Main components of free organic carbon generated by obligate chemoautotrophic bacteria that inhibit their CO₂ fixation

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Highlights
Small molecules are the main components of EFOC that inhibit the CO₂ fixation of SOBs
Amino acid and organic acid generated by SOB are the main inhibitors of its CO₂ fixation
Amino acid and organic acid inhibit CO₂ fixation by repressing cbb gene transcription
Developing targeted measures to remove main inhibitors profits CO₂ fixation of SOBs
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SUMMARY

Chemoautotrophic bacteria play an important role in combating the rise in global CO₂. However, recently it was found that extracellular free organic carbon (EFOC) generated by chemoautotrophic bacteria inhibits their CO₂ fixation. Although continuous-flow membrane bioreactor can remove EFOC and enrich bacteria, it may also remove beneficial bio-factors for bacterial growth. Finding out the main inhibitory factors and inhibitory mechanisms in EFOC can provide theoretical guidance for the development of targeted inhibitory component removal technology. The results show a significant negative correlation between the increasing proportion of small-molecule EFOC and the decreasing trend of CO₂ fixation efficiency, and simulation experiments confirm that the small molecule organics such as amino acids and organic acids are the main components of EFOC that inhibit CO₂ fixation by inhibiting ribulose bisphosphate carboxylase/oxygenase (RuBisCO) gene (cbb) transcription efficiency. Therefore, amino acids and organic acids are suggested to be recovered to promote efficient CO₂ fixation of autotrophic bacteria.

INTRODUCTION

Carbon capture, utilization, and storage (CCUS) have been regarded as a pivotal technological method for reducing carbon emissions and mitigating climate change.¹–³ The role of chemoautotrophic bacteria in CCUS is too significant to be ignored.⁴

Chemoautotrophic bacteria are widespread, and fix CO₂ without light in a variety of harsh habitats (including deep-sea hydrothermal vents, barren desert soil, and large-scale gas treatment reactors).⁵–⁸ However, the large-scale industrial use of chemoautotrophic bacteria to fix CO₂ is currently limited by their low growth rates and poor ability to fix carbon.⁹–¹¹

The internal factors affecting the growth rates of chemoautotrophic bacteria include the copy numbers of their ribosomal RNA (rRNA) genes, the protein synthesis rate of unit ribosomes, and the transcription efficiency of the key genes required for CO₂ assimilation, which provides the substrates for cytoskeleton synthesis.¹²–¹⁵ The Calvin-Benson-Bassham (CBB) cycle is considered the main pathway by which most aerobic chemoautotrophic bacteria assimilate CO₂.¹⁶ The transcription level of the cbb gene, which encodes the key enzyme of the CBB cycle pathway, ribulose bisphosphate carboxylase/oxygenase (RuBisCO), determines the CO₂ assimilation rate of chemoautotrophic bacteria, which in turn affects their growth rate and CO₂ fixation rate.¹⁷,¹⁸

In addition to these internal factors, the growth rate of chemoautotrophic bacteria is also affected by environmental factors, such as temperature, the carbon source, pH, O₂, and the energy source, which play important roles in the bacterial growth and the CO₂ fixation of autotrophic microorganisms.¹⁹,²⁰ It has also been demonstrated that external organic compounds generally have a negative impact on the CO₂ assimilation and cell growth of chemoautotrophic bacteria.²¹–²⁵

Importantly, the self-generated extracellular free organic carbon (EFOC) generated by chemoautotrophic bacteria has an inhibitory feedback effect on bacterial growth and CO₂ fixation.¹⁹,²⁶ According to several reports, membrane reactors promote bacterial growth and CO₂ fixation by separating EFOC from the...
confirming that EFOC has an inhibitory effect on the growth and CO2 fixation of autotrophic bacteria. Consequently, the inhibition exerted by self-generated EFOC contributes to the relatively low growth rate and low CO2 fixing efficiency of chemoautotrophic bacteria and may be the most important reason for their inability to sustain efficient autotrophic growth and CO2 fixation.

Although the inhibitory effect of EFOC on bacterial CO2 fixation has been estimated and a membrane filtration technology has been developed to release the inhibitory effect of EFOC, there are still several problems with membrane filtration technology, including membrane fouling, high membrane pressures, high energy consumption, and the difficulty of large-scale operation. Moreover, our understanding of the inhibitory components of EFOC that are responsible for low CO2 fixing efficiency is also limited, and there are no detailed data on the mechanism by which EFOC inhibits the CO2 fixation process of chemoautotrophic bacteria.

