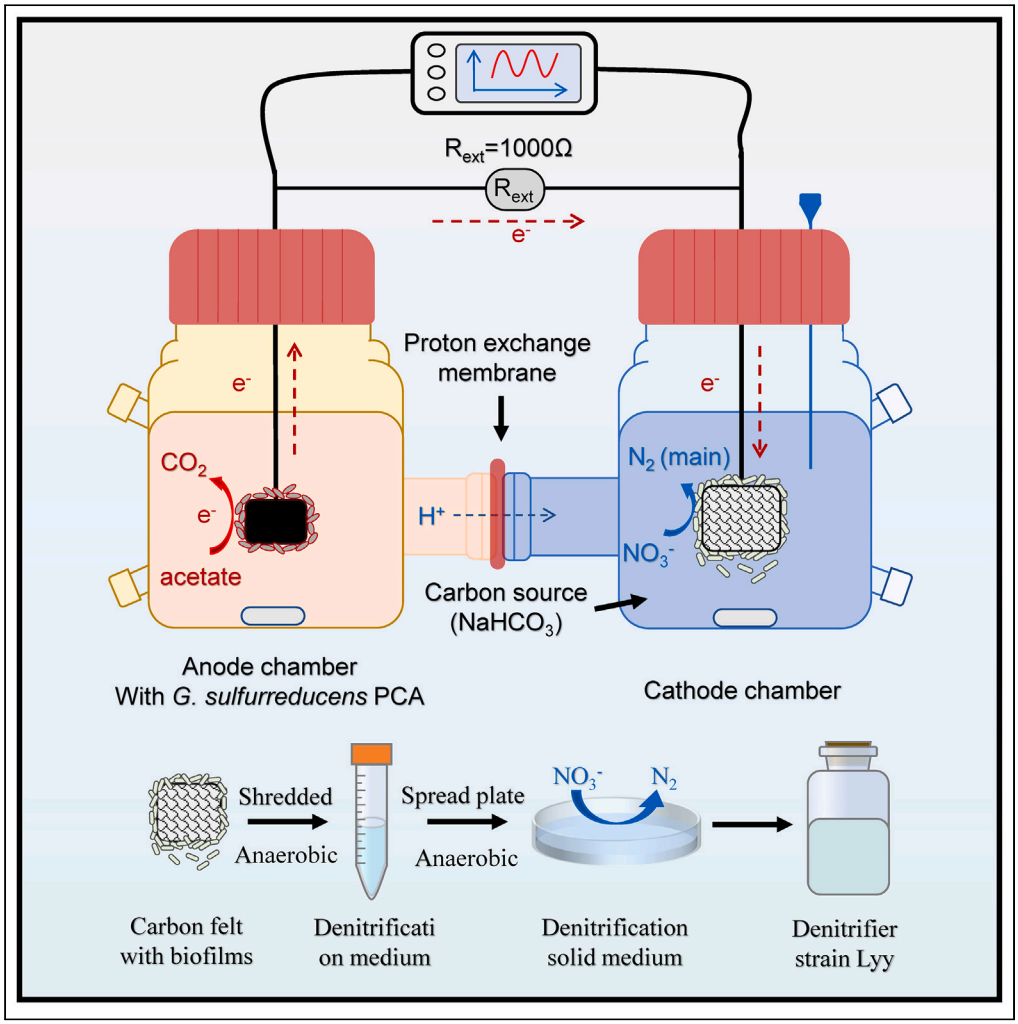
iScience

CellPress
OPEN ACCESS

Article

Efficient enriching high-performance denitrifiers using bio-cathode of microbial fuel cells



Ruitao Li, Xiang-peng Ren, Xinxin Fan, Zhen Zhang, Tian-peng Gao, Ying Liu

zkgtp@163.com (T.-p.G.)
liuy0512@hotmail.com (Y.L.)

Highlights

An efficient bio-cathode MFC method was reported to enrich denitrifying culture MBD

MBD showed a NO_3^--N removal efficiency of 69.99 ± 0.60% without added organic carbon

Paracoccus and *Pseudomonas* was the dominant genera in the MBD for NO_3^--N removal

An isolated strain Lyy (*S. stutzeri*) showed higher than 93% NO_3^--N removal value

Li et al., iScience 27, 110965
October 18, 2024
https://doi.org/10.1016/j.isci.2024.110965

iScience

CellPress
OPEN ACCESS

Article

Efficient enriching high-performance denitrifiers using bio-cathode of microbial fuel cells

Ruitao Li,[1,4] Xiang-peng Ren,[1,4] Xinxin Fan,[1] Zhen Zhang,[1] Tian-peng Gao,[2,3,*] and Ying Liu[1,5,*]

