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RESEARCH ARTICLE

CO_2 fixation in anaerobic biological treatment: amorphous carbon formation driven by electron bifurcation

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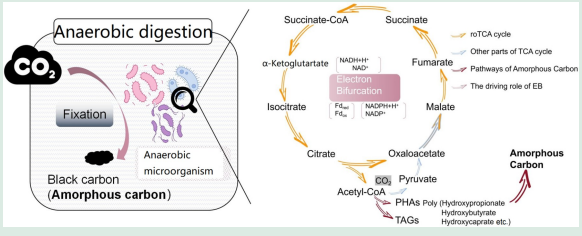
HIGHLIGHTS

- Anaerobic microorganisms immobilize CO_2 into amorphous carbon.
- Electron bifurcation provides energy for CO_2 biofixation in the roTCA cycle.
- Hydroxycaproicate is an intermediate in the formation of amorphous carbon.
- The REDOX equivalents generated by electron bifurcation support CO_2 biofixation.



ABSTRACT: Anaerobic digestion (AD) is a commonly used technology for facilitating carbon fixation by converting complex organic matter into volatile fatty acids and CH_4; however, the issue of CO_2 emission remains unresolved in AD. The formation of amorphous carbon has been identified as a more direct method of carbon fixation in AD. This study aimed to elucidate how amorphous carbon can be formed from organic matter or CO_2 by anaerobic microorganisms. The results showed that amorphous carbon was produced in the anaerobic digestion of inorganic and mixed carbon sources, with yields of 0.38 and 3 μg/10^5 cells, respectively. Its characteristics were analyzed using Raman microscopy. Isotope labeling revealed that CO_2 fixation into amorphous carbon primarily depends on the reversed oxidative tricarboxylic acid cycle (roTCA) and hydroxycaproate. Differential pulse voltammetry combined with gene abundance analysis indicated that flavin electron bifurcation (EB) is involved in electron transfer. The microbial isothermal calorimeter further measured the metabolic calorific value, demonstrating that anaerobic microorganisms can autotrophically fix CO_2 with energy provided by EB. Metagenomic analysis supported the large REDOX equivalents input from EB to sustain the roTCA cycle. This research contributes to understanding the mechanism of CO_2 fixation into solid carbon in anaerobic environments. Additionally, it provides new insights into the potential

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development of carbon-negative technologies in anaerobic biological treatment.

KEYWORDS: Anaerobic digestion, Amorphous carbon, Electron bifurcation, Anaerobic microbial, CO_2 fixation

1 Introduction

Anaerobic digestion (AD) is a low-carbon biological treatment technology commonly employed to convert organic matter in sewage and sludge into methane and volatile fatty acids (VFAs) (Zhang et al., 2023). However, the discovery of amorphous carbon highlights existing research gaps regarding the carbon sequestration potential of AD. The prevailing view has been that amorphous carbon results from incomplete combustion (Coppola et al., 2022). In contrast, a groundbreaking proposal by Allen suggested that anaerobic microorganisms, including methanotrophic archaea and methanogens, directly contribute to the formation of amorphous carbon from gaseous CO_2 (Allen et al., 2021). This hypothesis provides a novel perspective on CO_2 fixation in AD. The treatment of sewage and sludge is considered to be an important technology for the fixation of organic and inorganic carbon sources (Wang et al., 2024; Zheng et al., 2024), CO_2 fixation in anaerobic wastewater treatment contributes to mitigation of greenhouse gas emissions (Meng et al., 2024, Tong et al., 2024). In general, inorganic carbon fixation is facilitated through various autotrophic dark anaerobic pathways, such as the tricarboxylic acid (TCA) cycle, the Dicarboxylate/4-Hydroxybutyrate cycle, 3-Hydroxypropionate bicycle, and the photosynthetic acetyl-coenzyme A pathway (Santos Correa et al., 2023). Recent research shown the bioreactors under increased CO_2 pressure can be advantageous in autotrophic organisms with energetically efficient pathways, which is the reversed oxidative tricarboxylic acid cycle (roTCA) (Steffens et al., 2021). Given the intrinsic drive of microorganisms to optimize energy and matter utilization, the conversion of CO_2 to organic carbon can occur spontaneously (Thevasundaram et al., 2022; Mason-Jones et al., 2023). However, the production of solid inorganic carbon does not appear to play a significant role in microbial energy storage, nor is there a proven functional enzyme involved, despite the reaction being spontaneous, as illustrated by Eq. (1) below:

$$CO_2(g) + 2H_2(g) \rightarrow C(s) + 2H_2O(l) \quad \Delta G^0 = -79.8\ \text{kJ/mol} \tag{1}$$

The transition from CO_2, with an entropy of 213.75 kJ/mol, to graphite, which has an entropy of 5.74 kJ/mol, contradicts the law of entropy increase (Mackey, 1989). This highlights the significant activation energy required for this reaction, which is far from negligible. This energy expenditure is particularly noteworthy given the high energy demands of anaerobic microbes, which typically require one ATP to fix two CO_2 molecules. Crucially, unlike aerobic conditions, the anaerobic CO_2 fixation process does not produce surplus ATP (Ragsdale, 2018). This counterintuitive phenomenon makes the formation pathway of amorphous carbon particularly intriguing.

The roTCA cycle and electron bifurcation (EB) are key processes involved in the transforming of gaseous CO_2 into a solid form. Previous studies suggest that the Gibbs free energy differential provided by high pressure is sufficient to drive substrate phosphorylation, thereby enhancing CO_2 fixation in the roTCA cycle (Steffens et al., 2021). Furthermore, biological CO_2 fixation is a complex cascade of reactions involving multiple enzymes and intermediates. There must be significant driving forces that render metabolic processes biologically advantageous. Nunoura et al. proposed that substantial inputs of REDOX equivalents through EB may supply the necessary thermodynamic driving force for the roTCA cycle (Nunoura et al., 2018). Anaerobic bacteria and archaea utilize electron bifurcation, reverse electron transfer, and ATP energy coupling for growth and metabolism (Müller et al., 2018). Given the electron transfer rate of flavin hemiquinone in the picosecond range, EB has been identified as an efficient mechanism for energy coupling (Lubner et al., 2017). Specifically, the reduction of ferredoxin (Fd) by H_2 consumes one ATP equivalent for ATP hydrolysis, 0.58 ATP for reverse electron transfer, and 0.38 ATP for EB (Müller et al., 2018). EB can overcome energy barriers by regulating REDOX equivalents, such as Fd_{ox}/Fd_{red}, NAD^+/NADH, and $NADP^+$/NADPH, thereby conserving more energy (Buckel and Thauer, 2018). EB may also be potentially linked to biological CO_2 capture in AD. However, the precise enzymatic reactions involved in electron bifurcation within the roTCA cycle remain unknown. In addition, although Acetyl-CoA is the primary product of the roTCA cycle, how inorganic solid carbon is synthesized remains unsupported by existing research.

The production of amorphous carbon remains an enigmatic process, with an incomplete understanding of the underlying metabolic pathways and objectives.

This study aims to investigate the CO_2 fixation to amorphous carbon process during anaerobic digestion. A microorganism solution system was used to simulate CO_2 fixation under anaerobic conditions in order to: 1) explore this novel CO_2 fixation pathway and identify the metabolic pathways of amorphous carbon in AD; and 2) for the first time, examine the role of energy from EB in the formation of amorphous carbon.

