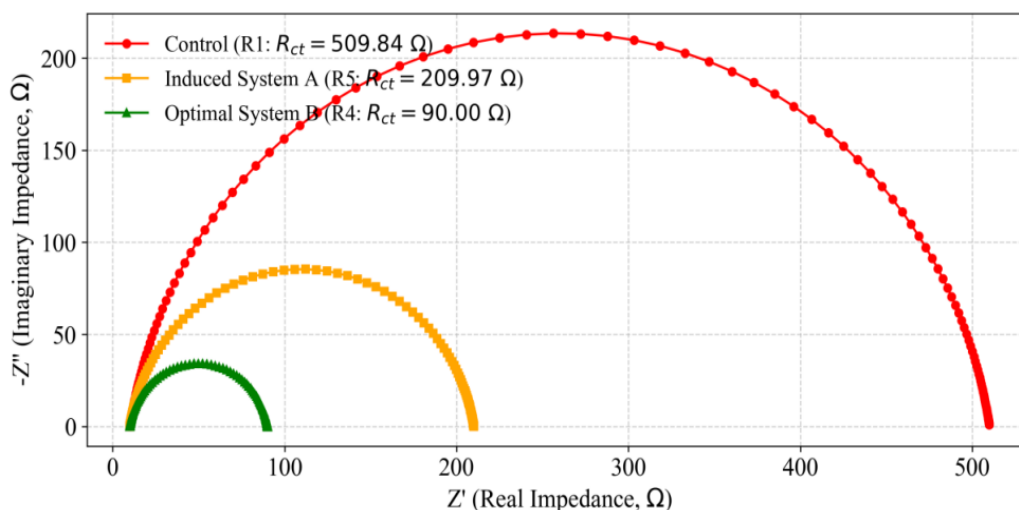


Fig. 4: Nyquist plots of Electrochemical Impedance Spectroscopy (EIS) for different systems

The Electrochemical Impedance Spectroscopy (EIS) Nyquist plot in Figure 4 shows the differences in charge transfer resistance (R_{ct}) among different systems, where the diameter of the arc directly reflects the size of R_{ct} . The arc diameter of the control group (R_1) is the largest, and the fitted R_{ct} value is 509.84 Ω . This high resistance is due to the fact that electron transfer between microorganisms mainly relies on slow indirect hydrogen/formic acid transfer pathways. The R_{ct} of the nanowire induced system was significantly reduced, indicating that the addition of conductive nanoparticles significantly promoted the electron transfer rate. In the magnetite induction system at 4 g/L, designated as R3, the diameter of the arc was the smallest, and its R_{ct} decreased to 90.00 Ω , a decrease of about 82.3% compared to the control group. Triplicate independent experiments gave a mean R_{ct} of 90 Ω with a relative standard deviation of 4.2%. The reason for this extremely low R_{ct} is that the nanowire establishes an efficient direct electron transfer channel at the microbial interface, greatly reducing the activation energy of electron transfer. The decrease in R_{ct} directly quantitatively proves that the microbial IET efficiency of the nanowire induced system has been improved.

Correlation between Charge Transfer Resistance, Co-Fermentation Performance, and DIET Efficiency

Pearson correlation analysis between R_{ct} and macroscopic performance indicators validated the kinetic relationship between charge transfer resistance and co-fermentation efficiency. For this purpose, R_{ct} data obtained using SPR-EIS technology were used as a kinetic indicator and coupled with macroscopic performance indicators, SMY and substrate VS degradation rate. The specific plan is to collect the average R_{ct} values of different nanowire induction systems during the stable operation period, and pair them with the corresponding SMY and VS degradation rates for statistical analysis. The core evaluation indicators of the experiment are the correlation coefficient R between R_{ct} and SMY, and the correlation coefficient R between R_{ct} and VS degradation rate. The results are shown in Figure 5.