SUMMARY

Recent advancements in microbial fuel cells (MFC) technology have significantly contributed to the development of bio-cathode denitrification as a promising method for eco-friendly wastewater treatment. This study utilized an efficient repeated replacement method to enrich a mixed bio-cathode denitrifying culture (MBD) within a bio-cathode MFC, achieving a stable maximum output voltage of 120 ± 5 mV and a NO_3^--N removal efficiency of 69.99 ± 0.60%. The electrotrophic denitrification process appears to be facilitated by electron shuttles. Microbial community analysis revealed a predominance of *Proteobacteria*, with *Paracoccus* and *Pseudomonas* as functional genera. Additionally, the isolated strain Lyy (belonging to *Stutzerimonas*) from MBD demonstrated exceptional denitrification efficiencies exceeding 98% when treating wastewater with a broad range of C/N (2–12) ratios and KNO_3 concentrations (500–3000 mg/L) within 60 h. These results demonstrated the effectiveness of the repeated replacement method in enriching bio-cathode denitrifiers and advancing MFC application in sustainable wastewater management.

INTRODUCTION

The growing prevalence of nitrogen pollution, driven by industrial development and enhanced living standards, emphasizes the urgent need for effective denitrification processes. The significance of the carbon-to-nitrogen (C/N) ratio as a critical parameter in denitrification is well-established, with practical ratios typically ranging from 8 to 15, in contrast to the theoretical optimum of 3.84.[1,2] Adding extra carbon as an electron donor and energy source can lead to resource depletion and the potential of secondary pollution. Therefore, minimizing carbon source input in denitrification processes is essential for the effective treatment of nitrogenous wastewater.

Microbial fuel cells have emerged as a promising technology for sustainable wastewater treatment, leveraging electroactive microorganisms to convert pollutants into electricity[3,4] and poised to significantly contribute to sustainable energy initiatives. While substantial progress has been made in the exploration of anode materials, structures, electroactive microorganisms, and electron transfer mechanisms, the efficiency of the cathode remains a primary bottleneck hindering the advancement of MFC technology.[5] The development of bio-cathode technology offers a compelling solution to these challenges, enhancing the overall performance of MFC. Bio-cathodes exhibit a diverse range of functionalities, including denitrification, chromium reduction, and the synthesis of value-added organic compounds.[6,7] By leveraging the unique ability of bio-cathodes and cathodic electrotrophic microorganisms to directly utilize electrons from electrodes as electron donors,[8] these systems facilitate the conversion of oxidized substances such as ammonia nitrogen, nitrate nitrogen, and nitrite nitrogen into harmless N_2 through microbial metabolic reactions.[9,10] With microorganisms acting as catalysts, bio-cathodes present a cost-effective alternative to conventional metal catalysts, significantly reducing operational and maintenance costs by preventing issues associated with poisoning and deactivation.[5] Moreover, bio-cathodes enable the utilization of electrons generated from the degradation of anode organic pollutants to drive electrotrophic reduction reactions, such as electrotrophic denitrification, thereby substantially decreasing the consumption of carbon sources. This capability holds tremendous promise for enhancing both the efficiency and environmental sustainability of MFC technology.

Recent research on bio-cathode denitrification has garnered significant attention, indicating the potential of nitrate and nitrite to not only facilitate current generation but also to eliminate organic and nitrogen-containing pollutants.[11,12] Many studies have concentrated on utilizing mixed microbial communities for bio-cathode denitrification, focusing on refining reactor design, optimizing operational parameters, and investigating

[1]Shaanxi Key Laboratory of Agricultural and Environmental Microbiology, College of Life Sciences, Northwest A&F University, No. 22 Xinong Road, Yangling, Shaanxi Province 712100, China
[2]The Engineering Research Center of Mining Pollution Treatment and Ecological Restoration of Gansu Province, Lanzhou City University, Lanzhou, Gansu Province 730070, China
[3]Xi'an Key Laboratory of Plant Stress Physiology and Ecological Remediation Technology, College of Biological and Environmental Engineering, Xi'an University, Xi'an, Shaanxi Province 710065, China
[4]These authors contributed equally
[5]Lead contact
*Correspondence: zkgtp@163.com (T.-p.G.), liuy0512@hotmail.com (Y.L.)
https://doi.org/10.1016/j.isci.2024.110965

the effects of pH and conductivity on reactor performance.[2,13,14] Additionally, research has explored the microbial community structure of biofilms to enhance the efficiency of bio-cathode denitrification.[15,16] Despite the promising catalytic roles of bio-cathodic microorganisms in cathodic reduction,[17] the slow acclimation process-potentially lasting up to two months[18,19]—poses a considerable challenge for understanding and improving MFC performance. A primary obstacle to this process is the sluggish growth of reducible electroactive bacteria on the electrode, often hindered by a negative charge that impedes their enrichment.[20] Currently, no standardized method exists to enhance bio-cathode performance and stability while concurrently reducing preparation time. Although a combination of selection, domestication, and applied voltage cultivation techniques has been explored,[21] studies indicate that cultivating under heterotrophic conditions at a reversing potential can reduce bio-cathode preparation time to under 30 days[20]—yet this duration remains substantial. Hence, there is a pressing demand for further research into methods for microorganism enrichment in bio-cathodes, which is essential for promoting MFC technology as a sustainable solution for wastewater treatment.