2 Materials and methods

2.1 Experimental design

The mixed microbial solution from sludge was put into 80 mL test tubes for culture, the extraction method is given in Support Information (SI). CO_2 was injected into the tubes for two ways (Circulate: 1 L/min for 1 min; Seal: 10 mL CO_2/50 mL microbial solution for 1 atm). The injection of hydrogen is continued and the final ratio of H_2:CO_2: 80%:20% is the headspace. Using acetic acid, nitrate and formic acid (GR, Aladdin, China) as electron donors (0.1 mmol/L) in others group. The Settings of carbon sources and electron donors for each experimental group are shown in Table 1.

2.2 Extraction and analysis of black materials containing amorphous carbon

The obtained part of the black microsphere were further analyzed by Raman image-scanning electron microscopy RISE-MAGNA (TESCAN, Czech Republic), confocal micro-Raman spectrometer Renishaw inVia Qontor (Renishaw, UK) and X-ray photoelectron spectrometer ESCALAB Xi+ (Thermo Fisher, USA) to determine the characteristic of elemental carbon. Excitation was provided by a 532-nm with maximum output power 25 mW, measurements were collected for 0.76 ms. And the ^{13}C abundance of carbon dioxide after combustion with oxygen. The extract method of black microspheres is given in SI. The growth density (weight/fluid volume) of amorphous carbon was calculated for each group of bacterial fluids before and after culture.

Table 1 The yield of amorphous carbon from different carbon sources

Reactor	Electron donor	CO_2 use	Carbon source	Yield of amorphous carbon (μg/10^5cell)
IC	H_2	Circulate	Inorganic	0.38
O	Glucose + H_2	None	Organic	0.38
MC	Glucose + H_2	Circulate	Mix	3
L	None	Circulate	Inorganic	17.44
H	H_2	Seal	Inorganic	1.13
N	Nitite	Seal	Inorganic	0.94
F	Formate	Seal	Organic	2.06
A	Acetate	Seal	Organic	1.5

2.3 General data

Microbial reactor operation data: Gas composition was analyzed using gas chromatography GC-2014C (Kratos Shimadzu, Japan), organic acid measured by gas chromatography Nexis GC-2030 (Kratos Shimadzu, Japan). Biological components data: electron carrier concentration, DPV determination of flavin activity was described in a previous article (Zhang et al., 2023).

2.4 Microbial isothermal calorimetry

The 1 mol/L (saturated concentration) ^{13}C-dissolved inorganic carbon (DIC) (MERYER, China) was configured with PBS and the concentrated sample was washed and suspended with PBS. The 500 μL sample was added to the reactor, the buffer solution was added to the other reactor, and the temperature of the detection chamber was set at a constant 35 °C. The change of heat value in the process of reaction was measured by Biological Calorimeter Micro Cavlet Ultra (SETARAM, France), ATP and calorimetric respiration ratio were calculated. Details are given in the SI.

2.5 Isotope labeling and polymer testing

The amorphous carbon was extracted and the elemental carbon was determined by elemental analysis isotope mass spectrometry TRACE1310/EA Isolink/PreCon/253Plus (Thermo Fisher, USA). The ^{13}C labeling results of organic acids were measured by gas chromatography-time-of-flight mass spectrometer 7890B/Pegsus BT (LECO, USA). The polymers were tested using Matrix-assisted Laser Desorption tandem time-of-flight mass spectrometry MALDI TOFTOF 7090 (Kratos Shimadzu, Shimadzu, Japan), details are given in the SI.

2.6 Metagenomics analysis

The E.Z.N.A. soil DNA Kit (Omega, M5635-02, USA) was used to extract DNA. Qubit 4.0 (Thermo, USA) was used to measure the DNA concertration.

Sequencing libraries were generated with the Hieff NGS® MaxUp II DNA Library Prep Kit for Illumina® (YEASEN, China). Details are given in the SI. The species annotation information and functional annotation information of genes were obtained by comparing gene sets with NR, KEGG, TCDB, and other databases. Functional abundance and species abundance were obtained according to gene set abundance.

3 Results

3.1 Discovery of amorphous carbon in anaerobic microorganisms

Hydrogen as electron donor, three anaerobic microbial reactors with 0.5 mol/L glucose (organic carbon source), CO_2 (inorganic carbon source) and 0.5 mol/L glucose + CO_2 (mixed carbon source) as substrate were designed. In the data of gas component, VFAs concertration and three-dimensional fluorescence (Figs. S1 and S2), it can be seen that CO_2 is almost completely converted but not balance with organic acid accumulation. The VFAs concertration of inorganic carbon source group may due to the conversion of carbon sources, but is more likely due to fluctuations in the data. While in the microbial organic metabolic region with mixed carbon sources, the fluorescence intensity is weak, and the carbon sources seem to be partially transferred to the unknown direction. The extraction of amorphous carbon was carried out after one month culture, and the method was referred to the previous research (Allen et al., 2021). In each reactor, a product resembling amorphous carbon is extracted, and the yield is given in Table 1. The observed variations in amorphous carbon yield across different electron donors can be primarily attributed to the intricate interactions within microbial consortia. Notably, acetophilic methanogens exhibit enhanced metabolic activity under acidic pH conditions, and acetic acid serves as a versatile substrate that can be metabolized by a broader spectrum of microorganisms. Formic acid plays a pivotal role in facilitating interspecies electron transfer processes, whereas the microbial populations capable of direct nitrite utilization for electron transfer are relatively restricted. Since formic acid and acetic acid can be obtained by decomposition of glucose, the carbon fixation pathways of them as electron donors are not discussed more. And both are represented by the mixed carbon sources group.

In Fig. 1(a), black particles (20 μm) can be seen in the optical electron microscope images. Characteristic peak D (disordered) at ~1350 cm^{-1} of the Raman spectrum represents the degree of disorder of amorphous carbon, and the peak G (graphite) at ~1575 cm^{-1}, represents the structure of graphene (Fig. 1(b)) (Allen et al., 2021). The product is not the graphene cause the activation energy required for the formation of graphite crystals is usually provided by high temperature and pressure rather than mild enzymatic reaction (Jedwab and Boulègue, 1984; Yamaoka et al., 2002). The level of order (crystalline carbon) compared to disorder (amorphous carbon) of the product assessing by the relative intensities of the D and G bands. The inorganic carbon source and mixed carbon source cultures also show obvious amorphous carbon characteristics. The valence band of amorphous carbon as the active material is 2.67 eV, its semiconductor properties are explained (Fig. 1(c)). The semiconductor characteristics of amorphous carbon represent the electron carrying and storage properties of amorphous carbon materials. Direct inter-specific electron transfer is a unique and important survival strategy in the coculture of anaerobic microorganisms. Semiconductors such as iron oxides, such as magnetite, can be transferred between species as electron carriers to promote microbial metabolism and growth. Moreover, the energy band of amorphous carbon is 2.67 eV, which is about equal to high-quality cadmium sulfide semiconductor particles. Although this "metabolite electron carrier" formed by microorganisms has a small output, it also has a good function of electron storage and transfer, which means that the formation of amorphous carbon has a certain significance for microbial electron transfer. It also exhibits a higher ion migration rate (Hu et al., 2023) (Fig. 1(d)), which is also explained by the test results of XPS. The C1s spectra from XPS (Fig. S3) identified a composition of 62.63% C−C and C=C bonds, 24.55% C−O and C−N bonds, and 12.82% C=O bonds, which is the residual protein on the amorphous carbon surface, and Table S1 shown the prevalence of elemental carbon in sample. To explore whether there are other electron donors other than H_2 in the mixed substrate that can participate in the electron bifurcation reaction, a repeated experiment with acetate, formate, H_2 and nitrite (Ragsdale, 2018; Allen et al., 2021) as electron donors and CO_2 as the only carbon source was set up according to previous studies, and the CO_2 conversion process was given in Figs. S1(a) and S1(b). According to the amount of amorphous carbon obtained in the CO_2 only reactor (L), it seems inevitable that CO_2 and biomass will eventually move to amorphous carbon even if no additional electron donor is added over a long period of operation.