Free organic carbon (FOC) is universally generated by chemoautotrophic bacteria during the assimilation of CO2 inside the cell, and can be secreted to the outside of the cell to form EFOC when too much FOC accumulates inside the cell. EFOC is a soluble microbial product with a wide distribution of molecular weight (MW), ranging from <5 kDa to >10 kDa, and contains several metabolites, mainly including carbohydrates, peptides, proteins, some lipids, and organic colloids. Large-molecule organic compounds (also known as “biomass-associated products”) are generated during cell lysis and decay. The organic compounds generated during the assimilation of CO2 are subsequently involved in the synthesis of the cytoskeleton. When the cytoskeleton synthesis rate cannot keep up with the CO2 assimilation rate, small-molecule organic compounds accumulate intracellularly and the excess diffuses through the cell membrane into the extracellular environment to form the small-molecule component of EFOC.

Because large and small molecules are generated via different production pathways and have different properties, the components of EFOC with different molecular weights may have different inhibitory effects on CO2 fixation, and the inhibitory targets of these EFOC components, which affect CO2 assimilation and protein synthesis, may also differ.

Consequently, in this study, we addressed the following questions. (i) What components of EFOC exert the main negative feedback inhibitory effect on bacterial CO2 fixation? and (ii) What are the main inhibitory targets of the major inhibitory components in EFOC on bacterial CO2 fixation?

We used typical sulfur-oxidizing bacteria (SOB) as the model organism, which have two environmental functions: fixing CO2 and removing sulfur pollutants in wastewater and gas, to (i) analyze the MW distribution characteristics of EFOC and its correlation with CO2 fixation in a control reactor, and verified the results with a membrane bioreactor, (ii) clarify the main components of small-molecule (or large-molecule) EFOC that are negatively related to bacterial CO2 fixation efficiency and their possible mechanisms of inhibition, (iii) verify the main inhibitory components of EFOC with simulation experiments and explore the inhibitory targets of their inhibitory effects on CO2 fixation.

In contrast to previous research, which mainly focused on the external factors that inhibit bacterial CO2 fixation, in this study, we investigated the components of self-generated EFOC that inhibit CO2 fixation by chemoautotrophic bacteria and their mechanisms. Our results provide a theoretical basis for developing targeted measures to remove the main inhibitory components from cultures of chemoautotrophic bacteria to improve their CO2 fixation, such as the affinity adsorption of small-molecule EFOC, and circulating flow technology with microfiltration membranes for large-molecule EFOC.

**RESULTS AND DISCUSSION**

**Molecular weight distribution of extracellular free organic carbon and its relationship to CO2 fixation efficiency in typical sulfur-oxidizing bacteria**

The large-molecule EFOC is mainly protein and the main component of cells, and its MW is generally more than 10 kDa, while small-molecule EFOC is mainly metabolic products and wastes with MW < 10 kDa. The MW distributions of the EFOC from the two strains in the control and membrane reactors and in the EFOC filtered from the membrane reactor during its operation were determined with gel filtration chromatography (Figure 1). In the control reactor, the proportion of small-molecule (MW < 10 kDa) EFOC for DSM
505 increased from 0.46% to 5.43% and that for DSM 15147 increased from 49.59% to 74.41% during the operation period, whereas the proportion of large-molecule (MW > 10 kDa) EFOC from both strains in the control reactor continued to decline (Figure 1A). For both strains in the control reactor, the concentration of small-molecule EFOC tended to increase, whereas the concentration of large-molecule EFOC increased for DSM 505, but barely changed for DSM 15147 (Figure 1B).

These results show that large-molecule organic matter was the main component of the EFOC from DSM 505, whereas small-molecule organic matter was the main component of the EFOC from DSM 15147. A more important conclusion is that for both strains, the proportion of large-molecule EFOC decreased as the culture time increased, indicating that neither strain in the reactor reached the death phase, because large-molecule organic compounds (also known as “biomass-associated products”) are generated during cell lysis and decay.26,33

The total apparent carbon fixation yields of DSM 505 and DSM 15147 in the control reactor increased rapidly in the early stage of culture and slowly in the late stage (Figure 2). In the control reactor, the CO₂ fixation efficiencies in the early and late stages for DSM 505 were 9.24 and 1.59 mg C/L/day, respectively, and those for DSM 15147 were 6.95 and 0.86 mg C/L/day, respectively. Therefore, the apparent CO₂ fixation efficiencies of both strains in the control reactor decreased with time.