Currently, *Stutzerimonas stutzeri (*also known as *Pseudomonas stutzeri)* from the *Pseudomonadaceae family*[22] has attracted considerable attention due to its versatile properties and biodegradation capabilities, particularly in marine and contaminated environments. Notably, Chen et al.[23] demonstrated that *S. stutzeri* A1501 could produce extracellular NH_4^+ without the need for chemical suppression or genetic manipulation, relying solely on an electrode as the sole electron donor. Additionally, Yamada et al.[24] investigated the extracellular electron transfer (EET) of *S. stutzeri* JCM5965, demonstrating its ability to convert NO_3^- to N_2 in a lithotrophic medium using Fe^{2+} as the electron donor. Despite its notable nitrogen fixation and denitrification potential, further exploration is required to elucidate its specific role in bio-cathode denitrification within MFC.

In this study, a mixed bio-cathode denitrifying culture named MBD was enriched from a consistently operational MFC bio-cathode. The MBD exhibited both electricity generation capability and efficient nitrogen removal in the bio-cathode without the addition of organic carbon sources. Furthermore, a facultative anaerobic strain named Lyy was isolated and identified from the MBD, and its denitrification characteristics under various culture conditions were investigated.

RESULTS AND DISCUSSION

The enrichment of mixed denitrifying cultures using bio-cathode MFC

The enrichment of the MDB was initiated by inoculating 10 mL of cultured sludge into a bio-cathode MFC, with residual acetate in denitrification medium (DM) serving as the sole additional organic carbon source in cathode during the enrichment process. Over several batch cycles, the cathode medium was replaced with DECA medium more than three times to effectively reduce the C/N ratio till 0, thereby shifting the electron donor from organic matter to electricity using the cathode.[7] Notably, the maximum output voltage of the MFC stabilized at 112 ± 3 mV over 100 h (Figure S1), indicating the successful enrichment of electroactive microorganisms on the cathode. Furthermore, replacing the medium without adding an organic carbon source proved effective in enhancing the relative abundance of electroactive microorganisms within the microbial community.[7] As a result, bacteria unable to utilize the cathode electrode as an electron donor were gradually phased out, contributing to the successful enrichment of MDB culture.

Following the enrichment phase, the carbon felt electrode containing the mixed culture was transferred from the cathode chamber, under anaerobic conditions, to a new cathode chamber for further investigation. After three consecutive medium replacements, the maximum output voltage stabilized at 120 ± 5 mV, maintaining this stability for over 380 h (Figure 1A), which indicated the robust stability of the MBD. Furthermore, it was observed that the MFC continued to generate electricity after replacing 90% of the anode medium (without replacing the cathode), with the voltage rising to 101 mV within 24 h (Figure S2). Concurrently, denitrification continued (Figure 1B), suggesting that the replenishment of the anode carbon source had minimal impact on bio-cathode denitrification, as this the process could persist as long as electrons were supplied. These findings indicated the stability of MBD and the effectiveness and robustness of the enrichment strategies within the bio-cathode MFC.

The nitrogen removal efficiency (calculated from the standard curve of Figure S6) of the bio-cathode MFC inoculated with the MBD-without additional electron donor=was analyzed and presented in Figure 1B. When the cathode served as the sole electron donor, the average removal rate and efficiency of NO_3^--N were 0.584 ± 0.008 mg/L/h and 69.988 ± 0.598%, respectively. These results indicated that the electrons generated from the anode were effectively transferred to the cathode via a copper wire, enabling the denitrifying microorganisms to utilize these electrons for denitrification. Notably, Ding et al.[7] demonstrated a comparable NO_3^--N removal efficiency of nearly 70% without the addition of organic carbon sources by gradually reducing the C/N ratio through fourteen consecutive medium replacements. In this study, a similar denitrification efficiency was achieved after only two batches of enrichments (totally six medium replacements), demonstrating the expedited effectiveness of the directly enrichment strategies without organic carbon sources, thereby significantly reducing the time required for enriching cathodic functional flora.

The electrotrophic denitrification reaction of the MBD was characterized using a three-electrode system. Chronoamperometry was conducted at a potential of −0.3 V (vs. SCE) (Figure 2A), while differential pulse voltammetry (DPV) scans were performed at different periods. As illustrated in Figure 2B, a peak was observed at -0.08 ± 0.05 V, which exhibited an increase during the operation. Based on relevant research, it is speculated that the MBD possesses the ability to secrete electron shuttles, such as phenazines.[23,25] The efficient removal of NO_3^--N indicated the robustness of the electrotrophic denitrification mediated by MBD, and the identification of potential electron shuttles phenazines could play a crucial role in electron transfer pathways.