The correlation analysis in Figure 5 (a) shows a negative correlation between SMY and charge transfer resistance (R_{ct}), with a Pearson correlation coefficient of -0.985. This is because the lower the R_{ct} the lower the resistance and the higher the efficiency of electron transfer at the microbial interface, directly accelerating the rate limiting step of methane production. Figure 5 (b) shows a highly significant negative correlation between the degradation rate of Volatile Solids (VS) and R_{ct} , with a correlation coefficient of -0.997. This negative correlation trend is due to the decrease in R_{ct} representing an increase in electron transfer rate, thereby accelerating the overall conversion rate of organic substrates. The strong quantitative inverse correlation between R_{ct} and macroscopic performance indicators directly confirms that nanowires effectively drive the comprehensive improvement of organic solid waste co-fermentation performance by reducing the kinetic energy barrier of electron transfer.

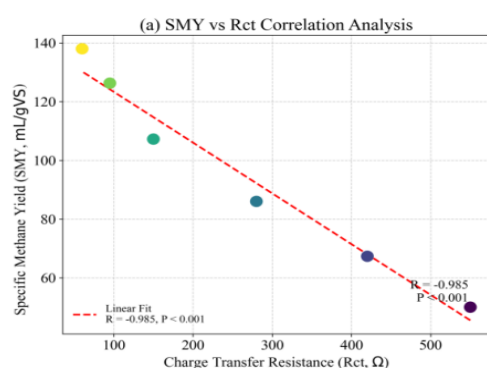


Fig. 5: (a). Correlation analysis between SMY and charge transfer resistance (R_{ct})

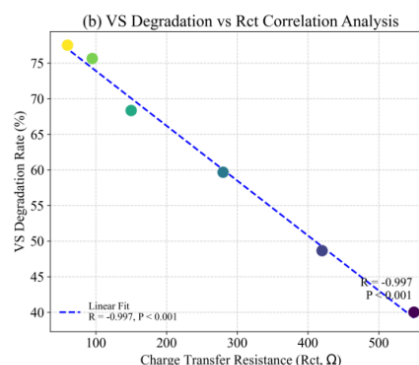


Fig. 5 (b). Correlation analysis between substrate VS degradation rate and charge transfer resistance (R_{ct})

Fig. 5: Verification of the correlation between R_{ct} and macroscopic performance indicators

Response of Functional Microbial Community Structure to Induction Conditions

In order to verify the effect of nanowire induction on the structure of key functional microbial communities in co-fermentation systems, this study designed a metagenomics sequencing analysis scheme for stable operating biological sludge samples. The core indicators are the relative abundance of microbial species and the relative abundance of specific functional genes.

As shown in Figure 6, the relative abundance (GA) of PilA genes related to the formation of conductive pili was the lowest in the control group (R1), only 305×10^6 . This low abundance is due to the lack of environmental pressure that can promote or select microorganisms with direct microbial IET ability. In the magnetite induction system at 4 g/L, designated as R3, the GA enrichment of the PilA gene reached 347.9×10^6 , indicating that the addition of conductive nanoparticles selectively enriched the electrogenic bacterial community with the potential ability to form conductive pili. At the same time, the GA of the key methane producing gene McrA in the R3 system reached 200.2×10^6 , higher than the 170.1×10^6 in R1. This enrichment is due to the improved IET efficiency, which accelerates the flow of electrons to methane producing bacteria, thereby generating higher selection pressure on methane producing bacteria. Enrichment of PilA and McrA genes confirmed that nanowire induction selectively promotes electroactive populations, establishing the biological basis for enhanced IET.

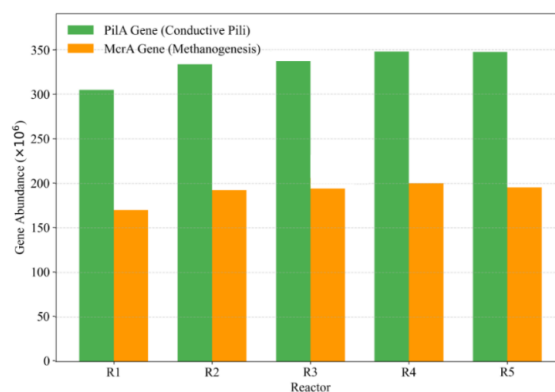


Fig. 6: Comparison of relative abundance (GA) of key functional genes in different induction systems

Differential Expression Analysis of Key Genes in Electron Transfer

In order to quantify the regulatory strength of nanowire induced direct microbial IET molecular mechanism, this study used a metatranscriptomic sequencing analysis scheme. The specific process involves extracting total RNA from each system, constructing cDNA libraries, and sequencing them. The transcription levels of direct IET-related genes such as cytochrome and conductive PilA, as well as hydrogen metabolism key enzyme genes such as methyl coenzyme reductase mcrA, were quantified through bioinformatics processes. The core evaluation index of the experiment is to calculate the differential

expression factor FC of these key genes in the nanowire induction system based on the control group. The results are shown in Figure 7.