To determine the main inhibitory factors affecting bacterial CO₂ fixation, the correlation coefficients between the apparent CO₂ fixation efficiency and the proportion of large molecules, the proportion of small molecules, the concentration of large molecules, the concentration of small molecules, or EFOC were calculated (Figure 3). For both strains, there was a significant negative correlation (at the 0.05 level)
between the proportion of small-molecule EFOC and the apparent CO₂ fixation efficiency, with a correlation coefficient of -0.999. However, the correlation coefficient between the proportion of large-molecule EFOC and the apparent CO₂ fixation efficiency in the control reactor was positive for both strains (Figure 3). The negative correlation between the apparent CO₂ fixation efficiency and the concentrations of small molecules, large molecules, and EFOC were not all significant for the two strains. However, the correlation coefficient between CO₂ fixation efficiency and the proportion of large-molecule EFOC in the control reactor was positive for both strains. This indicates that the components of large-molecule EFOC were not the main inhibitory factors of CO₂ fixation. Moreover, the membrane module separated out the EFOC and improved the bacterial CO₂ fixation by changing the MW distribution of the EFOC components in the reactor, especially by reducing the proportion of small-molecule EFOC. These observations indicate that the small molecules in EFOC are the main component inhibiting bacterial CO₂ fixation.

In the membrane reactor, the total apparent carbon fixation yields of DSM 505 and DSM 15147 were 235.19 mg C and 219.14 mg C, respectively, which were 3.72 times and 3.24 times that in the control reactor, respectively (Figure 2). The CO₂ fixation efficiencies of DSM 505 in the early and late stages of culture in the membrane reactor were 23.04 and 4.16 mg C/L/day, respectively, and those of DSM 15147 were 16.51 and 5.05 mg C/L/day, respectively (Figure 2). Therefore, the two typical SOB strains achieved higher CO₂ fixation in the membrane reactor than in the control reactor within the same operation time.

The proportion of small-molecule EFOC in the membrane reactor was 0% during the whole operation period for DSM 505 and decreased to 55.67% in the late stage for DSM 15147 (Figure 1C). The proportion of small molecules in the filtered EFOC in the membrane reactor decreased to 0% and remained there for DSM 505, and decreased in the late stage of culture for DSM 15147 (Figure 1D). This differs from the upward trend of the proportion of small-molecule EFOC in the control reactor and shows that the membrane module changed the MW distribution of the EFOC components in the membrane reactor, and especially reduced the proportion of small-molecule EFOC in the late stage of culture relative to that in the control reactor.

Overall, two typical bacteria with different growth characteristics and different MW compositions of EFOC showed some similar results. The proportion of small-molecule EFOC in the control reactor increased with time, with a corresponding downward trend in CO₂ fixation efficiency. The negative correlation between CO₂ fixation efficiency and the proportion of small-molecule EFOC in the control reactor was significant for both strains. The negative correlations between CO₂ fixation efficiency and the concentrations of small molecules, large molecules, and EFOC were not all significant for the two strains. However, the correlation coefficient between CO₂ fixation efficiency and the proportion of large-molecule EFOC in the control reactor was positive for both strains. This indicates that the components of large-molecule EFOC were not the main inhibitory factors of CO₂ fixation. Moreover, the membrane module separated out the EFOC and improved the bacterial CO₂ fixation by changing the MW distribution of the EFOC components in the reactor, especially by reducing the proportion of small-molecule EFOC. These observations indicate that the small molecules in EFOC are the main component inhibiting bacterial CO₂ fixation.
The proportion of small-molecule EFOC from DSM 15147 was always >30%, whereas that from DSM 505 was always <6% (Figure 1), which may be related to the different capacities for cytoskeleton synthesis of the two strains. During bacterial autotrophic growth, the small-molecule organic carbon produced via the CO2 fixation pathway is then used for protein synthesis and the formation of cellular components.26 The rRNA gene copy numbers of DSM 15147 and DSM 505 are five and seven, respectively,39 which suggests that the protein synthesis rate of DSM 505 is higher than that of DSM 15147. Therefore, compared with DSM 505, the small molecules synthesized by DSM 15147 are used to synthesize proteins at a lower rate, so more small molecules remain as FOC and EFOC. Furthermore, in the early stage of culture, the proportion of large-molecule EFOC from DSM 505 is high because the cytoskeleton synthesis process is faster than the generation of small molecules, rather than because the bacteria have entered the death phase.

Figure 3. The correlation coefficients of different indexes in control reactor
The correlation coefficients of different indexes of DSM 505 (A) and DSM 15147 (B) in control reactor at the early, middle, and late stages (* Correlation between apparent CO2 fixation efficiency and another index is significant at the 0.05 level.).