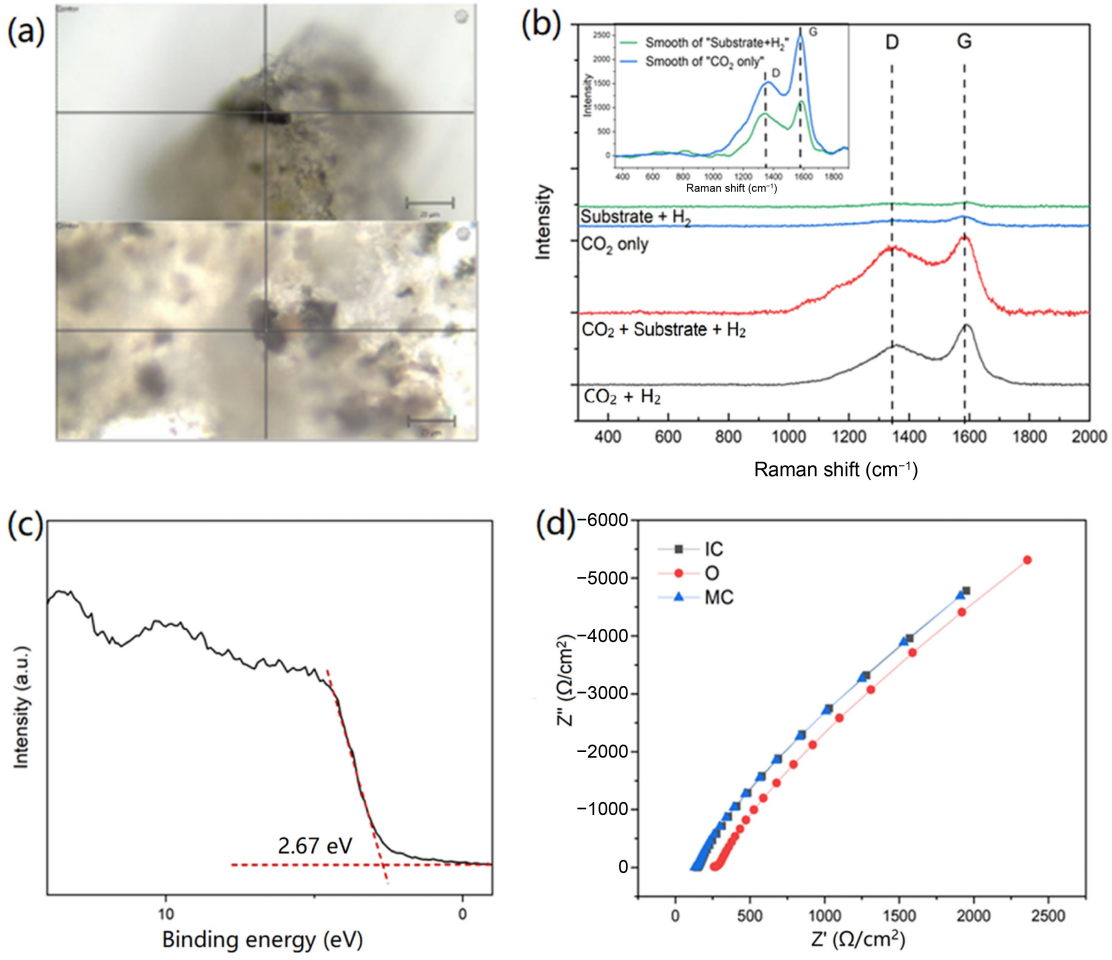

Fig. 1 Characterization of amorphous carbon. (a) Optical microscopy and (b) Raman spectroscopy. (c) Detection of amorphous carbon valence bands using X-ray photoelectron spectroscopy (XPS). (d) Electrochemical impedance spectroscopy (EIS) for microbial system detection.

3.2 Explore the formation path of amorphous carbon

In the isotope experiment, where soluble inorganic carbon (sodium bicarbonate) was used as the sole carbon source, a total of nine organic acids associated with the roTCA cycle were detected with ^{13}C labeling in two reactors. These included citrate, isocitrate, alpha-ketoglutarate, oxaloacetate, malate, fumarate, succinate, succinate-CoA, Acetyl-CoA and pyruvate (Ragsdale, 2018), as shown in Figs. 2(a) and 2(b). Lactate, as a product of pyruvate leaving the roTCA cycle, was only observed in the reactor with electron bifurcation. Notably, in mixed microbial systems, ^{13}C-NMR faces challenges due to spectral overlap and limited resolution, which can obscure specific metabolic pathways. In contrast, GC-MS offers superior sensitivity and the ability to simultaneously trace multiple metabolites, making it more suitable for detailed isotopic tracing in complex environments. Compounds in which ^{13}C replaces one carbon atom are designated as M + 1. Alpha-ketoglutarate exhibited the highest number and proportion of labeled C atoms, being three times greater in the IC reactor than in the MC reactor. Furthermore, 3-hydroxybutyrate, 3-hydroxypropionate, and 6-hydroxycaproate, previously identified as polymer monomers for intracellular carbon storage (Yang et al., 2019; Zheng et al., 2020), were also measured and found to carry a higher proportion of the ^{13}C in the IC group. As shown in Fig. 2(c), the appearance of 3-hydroxypropionate may be attributed to the fixation of inorganic carbon as HCO_3^- through a 3-Hydroxypropionate bicycle (Zarzycki et al., 2009). Finally, the percentage of labeled carbon atoms in the amorphous carbon cultured in the IC reactor was 2.16 times higher than in the MC reactor, with the corrected relative values of natural isotope abundance being 13342.76‰ and 67.96 ‰, respectively, as shown in Fig. 2(d).