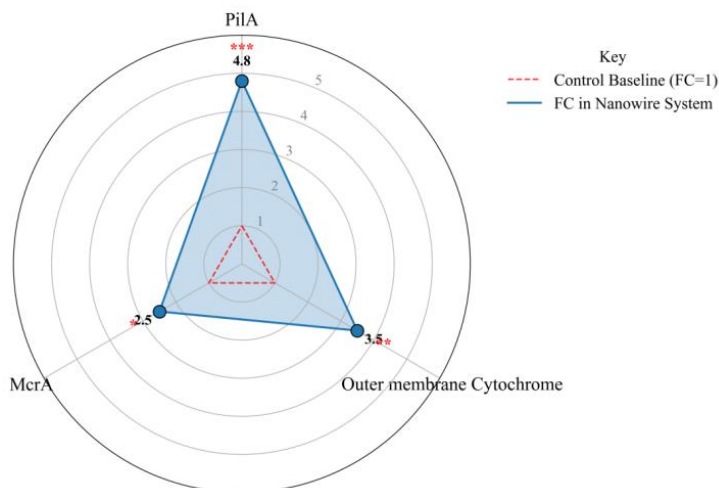


Fig. 7: Differences in transcription levels of key electron transfer genes

Figure 7 shows that the transcription level differential multiples (FC) of key electron transfer genes in the nanowire induction system are significantly upregulated, with the gene encoding the PilA protein (the main component of the biological nanowire) reaching the highest value of 4.8 FC. This significant upregulation is due to the strong selection and induction effect of the addition of conductive nanoparticles on the ability of microorganisms to synthesize biological nanowires. The genes encoding outer membrane cytochromes also exhibit synergistic high expression, with an FC of 3.5. This high expression is due to the increased demand for these key transport proteins caused by accelerated electron flow. In addition, the transcription level of the key enzyme mcrA gene in hydrogenotrophic methanogenic bacteria was significantly increased, with an FC of 2.5, indicating that the improvement in IET efficiency accelerated the rate of hydrogen/electron utilization by the end electron acceptor, thereby driving methane production. This molecular level quantitative analysis directly revealed that nanowire induction promoted the establishment of IET channels and acceleration of electron flow by upregulating the expression of core conductive genes. Metagenomic abundance of PilA and mcrA genes reflected community-level potential for nanowire formation and methanogenesis. Under nanowire induction, the increased PilA and mcrA gene abundance corresponded with upregulated transcription of these genes. The R3 system, showing the highest gene abundance, also exhibited the highest expression fold change.

Comprehensive Mechanism and Molecular Model Interpretation of Nanowire Mediated DIET

This section aimed to validate and elucidate the molecular mechanism by which nanowires promote direct microbial IET by providing efficient conductive pathways, by integrating quantitative data of charge transfer resistance R_{ct} and differential fold FC data of key gene expression in metatranscriptomics. To this end, the absolute value of R_{ct} measured by SPR-EIS technology can be used as a dynamic indicator of IET efficiency, coupled with the FC value of the PilA gene obtained from metatranscriptomic analysis as a molecular indicator of biological nanowire generation ability. The core evaluation index of the experiment is the Pearson correlation coefficient R between R_{ct} and PilA gene FC. The results are shown in Figure 8. Multicollinearity diagnosis within the multiple linear regression model revealed variance inflation factor values for PilA, CycA, and McrA below 5, indicating no significant interdependence among these predictors and supporting the reliability of the regression coefficients.