Possible inhibitory components of small-molecule extracellular free organic carbon
To further clarify the inhibitory components of small-molecule EFOC, the molecular and compositional diversity of the small-molecule EFOC generated by DSM 15147, whose proportion of small-molecule was higher than that of DSM 505, in the four stages of culture were determined with liquid chromatography-mass spectrometry. The composition of small-molecule EFOC and the elemental oxygen-to-carbon and
hydrogen-to-carbon ratios were in similar ranges in the early stage (day 2) and late stage (day 8) (Figures 4A and 4B). The molecular formulae (MF) of the components of small-molecule EFOC changed only slightly during culture, whereas there was a change in the relative intensities (circle size) of the corresponding mass peaks (Figures 4A and 4B). This indicates that the proportions and concentrations of the compound classes in small-molecule EFOC changed during the chemoautotrophic culture.

The molecular formulae could be divided into several compound classes according to the results of mass spectrometry. Most (53%-60%) of the molecules in small-molecule EFOC consisted solely of carbon, hydrogen, and nitrogen (CHN), and their proportion decreased with time, whereas the molecules that consisted solely of carbon, hydrogen, oxygen, and nitrogen (CHNO) or carbon, hydrogen, and oxygen (CHO) showed an uptrend in the proportion ranges of 27%-34% and 7%-14%, respectively (Figure 4C). This indicates that these molecular compound classes are the main compound classes of small-molecule EFOC. The peak area of the CHN molecules was highest on day 4 and decreased thereafter (Figure 4D), whereas the peak areas of the CHNO and CHO classes tended to increase with culture time (Figure 4D). The proportions and peak areas of other molecules were very small.

Figure 4. Molecular and compositional properties of small-molecule EFOC
(A and B) Molecular formulas (MF) of small-molecule EFOC of DSM 15147 on days 2 (A) and 8 (B) of cultivation with compound classes (colors) and relative intensity (circle size) of the corresponding mass peaks; Data are displayed as molecular hydrogen-to-carbon versus oxygen-to-carbon ratio.
(C and D) Proportion of molecule compound classes (C), peak area of molecule compound classes (D) of small-molecule EFOC generated by DSM 15147 in the control reactor in four chemoautotrophic cultivation stages (days 2, 4, 6, and 8).
The proportions and peak areas of the CHNO and CHO molecules correlated negatively with the CO2 fixation efficiency, whereas those of the CHN molecules did not correlate negatively with it at all, indicating that CHNO and CHO molecules might be the main inhibitory compound classes in small-molecule EFOC. The CHNO molecules mainly included organic acids, nucleosides, carnitine, and amino acids, and the CHO molecules included fatty acids and amino acids.

To clarify the specific compound types that constitute the inhibitory components of EFOC, small-molecular EFOC was divided into six compound types (organic acids, amino acids, nucleosides, fatty acids, lipids, and carnitine), according to the results of typical chromatograms and mass spectrometry (Figure 5A), with unrecognizable compound types were not considered. In the four culture stages, amino acids accounted for the largest proportion of these compounds (>53%) and increased with time, followed by organic acids (>23%) (Figure 5B). The proportions of nucleosides, fatty acids, and carnitine during the culture period were distributed in the ranges of 3%-11%, 1%-3%, and 0.9%-1%, respectively, and the proportion of lipids was zero in the first three stages (Figure 5B). This demonstrates that organic acids and amino acids are the main compounds in small-molecule EFOC.

There were also upward trends in the peak areas of both organic acids and amino acids in the small-molecule EFOC during chemosynthetic culture (Figure 5C), and these trends correlated negatively with CO2 fixation efficiency. The peak area of nucleosides showed an increasing trend at low
concentrations, whereas those of the other compound types were almost zero and changed little over time (Figure 5C). These results indicate that organic acids and amino acids, as the main compounds in small-molecule EFOC, may be related to the inhibitory effect of EFOC on CO₂ fixation during chemolithotrophic culture.

It has been reported that organic acids are toxic to the acidophile Thiobacillus ferrooxidans, because they affect its chemical permeability parameters. A substrate inhibitory effect of organic acids on Thiobacillus acidophilus has been observed in the presence of pyruvate. The addition of exogenous L-amino acids in inorganic culture environments disturbed the normal regulation processes and inhibited the growth of three obligately chemolithotrophic thiobacilli. Moreover, the growth of nitrifying bacteria (Nitrosomonas and nitrifying bacteria) is usually inhibited by relatively low concentrations of amino compounds.