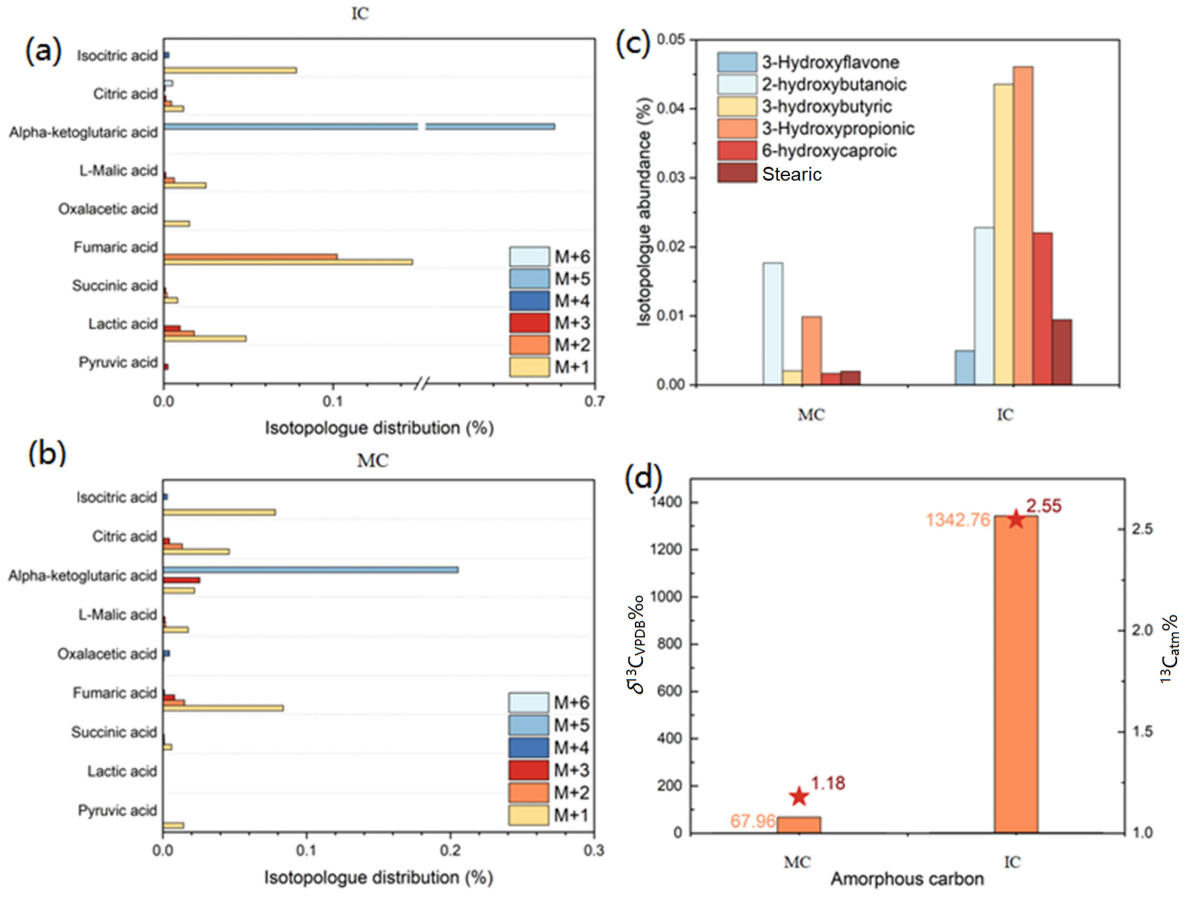

Fig. 2 The formation path of amorphous carbon. Organic acids and amorphous carbons labeled with the ^{13}C isotope were detected in MC and IC (a–d). Group IC: CO_2 only as carbon source, group MC: glucose and CO_2 as mixed carbon source.

The Gibbs free energy for the conversion of Alpha-ketoglutarate to subcitrate reaction is +19 kJ/mol (Nunoura et al., 2018), and similarly, the Gibbs free energy for the conversion of citrate to acetooxalic acid is +37 kJ/mol (Nunoura et al., 2018). These two steps are thermodynamic disadvantages that must be overcome in the formation of amorphous carbon. Given that Hydrolysis of ATP to ADP and Pi can release 7.3 kcal (30.5 kJ) free energy per mole (ATP = 30.5 kJ/mol), we give the minimum ATP assumed to be provided in rotca. For the overall reaction, as shown in Fig. 3, although the Gibbs free energy for the conversion of succinate to oxaloacetate is +55 kJ/mol, the Gibbs free energy for the other half of the reaction is –95 kJ/mol (Nunoura et al., 2018). The entire reaction is thermodynamically favorable and spontaneous. As previously mentioned, the rTCA cycle is one of the oldest and least ATP-demanding autotrophic pathways in evolution (Ragsdale, 2018).

In Fig. 4, the results of the Matrix-assisted Laser Desorption showed that the characteristic peaks of TAGs at 636 m/z (Mass to charge ratio) were obtained in the IC and MC reactors, respectively. The polymer characteristics of the repetitive unit approximate the molecular weight of hydroxycaproate were obtained. In the amorphous phase, the molecular chains in the polymer are not organized in any particular way. In combination with isotopic data, polymers with unsaturated hydroxycaproate as repeating units were considered.

3.3 REDOX activity of flavin-based electron bifurcation during CO_2 fixation

Further results support the correlation between electron bifurcation and CO_2 fixation in the roTCA cycle. Flavins, includes flavin mononucleotides (FMN) and flavin dinucleotides (FAD), act as electron transfer units in flavin-based electron bifurcation (Jia et al., 2021). In Fig. 5, the inorganic carbon source group (IC) exhibited excellent FAD-reduction and oxidation characteristics at −580 and −424 mV, respectively.

In our previous studies, the activity of flavin was used as an indicator of electron bifurcation activity

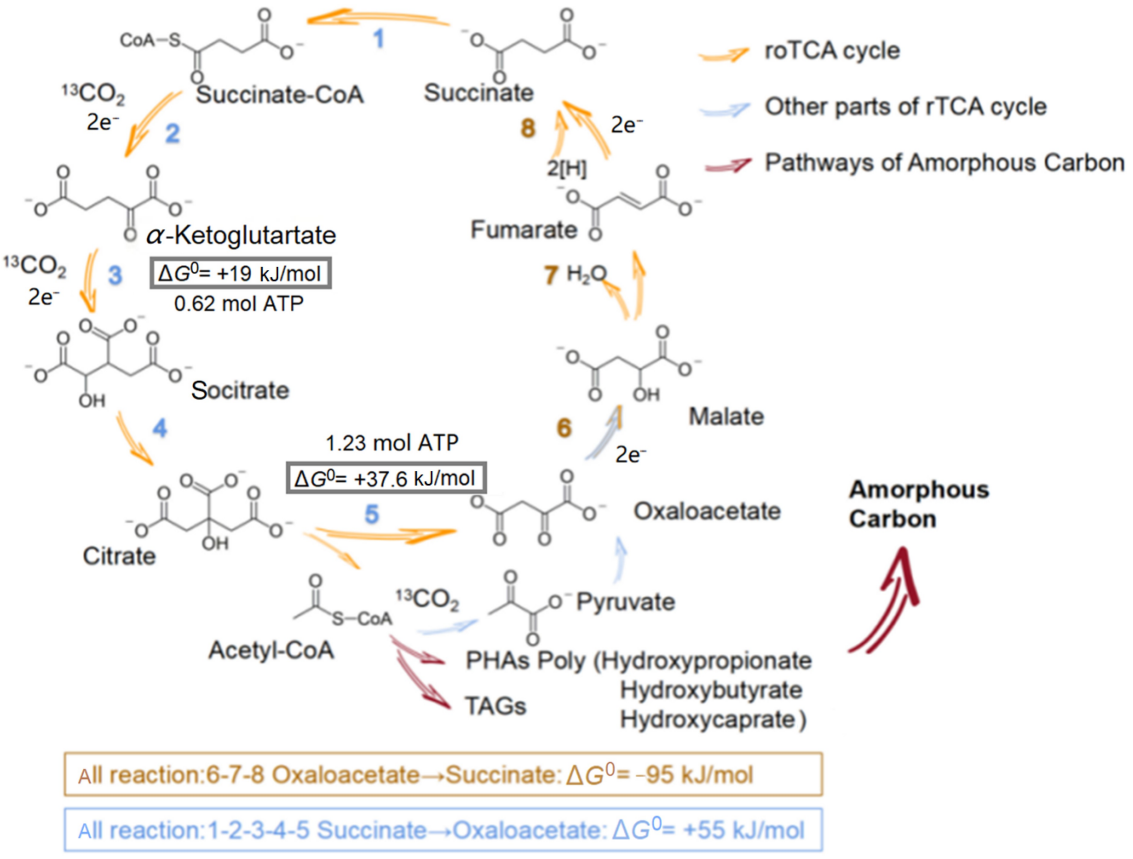

Fig. 3 A hypothesized metabolic pathway map of amorphous carbon produced by the roTCA cycle.