Microbial-community analysis of MBD

The microbial community composition of the MBD was investigated by using various molecular biology methods. The results revealed that *Proteobacteria* emerged as the dominant phylum, comprising 92.243% of the community, while *Paracoccus* (77.237%) and *Pseudomonas*

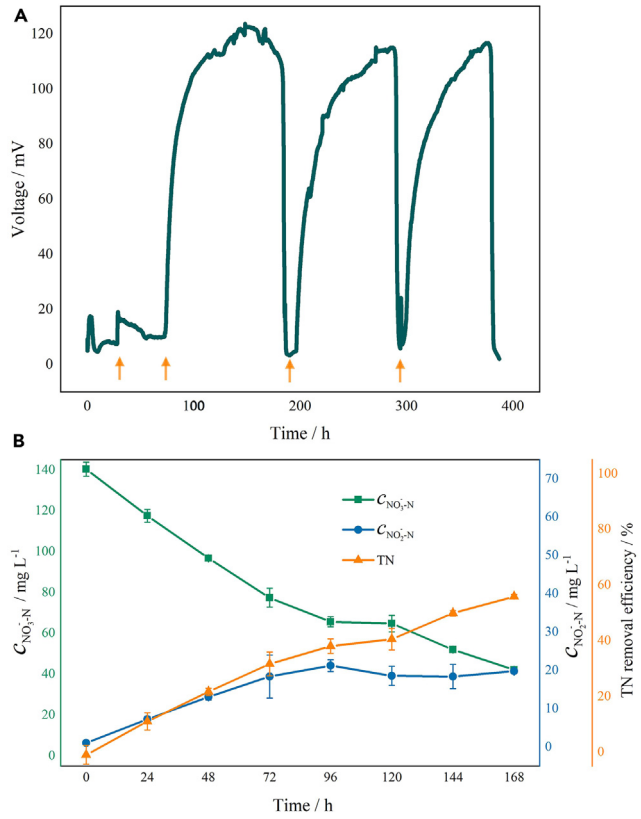


Figure 1. The performance of MBD in bio-cathode MFC (operated at a load resistance of 1000 Ω, the arrows indicating the medium replacement, and without organic carbon source in the cathode chamber)

(A) The voltage output of MBD.

(B) NO_3^--N concentration, NO_2^--N concentration, and TN removal efficiency of the MFC (values are means ± SD [error bars] for three replicates).

(13.448%) were identified as the predominant functional genera in the MBD (Figures 3 and S3). These findings align with the previous research[26–28] that has confirmed the prevalence of *Proteobacteria* as the primary bacteria in both the cathodic and anodic biofilms of MFC.[29,30] Additionally, a significant portion of autotrophic nitrifying bacteria belonging to the *Proteobacteria* phylum contributes critically to nitrogen cycling in MFC system.[30,31] *Paracoccus* has been recognized as a key functional bacteria associated with denitrification, with evidence indicating an increase in its relative abundance during the enrichment process,[27] suggesting its pivotal role in the denitrification process of MBD. Furthermore, *Pseudomonas*, a prominent member of the *Proteobacteria*, is well-known for its capability to degrade complex substrates and facilitate denitrification.[28,32] Certain strains of *Pesudomonas* can utilize various electron acceptors, including electron/NO_3^-/O_2/H_2, to directly reduce nitrogen into nitrogenous gases.[7,30] This indicates a significant role for *Pseudomonas* in electrotrophic reduction processes and in the inward EET within the bio-cathode.

Isolation and identification of facultative anaerobic denitrifier strain Lyy

The strain isolated and identified from the MBD through serial dilution, named Lyy, was confirmed to be *Stutzerimonas stutzeri* via PCR and 16S rDNA sequencing analysis. A comparison of the obtained sequences with those in the NCBI database was conducted using MEAG11, and a phylogenetic tree was constructed utilizing the maximum likelihood method (as shown in Figure 4). Results demonstrated a close relationship between strain Lyy and *S. stutzeri* DSM 4166 and *P. stutzeri* A1501, exhibiting 100% sequence similarity. Previous research has indicated that *S. stutzeri* strains may possess capabilities for denitrification, nitrogen fixation, and secretion of electron shuttles,[23,24] providing a basis for further exploration of the nitrogen cycle and electron transfer capabilities of strain Lyy.

Denitrification characteristics of strain Lyy under different conditions

Effect of C/N ratio on denitrification performance of strain Lyy

The effects of different C/N ratios on denitrification were evaluated in the growth medium, as illustrated in Figures 5A and S4A and detailed in Table S3. At the C/N ratio of 2, primarily due to limitations in carbon source,[33] the maximum optical density at 600 nm ($OD_{600\ nm}$) was only 0.07 ± 0.003. Despite achieving a notable 98.18 ± 0.39% removal of NO_3^--N, the accumulation of NO_2^--N reached