Exogenous organic acids and amino acids inhibit the growth of chemolithotrophic bacteria. Our experimental results show that organic acids and amino acids are the main compounds in small-molecule EFOC and that small-molecule EFOC is the main fraction of EFOC inhibiting CO₂ fixation. It can be inferred that organic acids and amino acids are the main components of small-molecule EFOC that inhibit CO₂ fixation during chemoautotrophic culture.

**Verification of the inhibitory effects of self-generated organic acids and amino acids on CO₂ fixation by sulfur-oxidizing bacteria**

DSM 505 produced a smaller proportion of small-molecule EFOC than DSM 15147, so the use of DSM 505 to verify whether self-generated small molecule organic acids and amino acids inhibit CO₂ fixation could demonstrate both the inhibitory effect of self-generated small molecule organic acids and amino acids and the applicability of the inhibition to different bacteria. The effects of organic acids, amino acids, and their typical metabolites (e.g., carbohydrate, protein) on CO₂ fixation by DSM 505 were studied.

Notably, the TOC fixed in the presence of pyruvate, oxaloacetate, fructose, and with no supplementation (control) was $-2.47, -1.37, 2.94$, and $1.82$ mg C/L, respectively, in the early stage of culture (Figure 6A). This indicates that organic acids are utilized by bacteria for cytoskeleton synthesis and that the immediate inhibitory effect of organic acids on CO₂ fixation is greater than that of carbohydrates. Furthermore, DSM 505 utilizes organic acids at a higher rate than it utilizes fructose.

The TOC fixed in the middle and late stages of culture in the presence of pyruvate or oxaloacetate was still lower than that fixed in the presence of fructose or in the control (Figure 6A). This demonstrates that the
inhibitory effect of organic acids on CO₂ fixation is greater than that of carbohydrates in all stages of culture, indicating that organic acids are one of the main inhibitory components of EFOC.

The effects of amino acids (alanine or aspartic acid) and their typical metabolites (protein) on CO₂ fixation by DSM 505 were investigated, as shown in Figure 6B. The TOC fixed in the early stage of culture in the presence of alanine, aspartic acid, protein, and in the control was 0.21, 0.03, 0.99, and 1.82 mg C/L, respectively (Figure 6B). Simultaneously, the peak areas and proportions of amino acids in small-molecule EFOC in the presence of alanine or aspartic acid were greater than in the presence of protein (Figures 7A and 7B). This shows that amino acids and protein inhibited CO₂ fixation relative to that in the control group, and the immediate inhibitory effect of amino acids on CO₂ fixation was more obvious than that of protein.

In the middle stage of culture, the TOC fixed in the presence of alanine, aspartic acid, protein, and in the control was 6.53, 6.33, 4.26, and 8.87 mg C/L, respectively (Figure 6B), and the amino acid peak areas and proportions of small-molecule EFOC in the presence of protein increased and exceeded that in the presence of alanine or aspartic acid (Figure 7B). This indicates that amino acids and protein inhibit CO₂ fixation in the middle stage compared with the control group, and the inhibitory effect of amino acids on carbon fixation in the middle stage was weaker than that in the early stage, while the inhibitory effect of proteins on carbon fixation in the middle stage was stronger than that in the early stage. This may be related to the decomposition of proteins into amino acids, which has stronger inhibition effect on carbon fixation, in the middle stage.

In the late stage of culture, the TOC fixed in the presence of alanine, aspartic acid, protein, and in the control was 8.58, 7.05, 8.45, and 12.04 mg C/L, respectively (Figure 6B). The amino acid peak areas and proportion of small-molecule EFOC in the presence of amino acids were slightly smaller than in the presence of protein in the late stage (Figures 7A and 7B). These results indicate that the long-term effects of amino acids and protein on CO₂ fixation are similar, because of the metabolic balance between protein and amino acids.

Thus, the immediate inhibitory effect of amino acids on CO₂ fixation was greater than that of protein in the early stage of culture, whereas the inhibitory effect of protein was greater in the middle stage. However, the long-term effects of both on CO₂ fixation were similar, which is related to the dynamic balance between protein hydrolysis into amino acids and the synthesis of protein from amino acids. Since bacteria produce amino acids in EFOC continuously, the inhibitory effect of amino acids is always maintained, so amino acids are one of the main inhibitory components of EFOC.
The total cbb gene transcription is an important factor influencing bacterial CO₂ fixation by chemoautotrophic bacteria. However, previous studies have mainly focused on the cytotoxicity of organic acids and amino acids to bacteria, and few studies have examined the effects of organic acids and amino acids on the cbb gene transcription. Therefore, the efficiency of the cbb gene transcription in DSM 505 in the middle stage of culture in the presence of different typical metabolites was analyzed to investigate the effects of organic acids and amino acids on cbb gene transcription.