(Zhang et al., 2023). FAD has been frequently referenced in many enzyme activity studies of electron bifurcation (Buckel and Thauer, 2018; Peters et al., 2018; Kayastha et al., 2021). Our results indicate that the electrochemical activity of FAD favors inorganic carbon utilization by anaerobic microorganisms in hydrogen as an electron donor over other types of carbon source utilization (organic or mixed). Microorganisms cultured with an inorganic carbon source exhibited higher FAD (Flavins) REDOX activity, while showing weaker ascorbic acid oxidation activity.

3.4 Thermodynamic conditions support by electron bifurcation

The biological process of fixing carbon dioxide as a solid carbon does not involve complex extracellular electron transfer but instead requires high energy metabolism within the cell. The microbial calorific results provide insight into why microbial metabolism with EB can support this carbon fixation process. Microbial isothermal calorimetry (MIC) was employed to characterize the metabolic activity of anaerobic microorganisms, typically presented as plots of power (μW) versus time (*t*) (Velázquez‐Campoy et al., 2004; Corvec et al., 2020). The heat flow data from the Isothermal microcalorimeter support the energy supply shown in Fig. 6 and SI text. The M + DIC group, using DIC as the background calorific value, represents the reaction heat upon interaction between microorganisms and inorganic carbon. The actual reaction calorific value for a 500 μL cell sample is the integral area multiplied by time, yielding 0.035 J for microbes without carbon source substrate. The reaction calorific value for the inorganic carbon source is 0.143 J at 37 °C, and the combined reaction calorific value for microbial and inorganic carbon source is 0.521 J. When the cell concentration was doubled, the response rate also doubled, while the heat dissipation decreased. These results indicate that the energy of microbial reaction is fixed with constant substrate concentration, and the remaining ATP primarily supports their own growth and reproduction, thereby increasing carbon sequestration efficiency.

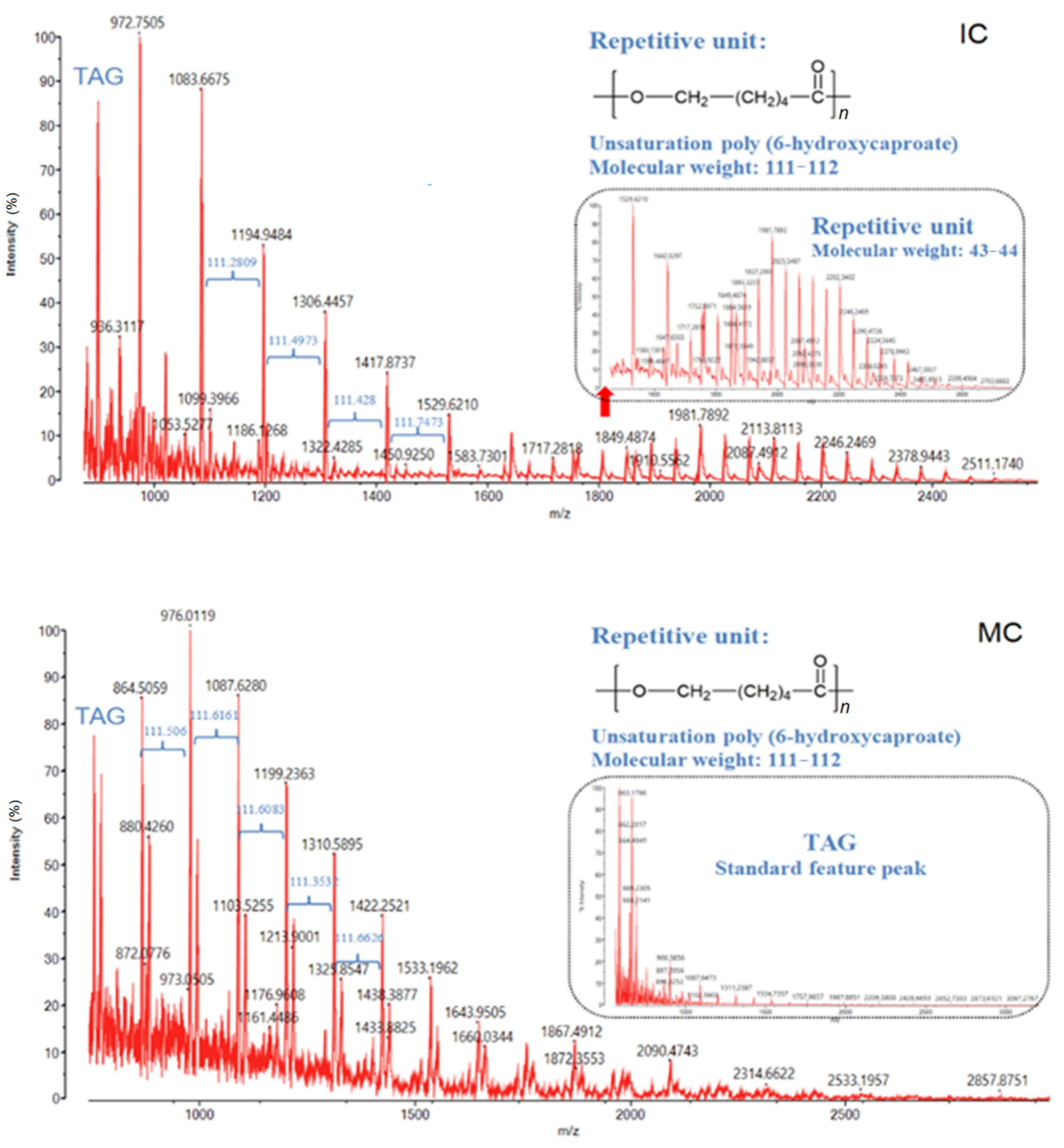

Fig. 4 Polymers characteristics in microbial reactors.