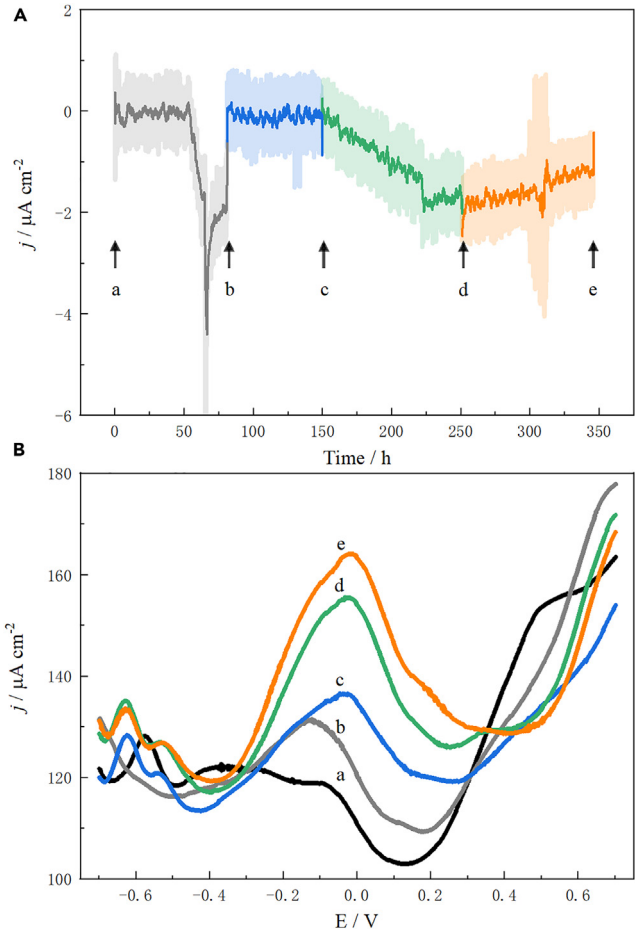


Figure 2. The electrochemical properties of MBD (with nitrate as electron acceptor, in a three-electrode system, −0.3 V, vs. SCE)
(A and B) Chronoamperometry. (B) DPV.

113.28 ± 0.19 mg/L, resulting in a total nitrogen (TN) removal efficiency of less than 21%. These findings suggested that under carbon limitation conditions, NO_3^--N was preferentially reduced before NO_2^--N, which corroborates observations in *Pseudomonas stutzeri* HK13.[34] As the C/N ratios increased—specifically at ratios of 4, 6, 8, 10, and 12—nitrogen removal efficiencies exceeded 98%. The results indicated that the most rapid consumption of NO_3^--N occurred at a C/N ratio of 4, signaling this ratio as optimal for denitrification of strain Lyy. Previous studies have demonstrated the significantly impact of C/N ratios on pollutant removal and by-product formation. Huang et al.[35] observed that increasing the C/N ratio enhanced nitrate removal in MFC while simultaneously inhibiting nitrite accumulation. These findings suggested that the denitrification capability of strain Lyy can be optimized with a lower optimal C/N ratio of 4, as opposed to other reported strains that typically require a C/N ratio of 8[34]. This characteristic could potentially reduce carbon source consumption and processing costs, making strain Lyy a promising candidate for applications in sustainable wastewater treatment.

Effect of initial pH on denitrification performance of strain Lyy

pH plays a significant role in influencing both the denitrification ability and cell growth. As illustrated in Figures 5B and S4B and outlined in Table S3, the optimal pH for denitrification by strain Lyy was determined to be 8. Under these conditions, a TN removal efficiency of 97.70 ± 0.16% was achieved within 36 h, with an average NO_3^--N removal rate of 2.27 ± 0.01 mg/L/h, which surpassing the rate observed for *P. tolaasii* Y-11 (1.99 mg/L/h).[36] Conversely, lower pH levels negatively impacted both the growth and denitrification capabilities of strain Lyy, consistent with findings from the previous studies.[34,37] Limited cell growth was noted at pH levels of 4, 5, and 6, where the TN removal efficiency fell below 16% over 60 h. In contrast, efficient denitrification by strain Lyy was observed within a pH range of 7∼9, in which TN removal efficiency consistently exceeded 93% within 60 h with no detectable nitrite accumulation. Alkaline growth conditions were beneficial for enhancing the denitrification performance of strain Lyy. However, increasing the pH to 9 resulted in a decrease in TN removal efficiency (93.28 ± 0.17%) and a lower $OD_{600\ nm}$ (0.11 ± 0.003), in comparison to pH 8, where the TN removal efficiency was 97.70 ± 0.16% and $OD_{600\ nm}$