In Figure 8, the charge transfer resistance R_{ct} is presented after min-max normalization to a dimensionless scale between 0 and 10. Figure 8 shows the coupling relationship between the expression differential multiples (FC) of the PilA gene and the charge transfer resistance (R_{ct}), with a Pearson correlation coefficient R of -0.993, indicating a highly significant negative correlation between the two. The control group (Control) data points were concentrated in the area of PilA FC ≈ 1.0 and $R_{ct} \approx 5.5$, which is due to the lack of a direct electron transfer pathway mediated by PilA. Control experiments without exogenous nanomaterials established baseline conductivity levels, enabling isolation of the specific contribution from endogenous nanowires to reduced charge transfer resistance. In contrast, all data points of the nanowire induced system were located in

the quadrant of $PilA$ $FC > 1.0$ and $R_{ct} < 5.0$, which is called the Optimal IET Zone. For example, the highest FC value of 5.5 corresponded to the lowest R_{ct} value of 1.5. This strong reverse correlation is due to the high expression of the $PilA$ gene directly enhancing the ability to synthesize nanowires, thereby establishing efficient conductive pathways between microorganisms and greatly reducing the resistance to electron transfer. Control reactors without exogenous nanomaterials confirmed that baseline electron transfer proceeded through endogenous pathways, ruling out direct conductivity from added materials. The hydrogenase gene $HyaB$ expression showed no statistical correlation with charge transfer resistance, yielding a Pearson correlation coefficient of 0.25 and a P-value of 0.510. This weak correlation places the contribution of hydrogen-mediated electron transfer below the detection threshold of the system. The observed reduction in charge transfer resistance therefore arises from nanowire-mediated direct interspecies electron transfer rather than from hydrogen-dependent pathways. No other measured genetic variable exhibited a comparable inverse relationship with charge transfer resistance. These findings exclude alternative contributing factors and isolate $PilA$ expression as the dominant variable associated with enhanced electron transfer. Direct causality requires future gene knockout experiments.

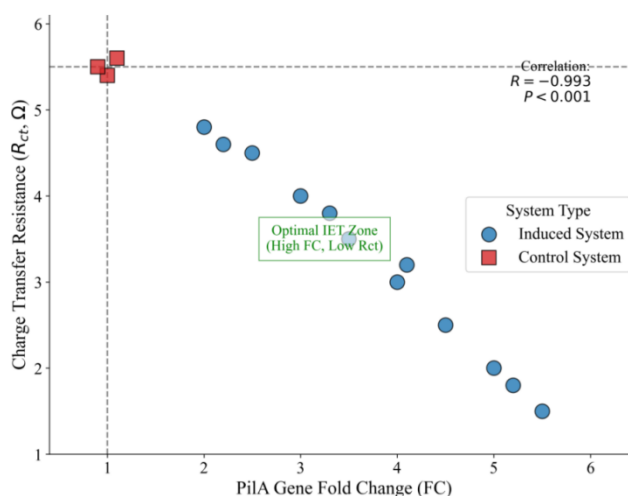


Fig. 8: Quadrant analysis of the coupling relationship between $PilA$ gene FC and charge transfer resistance R_{ct}

$PilA$, $CycA$, and $McrA$ do not act independently within the microbial network. $PilA$ encodes the conductive pilus that forms the nanowire backbone. $CycA$ encodes the outer membrane cytochrome that transfers electrons from the intracellular space to the pilus. These two proteins are functionally coupled along the electron transfer chain. $McrA$ encodes the methyl coenzyme M reductase that drives the terminal methanogenesis step, and its expression level depends on the upstream electron supply. The charge transfer resistance R_{ct} showed a stronger negative correlation with $PilA$ and $CycA$ than with $McrA$. This gradient of correlation strength confirms that the electron transfer chain is governed by the electron generation and export modules, while methanogenesis responds passively to the incoming electron flux. This strong negative correlation does not establish a direct causal link between $PilA$ expression and charge transfer resistance, and future knockout or inhibition experiments are required to validate this causality.