3.5 Microbial metagenome analysis

Figure 7(a) presents the annotated results from the KEGG gene database, illustrating the abundance of key microorganisms associated with the reverse tricarboxylic acid (roTCA) cycle and their corresponding functional genes. *Ethanoligenens harbinense* is considered the primary microorganism responsible for electron bifurcation-driven CO_2 fixation into amorphous carbon. As illustrated in Fig. 7(b), Sporolactobacillus putidus emerged as the dominant species in both the MC (63.17%) and IC (58.46%) groups. Notably, the abundance of *Ethanoligenens harbinense*, a key hydrogen-producing bacterium, exhibited a significant increase in the IC group (5.97%) compared to the MC group (0.45%), suggesting its potential role in enhancing hydrogen metabolism under specific conditions. Similarly, *Lacticaseibacillus paracasei* showed a contrasting trend, with higher abundance in the MC group (5.24%) than in the IC

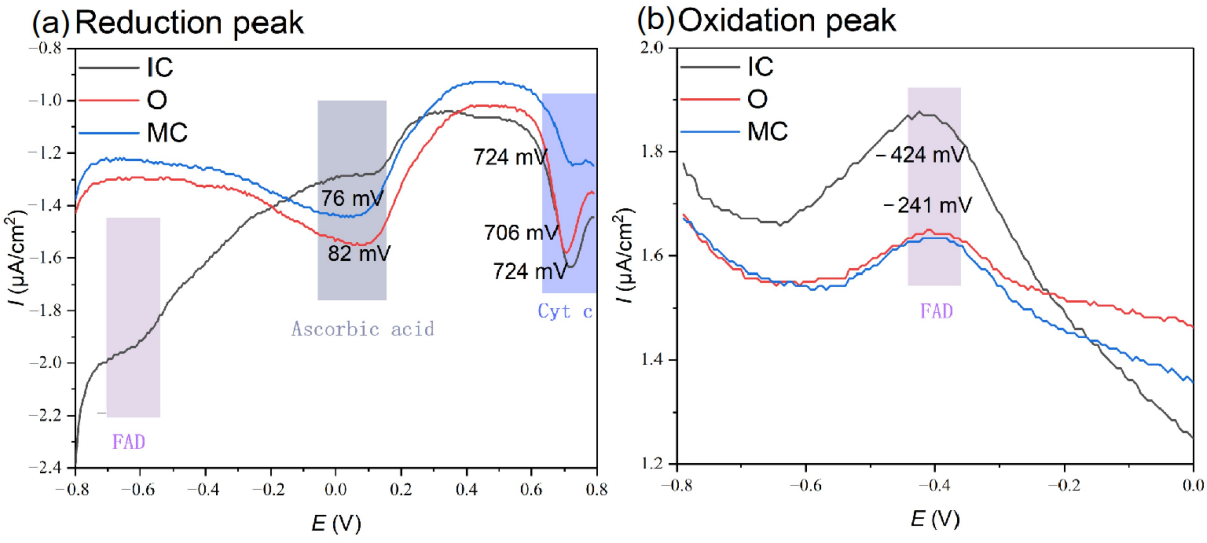

Fig. 5 DPV test for flavins and ascorbic acid. Reduction peak (a) and oxidation peak (b). Group O: glucose only as substrate, group IC: CO_2 only as carbon source, group MC: glucose and CO_2 as mixed carbon source.

group (0.04%). In groups with different electron donors, hydrogen promoted the dominance of *Methyloversatilis universalis* (33.12%) and *Methyloversatilis* sp. (11.17%) in the IC group, indicating their involvement in methylotrophic metabolism. Conversely, acetic acid favored the growth of *Azospira oryzae* (16.66%) and unclassified *Azospira*species (11.31%), highlighting their adaptability to organic acid utilization. These findings underscore the influence of electron donors on shaping microbial community structure and metabolic functions. As shown in Table S2, the genes K00265 and K00266 derived from *Ethanoligenens harbinense* were annotated. The two units of Glutamate synthase (NADPH), large and small chain are closely related to EB due to the NADP/NADPH (Swuec et al., 2019). The iron sulfide clusters, FAD and FMN transfer electrons from NADPH to Alpha-ketoglutarate and the amino group NH_3^- from L-glutamine, and finally form L-glutamate. Although this is not a direct electron bifurcation reaction, electron bifurcation regulates the REDOX equivalent, serving as an electronic currency to accelerate the process. There was a large difference in abundance between IC and MC (3.86% vs 0.24% and 7.11% vs 0.46%). Additionally, differences in the abundance of K04072: Acetaldehyde dehydrogenase/ alcohol dehydrogenase, a double-branching functional enzyme with associated energy potential (Camargo et al., 2023), were noted.

Figure S4 shows the annotated proteins in the TCDB database. The predominance of Iron-Sulfur Protein (ISP) Superfamily in IC verifies the claim that microbes rely on Iron-Sulfur cluster for electron transfer in the absence of substrate energy (Zanello, 2018). Interestingly, the P-type ATPase (P-ATPase) Superfamily (Chan et al., 2010) also showed an abundance advantage in groups other than MC. The two functions of ATP hydrolase and EB, previously thought to be opposite, may occur under distinct nutritional conditions, and the underlying mechanisms warrant further investigation. In Figs. 8(a) and 8(b), based on the abundance of genes in the TAGs synthetic pathway, it appears that hydrogen as an electron donor is more conducive to the storage of intracellular carbon sources as lipids. In comparison to mixed carbon sources, inorganic carbon sources are more conducive to the transformation of acetyl-CoA into more complex biological precursors. A more pronounced gene abundance related to PHBs synthesis appeared in the IC group. In Fig. 8(c), pathways such as M00173: Reductive citrate cycle, M00374: Dicarboxylate-hydroxybutyrate cycle and M00376: 3-Hydroxypropionate bi-cycle represent the carbon sequestration pathway favored by the microbial community. The abundance comparison of fixed carbon pathway is shown in Table S3. The genes in the flavin and quinone synthesis pathways are shown in Fig. 8(d), as a comparison, with an abundance comparison of energy-related genes shown in Fig. S5.

4 Discussion

4.1 Conditions for the formation of amorphous carbon

Recent studies have demonstrated that amorphous carbon is a significant product of certain newly