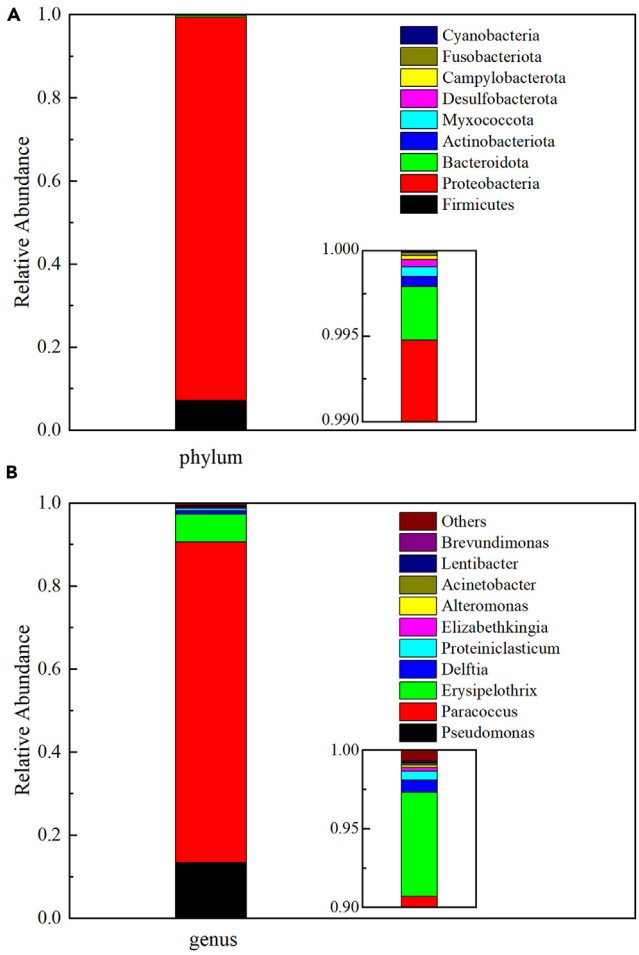


Figure 3. The species relative abundance of the top 10 microorganisms in MBD

(A and B) phylum level.

(B) genus level.

was 0.12 ± 0.01. These observations align with previous studies indicating that both nitrate removal and cell growth declined when the pH exceeds 8.6.[38]

Effect of incubation temperature on denitrification performance of strain Lyy

In Figures 5C and S4C and Table S3, it is evident that strain Lyy achieved its highest denitrification efficiency at a temperature of 35°C, with NO_3^--N and TN removal efficiency reaching 99.41 ± 0.04% and 99.34 ± 0.05% within 60 h, respectively. Notably, even at a lower temperature of 20°C, strain Lyy demonstrated notable NO_3^--N removal efficiency of 98.37 ± 0.17% within 84 h (Figure S5), demonstrating its ability to grow and denitrify effectively at lower temperatures. Conversely, at elevated temperatures, such as 45°C, both the NO_3^--N removal efficiency and cell growth of strain Lyy were significantly hindered due to the denaturation of enzyme structure. At 40°C, the $OD_{600\ nm}$ value of strain Lyy decreased to 0.05 ± 0.004, while the NO_3^--N removal efficiency remained high at 100%, the TN removal efficiency dropped to only 20.43% in 60 h, primarily due to the accumulation of NO_2^--N. These results indicated that nitrite reductase is more temperature-sensitive than nitrate reductase, with elevated temperature exerting a more pronounced effect on nitrite reductase activity, being consistent with previous findings.[39] Furthermore, strain Lyy exhibits a broader temperature range (20–40°C) for growth and denitrification compared to *P. stutzeri* XL-2 (25–35°C).[40]

Effect of NO_3^--N concentration on denitrification performance of strain Lyy

As illustrated in Figures 5D and S4D and Table S3, strain Lyy exhibited varying denitrification capabilities across a range of initial KNO_3 concentrations (500–3000 mg/L). The optimal denitrification and growth performance was observed at the KNO_3 concentration of 2000 mg/L, where the maximum $OD_{600\ nm}$ reached 0.16 ± 0.01, and the NO_3^--N and TN removal efficiency were 98.58 ± 0.03% and 98.47 ± 0.03%, respectively. The denitrification rate gradually increased with the increasing concentration of KNO_3 (500–3000 mg/L), with NO_3^--N removal