The total cbb gene transcription efficiency in the middle stage of culture in the presence of pyruvate, oxaloacetate, or fructose was lower than in the control group, and total cbb gene transcription efficiency in the presence of pyruvate or oxaloacetate was lower than in the presence of fructose (Figure 8A). These data show that pyruvate and oxaloacetate inhibit cbb gene transcription more efficiently than fructose.

The total cbb gene transcription efficiency in the middle stage of culture in the presence of alanine, aspartic acid, or protein was lower than in the control group (Figure 8B); and was lower in the presence of protein than in the presence of alanine, although in the presence of protein, it was similar to that in the presence of aspartic acid (Figure 8B). These results indicate that the inhibitory intensity of protein on cbb transcription is similar to that of amino acids in the middle stage of culture and even stronger than that of some amino acids, which...
may be due to the increase in the amino acid peak area and the proportion of amino acids in small-molecule EFOC in the presence of protein.

In summary, organic acids exert a stronger inhibitory effect on CO₂ fixation than carbohydrates by inhibiting the cbb gene transcription and by acting as substrates for cytoskeleton synthesis. The inhibitory effects of amino acids on CO₂ fixation are stronger than that of protein in the early stage of culture. However, when protein is decomposed to amino acids in the middle stage of culture, the increases in the concentration and proportion of amino acids in small-molecule EFOC increase the inhibitory effect on CO₂ fixation by inhibiting the cbb gene transcription. The similarity of the long-term inhibitory effects of amino acids and proteins on carbon fixation is related to the metabolic balance maintained between amino acids and proteins.

Technical perspectives on the removal of organic small molecules to improve CO₂ fixation by chemoautotrophic bacteria

Therefore, to enhance bacterial CO₂ fixation, we recommend the removal of the small molecule EFOC, especially amino acids and organic acids, during their chemoautotrophic culture. The membrane filtration technology is an feasible option to remove EFOC for continuous cultivation of chemoautotrophic bacteria, but the problems of membrane fouling, high membrane pressure, and high energy consumption associated with membrane filtration technology need to be addressed.

The removal of dissolved organic small molecules by adsorption is low-energy and efficient. As a promising adsorption material with nanoscale pore size and designed functional sites, covalent organic frameworks (COFs) may carry out specific intra-pore adsorption for small molecules, while cells and macromolecules are not adsorbed by COFs, thus isolating small molecules and cells and eliminating feedback inhibition of small molecules. When nanopore adsorption-culture technology with its simple operation is used for batch culture of chemoautotrophic bacteria, there are advantages that the adsorbent can be regenerated and reused, and the adsorbed small-molecule amino acids and organic acids can be utilized, thus making it a cost-controllable technology.

Nanopore adsorption-culture technology for the adsorption of small-molecule EFOC can enhance CO₂ fixation by chemoautotrophic bacteria, which broadens the application of adsorbent materials and enables the reuse of fixed organic carbon products.

Conclusions

When EFOC is analyzed in terms of the molecular weight of its components, the proportion and concentration of the small molecules in EFOC increase during chemoautotrophic culture, and the small molecules are the main components of EFOC that inhibit CO₂ fixation by chemoautotrophic bacteria. In terms of the types of compounds in small-molecule EFOC, amino acids and organic acids are the main inhibitory components. The inhibitory effects of amino acids and organic acids on CO₂ fixation are stronger than those of protein and carbohydrate, respectively, because they also inhibit the transcription of the cbb gene. Therefore, nanopore adsorption-culture technology for the adsorption of amino acids and organic acids is suggested to enhance CO₂ fixation by chemoautotrophic bacteria.

Limitations of the study

The prospect of nanopore adsorption-culture technology for enhancing CO₂ fixation by chemoautotrophic bacteria needs further experimental testing.