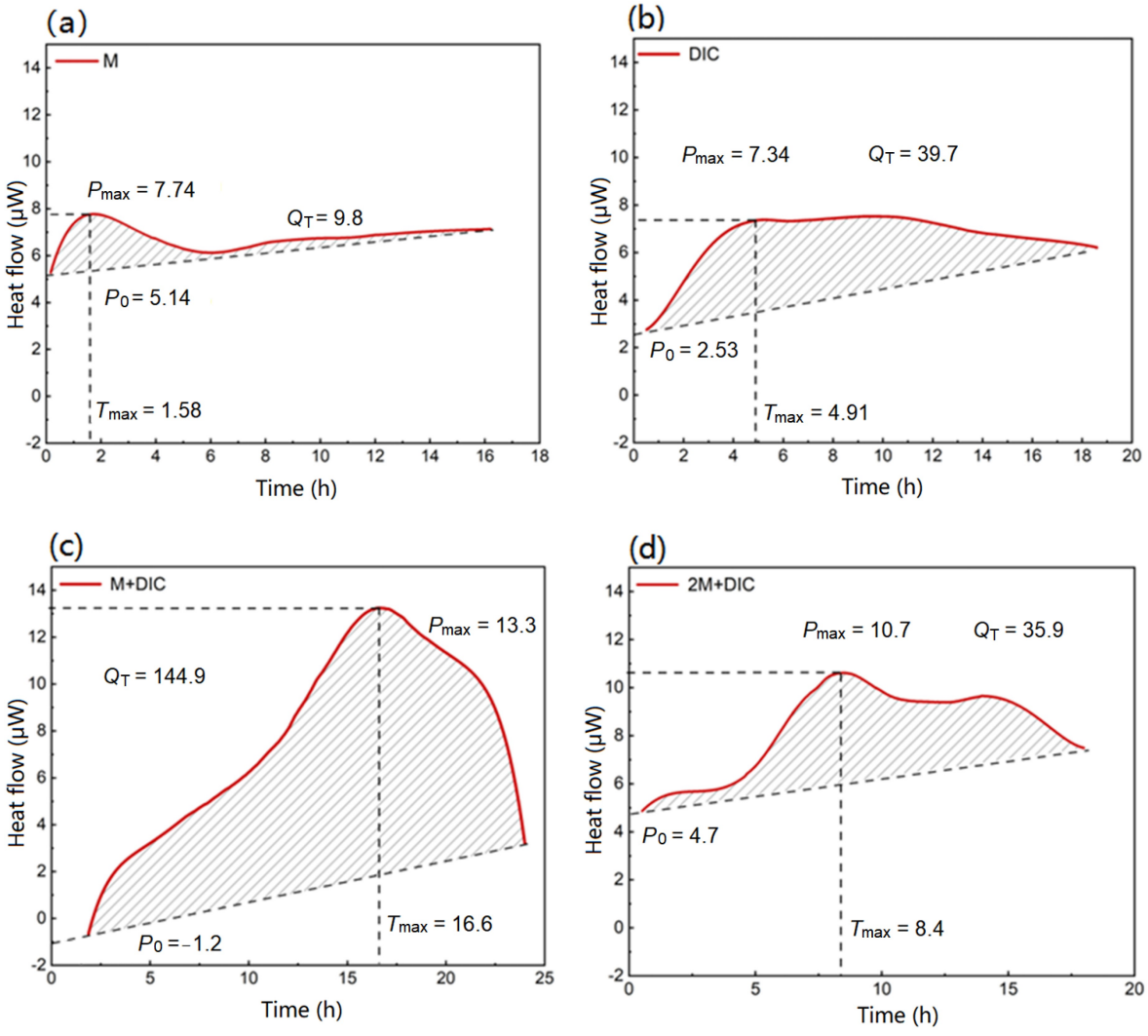

Fig. 6 Microbial heat change in the CO_2 conversion process. (a) Microbial only, (b) DIC: Soluble inorganic carbon + inactivated microorganism, (c) microorganism + DIC: Microorganism and soluble inorganic carbon, (d) double microorganism + DIC.

identified anaerobic microorganisms, although the specific metabolic pathways and conditions driving this process remain undefined (Allen et al., 2021). The production of solid carbon within anaerobic environments represents a noteworthy finding, suggesting that cells, which already expend considerable effort to sustain themselves, must also navigate thermodynamically unfavorable conditions to produce compounds that, at present, do not seem to confer any direct benefit to them. The primary focus of our study is to investigate the mechanism by which anaerobic microorganisms fix carbon dioxide as amorphous carbon and the metabolic processes that drive this reaction, with the aim of elucidating and discussing these phenomena.

It can be inferred that H_2 and CO_2 are essential for the formation of amorphous carbon. The inorganic carbon source shares distinct amorphous carbon characteristics with mixed carbon sources, indicating that the additional ATP from organic carbon sources has a minimal correlation with the development of disordered structures. EB is currently considered the most efficient energy coupling mechanism for anaerobic microorganisms (Müller et al., 2018). Given that H_2 serves as a direct electron donor in EB, it is hypothesized that electron bifurcation could be activated under substrate-limited conditions to facilitate CO_2 fixation. In contrast, glucose cultures exhibit a higher of amorphous carbon production, implying that H_2 might not be the sole electron donor capable of

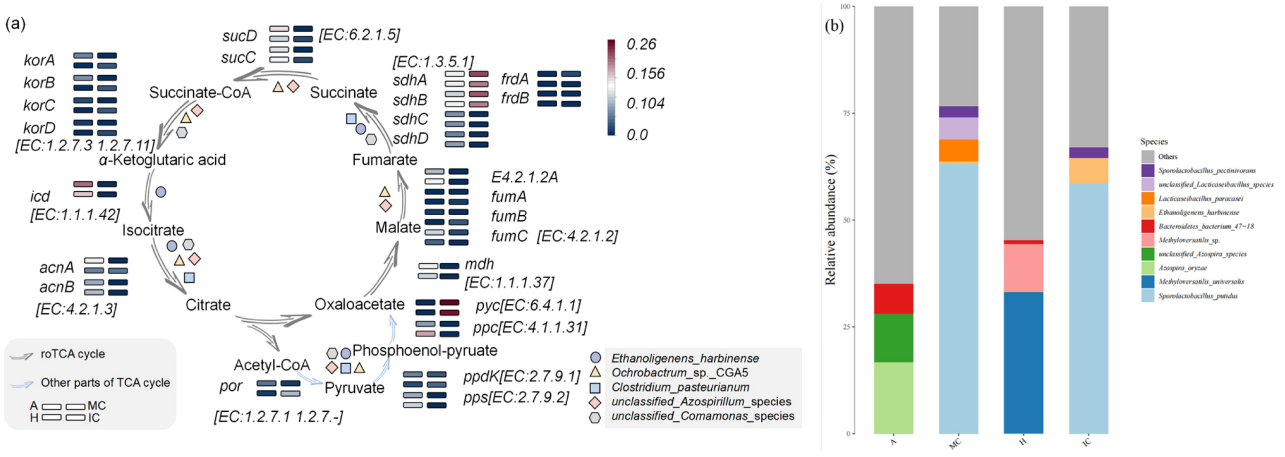

Fig. 7 The abundance of genes associated with amorphous carbon formation pathways. (a) Microorganisms participating in the roTCA metabolic pathway and their gene abundance, (b) abundance of microbial in species levels.

promoting CO_2 fixation into amorphous carbon. Furthermore, as a unique byproduct of anaerobic digestion, the broad valence band of amorphous carbon, similar to the effective catalyst cadmium sulfide, could act as an electron transfer intermediary between cells. The role of this process in enhancing the system's electrical conductivity, manifested through ionic mobility (Hu et al., 2023).

4.2 The formation path of amorphous carbon

A proposed metabolic pathway for the biological fixation of CO_2 is outlined. The detection of ^{13}C in organic acids and amorphous carbon suggests that the inorganic carbon source is partially fixed via the roTCA to a certain degree. Alpha-ketoglutarate, a key intermediate in the roTCA, and the NADPH driven enzymatic reaction not only reflect the inversion of the TCA cycle but also emphasize the REDOX equivalence associated with EB. The enhanced efficiency of Alpha-ketoglutarate metabolism, accelerated by electron bifurcation, promotes CO_2 fixation within the roTCA (Swuec et al., 2019). CO_2 fixation is coupled with the synthesis of acetyl-CoA as valuable biochemicals, leading to the formation of TAGs and longer chain hydroxyl polymer monomers, such as hydroxybutyrate and hydroxypropionate. Notably, in addition to polyhydroxycaproate, another polymer characterized by repeating units has been identified in the IC-reactor. Given the slightly higher metabolic rate in IC compared to MC, this compound may represent a previously unrecognized step in the polymer evolution toward amorphous carbon. The molecular weight of this repeating polymer unit is 44, and the lack of identifiable ^{13}C-labeled short-chain acids suggests it might be an unsaturated repeating unit monomer, likely resulting the removal of some functional groups, with a carbon chain backbone. Amorphous carbon, its intracellular existence is similar to that of a magnetosome, might be enveloped by protein membranes and exported from cells through exocytosis or released upon cell fragmentation via apoptosis.