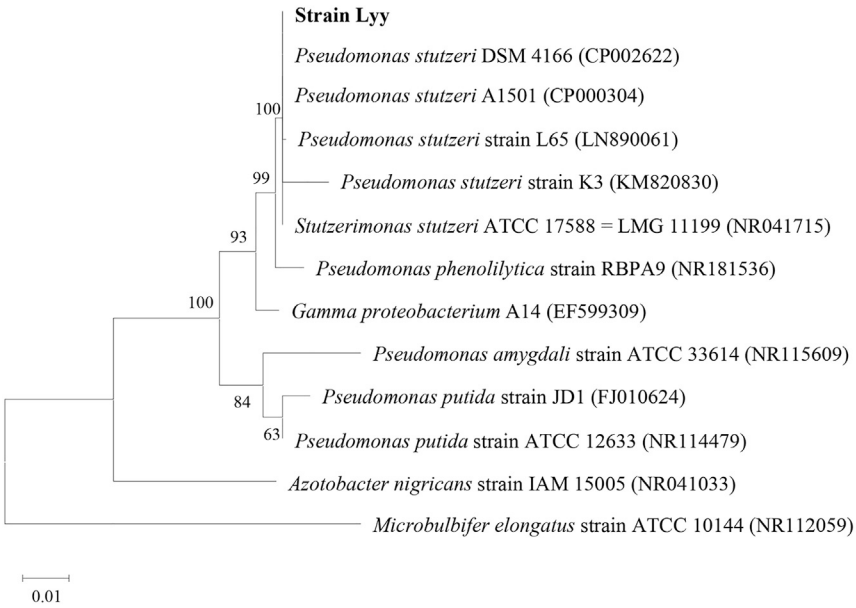


Figure 4. Phylogenetic trees of strain Lyy based on 16S rDNA sequences

efficiencies consistently exceeding 95%. Complete TN removal was achieved at concentrations below 2000 mg/L of KNO_3 within 60 h. However, in the concentration range of 2000∼3000 mg/L, preferential removal of NO_3^--N occurred due to its higher redox potential compared to NO_2^--N. This preferential removal led to concentration-dependent accumulation of NO_2^--N, further NO_3^--N and TN removal efficiency of 97.05 ± 0.04% and 53.09 ± 0.06%, respectively, at 3000 mg/L of KNO_3. These results suggested that strain Lyy can effectively perform denitrification across a broad of KNO_3 concentration range (500–3000 mg/L), while concentration inhibition did not significant impact the related performance, and higher concentration required longer reaction times.

Effect of dissolved oxygen on denitrification performance of strain Lyy

Table S3 illustrated the impact of dissolved oxygen on the denitrification process for strain Lyy. Under aerobic conditions, with a continuous oxygen supply, strain Lyy reached a maximum $OD_{600\ nm}$ of 0.50 ± 0.003. However, the removal efficiencies for NO_3^--N and TN were relatively low at 29.53 ± 0.05% and 22.42 ± 0.05%, respectively. In contrast, under anaerobic culture conditions, strain Lyy exhibited a lower maximum $OD_{600\ nm}$ of 0.07 ± 0.004, but significantly higher TN removal efficiencies of 98.53 ± 0.16% within 60 h, with no detectable NO_2^--N accumulation. The average TN removal rate, maximum TN removal rate and TN removal efficiency under anaerobic condition were notably higher—3.06, 3.26, and 3.34 times, respectively—compared to aerobic conditions. This indicates the superior denitrification performance of strain Lyy in anaerobic environments. The presence of dissolved oxygen facilitated cell growth but also consumed carbon sources as the primary electron acceptor, resulting in lower nitrogen removal efficiency.[34] These results indicated that strain Lyy is a facultative anaerobic denitrifier capable of thriving and denitrifying efficiently in both aerobic and anaerobic conditions, with a clear preference for denitrification under anaerobic setting.

Overall, the results indicated that strain Lyy demonstrated efficient denitrification ability, achieving a NO_3^--N removal efficiency over 93% across various conditions, including C/N ratio of 2∼12, pH levels of 7∼9, temperature ranging from 20°C to 40°C, initial KNO_3 concentration of 500∼3000 mg/L, and under anaerobic conditions with acetate as the carbon source. The optimal conditions for achieving complete denitrification (TN removal efficiency >98%) were identified as the C/N ratio of 4, pH of 8, temperature of 35°C, and KNO_3 concentration of 2000 mg/L. These findings indicated the significant potential of strain Lyy for highly efficient denitrification in wastewater treatment, particularly with low C/N ratios (2–6) and high-nitrate concentrations (KNO_3 500–3000 mg/L), making it a promising candidate for nitrogenous wastewater treatment.

Conclusion

In this study, an effective method of continuous replacement was used to enrich a mixed bio-cathode denitrifying culture (MBD) within a bio-cathode MFC. The MBD exhibited remarkable stability, maintaining a maximum output voltage of 120 ± 5 mV over 400 h under a load resistance of 1000 Ω. Furthermore, the MBD achieved a notable NO_3^--N removal efficiency of 69.99 ± 0.60% without requiring supplementary organic carbon sources. This finding suggests that electron shuttles likely play a crucial role in the electrotrophic denitrification process through the bio-cathodes. Microbial-community analysis revealed that *Proteobacteria* was the dominant phylum, with *Paracoccus* and *Pseudomonas* identified as the dominant genera within the MBD. Additionally, a facultative anaerobic denitrifier strain, named Lyy, was