STAR METHODS

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AUTHOR CONTRIBUTIONS

Saiwei Zhang: Writing-original draft preparation, methodology, software, validation, data curation, and visualization.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| L-alanine | HUSHI | Cat#62001332 |
| L-aspartic acid | OKA | Cat#62004780 |
| Oxaloacetate | OKA | Cat#XW03284271 |
| Pyruvate | HUSHI | Cat#30161435 |
| **Critical commercial assays** | | |
| DNeasy PowerSoil Pro Kit | QIAGEN | Cat#47014 |
| RNAiso Plus | TaKaRa | Cat#9108 |
| PureLink™ RNA Mini Kit | Invitrogen™ | Cat#12183018A |
| PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real time) | TaKaRa | Cat#RR047A |
| TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) | TaKaRa | Cat#RR820B |
| **Experimental models: Organisms/strains** | | |
| Thiobacillus thioparus | DSMZ | DSM 505 |
| Halothiobacillus neapolitanus | DSMZ | DSM 15147 |
| **Oligonucleotides** | | |
| cbbL(DSM 505) _K2F 5'-ACCAYCAAG CCSAAGCTSGG-3' | Synthesized by Wcgene Biotechnology | N/A |
| cbbL(DSM 505) _V2F 5'-GCCCTTCSA GCTGCCSACCRC-3' | Synthesized by Wcgene Biotechnology | N/A |
| cbbL(DSM 15147) _15147L-F 5'-CGT GTGGACGGATTTGCTGA-3' | Synthesized by Wcgene Biotechnology | N/A |
| cbbL(DSM 15147) _15147L-R 5'-CTTC AGCAGTTTCGGTCGC-3' | Synthesized by Wcgene Biotechnology | N/A |
| cbbM _cbbM-F 5'-TTCTGGCTGGGGGBGG HGAYTTYAYAARAYGACGA-3' | Synthesized by Wcgene Biotechnology | N/A |
| cbbM _cbbM-R 5'-CGTGTRCRRGCVC GRTGGTARTG-3' | Synthesized by Wcgene Biotechnology | N/A |
| Bacterial 16S rRNA, 338F 5'-ACTCCTAC GGAGGCCAGCAG-3' | Synthesized by Wcgene Biotechnology | N/A |
| Bacterial 16S rRNA, 518R 5'-ATTACCGCGGCTGCTGG-3' | Synthesized by Wcgene Biotechnology | N/A |
| **Recombinant DNA** | | |
| Plasmid: pUC57-cbbL | Synthesized by Wcgene Biotechnology | N/A |
| Plasmid: pUC57-cbbM | Synthesized by Wcgene Biotechnology | N/A |
| Plasmid: pUC57-16S rRNA | Synthesized by Wcgene Biotechnology | N/A |
| **Software and algorithms** | | |
| Origin 2017 | OriginLab | https://www.originlab.com/ |
| Excel | Microsoft | N/A |
| IBM SPSS Statistics 20 | IBM | https://www.ibm.com/ |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lei Wang (celwang@tongji.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Microbe strains and culture conditions
Two obligate autotrophic SOB strains Thiobacillus thioparus DSM 505 and Halothiobacillus neapolitanus DSM 15147 were used as the model organism and gained from the German Collection of Microorganisms and Cell Cultures (DSMZ; www.dsmz.de). The optimum medium components (g/L) for DSM 505 and DSM 15147 were provided with Medium No. 36 and DSMZ Medium No. 68 from DSMZ, respectively. Detailed bacterial culture conditions refer to Zhang et al. The batch culture experiments were performed in triplicate.

METHOD DETAILS

Reactor design and operation
The design and operation conditions of the laboratory-scale membrane reactor system have been described by Zhang et al. The control reactor was set up without flat membrane. Besides, the control and membrane reactors were aerated at a rate of 1 L/min. The necessary energy and carbon sources were sufficient during the entire operation period, because the control reactor was supplemented with 5 g of Na2S2O3, 0.5 g of Na2CO3, and 0.5 g of NaHCO3 every other day and 0.75 g/L Na2CO3 and 0.75 g/L NaHCO3 was added to the autotrophic medium of membrane reactor.

Testing the effects of different EFOC components on CO2 fixation by SOB
Pyruvic acid, oxaloacetic acid, and fructose (at same concentration, 10 mg carbon (C)/L) were used as typical organic acids and their metabolites (carbohydrates), and aspartic acid, alanine, and protein (all 10 mg C/L) were used as typical amino acids and their metabolites (protein) to investigate the effects of different metabolites on bacterial CO2 fixation. Each compound was added to a separate 150 mL serum bottle containing 60 mL of autoclaved inorganic optimum medium for DSM 505 (Medium No. 36 from DSMZ), respectively, to achieve a concentration of 10 mg C/L of each compound. DSM 505 inoculated into the medium at 2.5% (V/V), and the serum bottles were filled with 10% CO2 and 21% O2. A control reactor with no added metabolite was also set up simultaneously as the control. Three duplicate samples were included in these experiments.