4.3 Energy and electron transfer for amorphous carbon formation

We further explore the energy metabolism and electron transfer mechanisms in microorganisms. Interaction between microbial and soluble inorganic carbon exhibits an exothermic reaction, promoting energy accumulation during the autotrophic CO_2 fixation. This observation underscores the hypothesis that the energy generated by electron bifurcation in the reversed oxidative tricarboxylic acid (roTCA) carbon sequestration pathway is ample to sustain the cycle, with a resultant surplus of energy being released. The yield of amorphous carbon is quantified at 0.38 μg per 10^5 cells, and the energy dissipated during the fixation of CO_2 into amorphous carbon amounts to 0.417 J/μg of amorphous carbon. Notably, when the concentration of cells doubled, the reaction rate of also doubled, leading to a reduction in heat dissipation.

The primary focus of this research is to clarify the catalytic role of electron bifurcation in the reversed oxidative tricarboxylic acid (roTCA) cycle. The conversion of Alpha-ketoglutarate into glutamate is posited as a crucial step in facilitating the roTCA cycle. Within the roTCA cycle, the conversion of succinic acid to succinyl-CoA represents a significant energy-consuming phase. The reduced product concentration

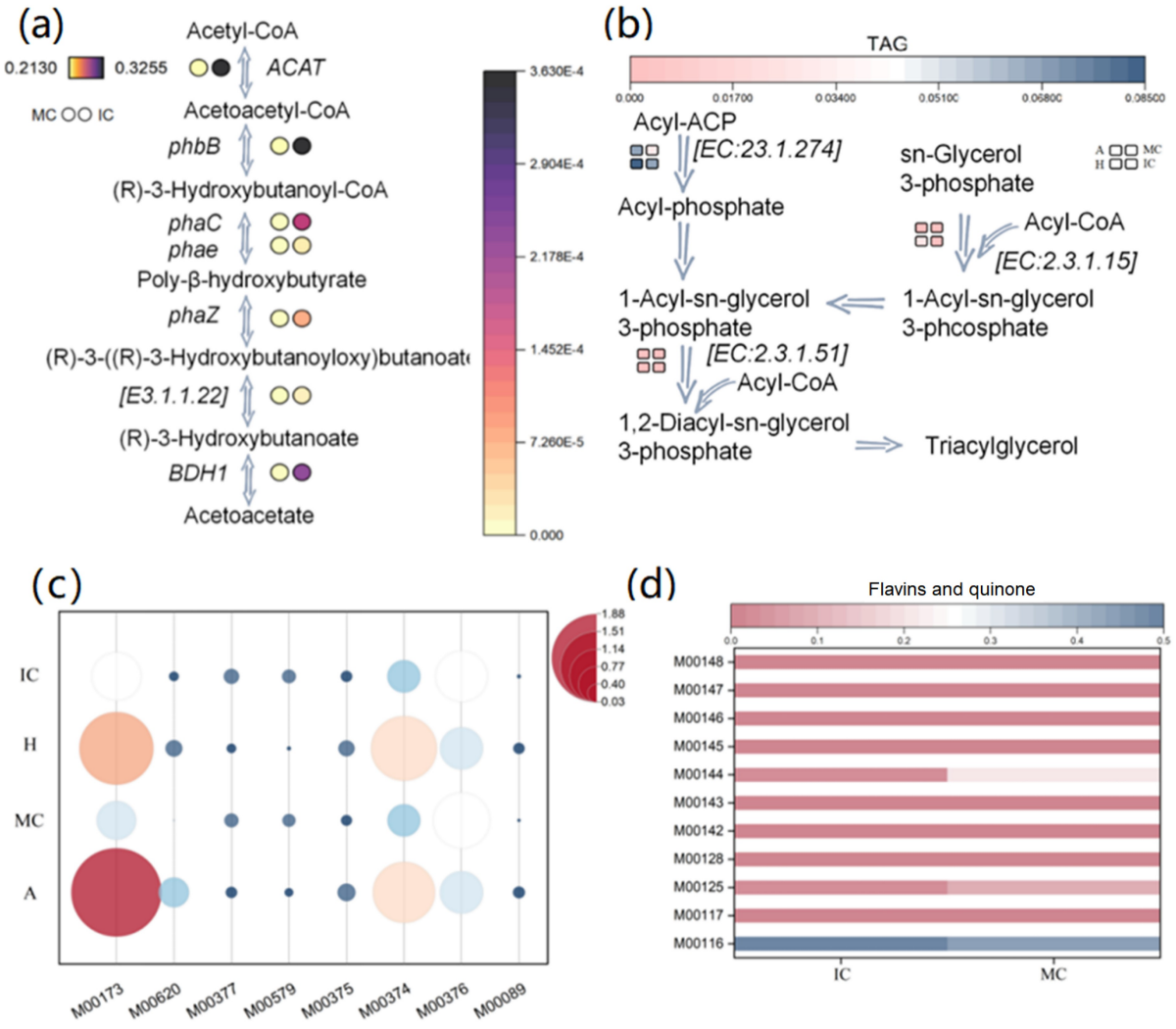

Fig. 8 The abundance of (a) TAGs synthesis pathway, (b) PHBs synthesis pathway, carbon sequestration and (c) triacylglycerol biosynthesis pathway, the abundance data are given in the Table S3, and (d) flavin and quinone gene abundance.

aids the conversion of succinate-CoA back to CO_2, simultaneously mitigating the ATP requirement of the preceding reaction. Moreover, other rate-limiting enzymes reactions may also rely on the REDOX equivalents (electron pairs: NADPH, NADH, and Fd) from the electron bifurcation (Buckel and Thauer, 2018). Due to the phylogenetic proximity of these anaerobic microorganisms, most are likely capable of electron bifurcation, potentially forming a symbiotic multi-cellular system for amorphous carbon production. In conclusion, while black carbon is conventionally believed to enter oceans via aerosols and riverine deposits, the amorphous carbon characterized in this study shares similar properties and morphology with black carbon. The multicellular amorphous carbon production system be applicable to anaerobic environments such as oceans and soils (Coppola et al., 2022). Biologically sourced black carbon may complicate the measurements of black carbon derived from petroleum combustion, thereby affecting our understanding of CO_2 fixed and black carbon formation in anaerobic environments.

5 Conclusions

This study presents the yield and characterization of amorphous carbon collected from anaerobic microbial solutions, and discusses its formation process and energy requirements. The formation of amorphous carbon can be summarized as follows: the CO_2 fixation process to amorphous carbon under the reversed roTCA

cycle, facilitated by electron bifurcation, with polyhydroxycaproate serving as an intermediate product. The discovery of amorphous carbon suggests that anaerobic digestion may have untapped potential for carbon sequestration. As a result, the development of direct anaerobic biological CO_2 fixation technologies is expected to gain attention in the future.

CRediT Authorship Contribution Statement

Tengyu Zhang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing-original draft, Writing-review and editing. **Jingxin Zhang:** Conceptualization, Funding acquisition, Project administration, Resources, Validation, Writing -review and editing. **Pengshuai Zhang**: Writing-review and editing. **Yen Wah Tong**: Conceptualization, Resources. Yiliang He: Conceptualization, Resources. **Qing Yang**: Conceptualization.

Conflict of Interests 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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