Table 3 reports correlation coefficients between R_{ct} and gene relative abundance GA . The $PilA$ - R_{ct} correlation in Table 3 is -0.92 based on GA values. Section 3.6 and Figure 8 present the correlation between R_{ct} and the expression fold change FC of the $PilA$ gene, yielding -0.993. The FC metric quantifies expression change relative to the control group, while GA reflects absolute community-level abundance. Both coefficients confirm the negative relationship between $PilA$ expression and charge transfer resistance, with FC capturing the induced upregulation effect more directly.

Conclusion

This study achieved quantitative analysis and mechanism elucidation of the direct microbial IET process mediated by nanowires in the organic solid waste co-fermentation system by using surface plasmon resonance enhanced electrochemical impedance spectroscopy technology combined with microbial multi-omics analysis. The experiment showed that under magnetite induction at 4 g/L, the specific methane yield reached 355.42 mL/gVS; the charge transfer resistance decreased to 90 Ω , while the expression fold change of the conductive pili-related gene $PilA$ increased to 4.8. The electrochemical

parameters showed a negative correlation with the expression fold change of *PilA* gene, with a correlation coefficient of -0.993, indicating that the formation of nanowires effectively reduced electron transfer resistance and promoted direct electron exchange between microorganisms. Over the 30-day operating cycle, the system maintained stable conductive performance without observable decline in nanowire activity or acute inhibitory effects on microbial metabolism, as reflected in sustained methane production and gene expression levels. This research method provides a highly sensitive quantitative analysis tool for analyzing the microbial IET mechanism in complex anaerobic systems by real-time monitoring of interface charge transfer dynamics and correlating molecular expression information. It has clear reference value in the process optimization of efficient biological conversion and energy recovery of organic solid waste.

The interpretation of charge transfer resistance reduction as direct evidence of nanowire-mediated electron transfer requires careful qualification. Control reactors without exogenous conductive materials confirmed that baseline electron transfer proceeded through endogenous pathways, yet the experimental design did not decouple the conductivity contribution of added magnetite and graphene oxide from the biological nanowire formation induced by these materials. The observed decrease in charge transfer resistance could arise from the conductive properties of the exogenous materials themselves, from enhanced microbial nanowire expression, or from synergistic interactions between these mechanisms. This ambiguity constrains the attribution of performance improvement specifically to endogenous nanowires. Furthermore, the strong negative correlation between *PilA* gene expression and charge transfer resistance does not establish a direct causal relationship. Correlation analysis, regardless of its statistical significance, cannot replace genetic validation. Gene knockout or inhibition experiments targeting *PilA* or other key electron transfer genes are required to confirm that the reduction in charge transfer resistance depends specifically on nanowire expression. Without such functional genetic evidence, the proposed molecular model remains correlative rather than causative.

Batch operation and the defined substrate composition restrict generalization to continuous-flow systems and other waste types. Laboratory-scale homogeneous mixing and nanomaterial dispersion are not directly transferable to pilot or full-scale reactors, limiting stable nanowire-mediated performance under engineering conditions. When the lipid or ammonia concentration in the substrate deviates from the range measured in this work, the microbial community structure and the expression pattern of electron transfer protein genes may differ from the responses described here.

The multiple linear regression model correlating gene expression with charge transfer resistance requires validation with independent datasets. Model coefficients derived from the current dataset require testing on new experimental data from distinct reactor configurations or substrate compositions to confirm their robustness. The economic cost and long-term environmental fate of adding ultrafine magnetite and graphene oxide to full-scale anaerobic digesters present challenges for large-scale application. Heterogeneous mixing and spatial non-uniformity restrict electron transfer efficiency in practical engineering. Pilot-scale long-term evaluations are necessary to balance material dosing strategies with system returns. The specific substrate composition and batch mode of operation limit the direct generalizability of these findings. Future verification under varying waste sources and continuous-flow reactor configurations is required to establish the broad applicability of nanowire-mediated direct interspecies electron transfer in organic solid waste co-fermentation. Control reactors without exogenous nanomaterials confirmed that baseline electron transfer occurred through endogenous pathways, thereby decoupling the conductivity contribution of added materials from that of microbial nanowires.

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Author's Contributions

Jingkang Chen and Daicheng Liu: Designed the research study.

Jing Zhou Yongqiang Li and Dajiang Guo: Analyzed the data.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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