Estimation of CO2 fixation efficiency and EFOC yield
After the samples were pretreated with an ultrasonic cell disruptor (Haishu Kesheng Ultrasonic Equipment Co., Ltd, Ningbo, China) for 10 min and the pH of samples was adjusted to 4.0 to reduce any interference by inorganic carbon, the total organic carbon (TOC) of samples was determined with a TOC analyzer (TOCVPCH; Shimadzu Seisakusho Co., Ltd, Kyoto, Japan). The samples of reactors were centrifuged at 13,000 x g for 10 min and the supernatants were obtained as EFOC. EFOC was assayed with the TOC analyzer after EFOC was disrupted with a cell disruptor and its pH was adjusted to 4.0.

The carbon fixation yield within a specific period directly reflects the bacterial CO2 fixation efficiency. For the calculation method of total carbon fixation yield in membrane reactor and control reactor, please refer to Zhang et al. The samples treated with different metabolites were collected from the serum bottles on
days 1, 4, and 7. The TOC fixed by DSM 505 under different metabolites conditions at a concentration of 10 mg C/L was calculated by subtracting the initial TOC from the measured TOC at different culture times.

**Determination of MW distribution of EFOC**

The gel filtration chromatography (LC-20AD, Shimadzu Co., Ltd) was used to determine the MW distribution of EFOC on day 3, day 6, and day 9 to represent the MW distribution of the early, middle and late stages, and all samples were filtered through a 0.22 μm membrane. Gel filtration chromatography was performed with polyethylene oxide/glycol easivials of varying MWs as standards, using ultrapure water as the mobile phase, analysis time of 25–30 min, evaporative light scattering detector and TSKgel G4000PWXL column. The small-molecule and large-molecule organic carbon yield were calculated based on the proportion of small molecules and large molecules and EFOC in the control reactor.

**Determining the composition of small-molecule EFOC**

Samples were collected from the control reactor on days 2, 4, 6, and 8 of operation. All the samples were filtered through 0.22 μm membranes before analysis. The composition of the small-molecule EFOC in all the samples was analyzed with liquid chromatography (Vanquish™ Flex, Thermo Scientific, USA)—mass spectrometry. An Xbridge BEH C18 chromatographic column (2.1 × 100 mm, 2.6 μm) was used for liquid chromatography, with a column temperature of 40°C, a flow rate of 0.3 mL/min, and an elution phase of 0.1% formic acid–water and acetonitrile. The gradient conditions were 98%, 98%, 60%, 5%, 5%, 98%, and 98% of 0.1% formic acid–water at elution times of 0, 1.5, 10, 17, 22, 22.1, and 25 min, respectively. The scan type full MS/ddMS2 was used for mass spectrometry (Q Exactive, Thermo Scientific), with positive/negative polarity, a sheath gas flow rate of 45 arbitrary units, a gas flow rate of 10 arbitrary units, a spray voltage of 3.5 kV, a capillary temperature of 275°C, and a gas heater temperature of 450°C. The peak area under the same experimental conditions indicated the concentration of the substance.

**DNA and RNA extraction, and reverse transcription**

Bacterial cell samples were collected from serum bottles containing supplementary pyruvate, oxaloacetate, fructose, aspartic acid, alanine, or protein in the middle stage of culture (day 4), and filtered through 0.22 μm membranes to collect cell. The UltraClean® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) and the TRIzol™ Plus RNA Purification Kit (Invitrogen, USA) were used to extract DNA and RNA according to the manufacturer’s instructions. The concentration and purity of DNA and RNA were measured with a spectrophotometer (NanoDrop ND-2000; Thermo Scientific).

The gDNA Eraser (Takara, Japan) was used to eliminate DNA from the RNA samples, and then the purified RNA was reverse transcribed into cDNA with the PrimeScript™ RT Reagent Kit (Takara) for cbb gene transcription analysis, according to the manufacturer’s instructions.

**Determination of abundance and transcription level of the cbb gene**

Since DSM 505 contains the cbbL and cbbM genes, the abundance and transcription level of the total cbb genes (cbbL and cbbM) was measured by using real-time quantitative PCR (qPCR) (Applied Biotechnologies, Carlsbad, CA, USA), with 20 μL of SYBR Premix Ex Taq™ System (TaKaRa) containing 2 μL of template. The primers, cycling conditions and qPCR methods used to determine cbb gene abundance and transcription level were those reported by Wang et al.11

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The Bivariate correlations relationships between the proportion and concentration of small-molecule EFOC and large-molecule EFOC and the apparent CO2 fixation efficiency were investigated with the SPSS 20.0 statistical analysis software (IBM, Armonk, NY, USA). One-way analysis of variance (One-way ANOVA) has been used to analyze the difference between the control group and the experimental group. Where an * is indicated in figures, difference is significant (p < 0.05).