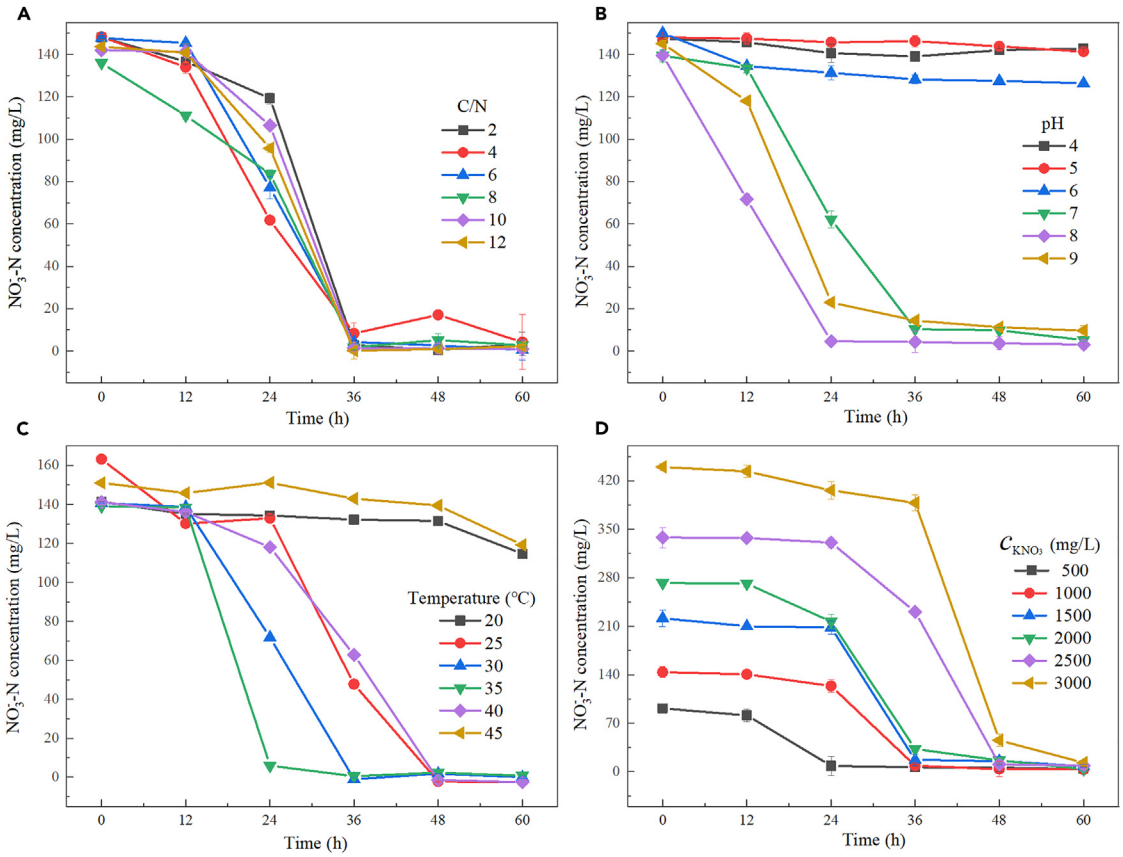


Figure 5. Effect of different conditions on the concentration changes of NO_3^--N (Values are means ± SD [error bars] for three replicates)
(A–D) different C/N, (B) different pH, (C) different temperature, and (D) different NO_3^--N concentration.

successfully isolated from the MBD and identified as *Stutzerimonas stutzeri* through 16S rRNA analysis. Strain Lyy exhibited impressive denitrification capabilities, achieving NO_3^--N removal efficiencies exceeding 93%, effectively treating wastewater with wide range of C/N ratios (2–12) and KNO_3 concentrations (500–3000 mg/L) in 60 h. These results indicated the efficiency and convenience of the enriching method employed using bio-cathode MFC system, demonstrating its promising application in sustainable wastewater treatment in the future.

Limitations of the study

While convenient methods have successfully facilitated the enrichment of bio-cathode denitrifying cultures, there remains a significant gap in our understanding of intra-community cooperation and reverse extracellular electron transfer. These interactions could provide insights into how microbial communities function synergistically in the bio-cathode system. Additionally, the bio-cathode system has considerable optimization potential that has yet to be fully realized. Future research should focus on exploring the effectiveness of bio-cathode systems in treating real wastewater over extended operational durations. Furthermore, integrating diverse functionalities in the anodes, such as the ability to degrade cellulose or remove phenolic compounds, is critical for enhancing treatment efficacy across various polluted environments. Ultimately, a deeper understanding of microbial interactions, along with targeted optimizations, could significantly advance the development and application of bio-cathode MFCs in sustainable wastewater treatment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ying Liu (liuy0512@hotmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This article does not report original code.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact on request. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ying Liu (liuy0512@hotmail.com).

ACKNOWLEDGMENTS

The research was funded by National Natural Science Foundation of China (no. 21375107) and Key Research and Development Program of Gansu Province (20YF3FA037) and Shaanxi Province (2020ZDLSF06-06).

AUTHOR CONTRIBUTIONS

R.L.: data curation, original draft, investigation, formal analysis, visualization. X.R.: data curation, original draft, investigation, formal analysis. X.F.: investigation. Z.Z.: investigation. T.G.: conceptualization, resources, funding acquisition. Y.L.: conceptualization, resources, review and editing, supervision, funding acquisition.

DECLARATION OF INTERESTS

The authors have no relevant financial interests in this article and no potential conflicts of interest to disclose.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Culture mediums and enrichment of MBD
  - System construction and operation
  - Isolation of single strain and 16S rRNA gene-based identification
  - Denitrification characteristics under different conditions
  - Analysis of nitrogen removal
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110965.

Received: June 11, 2024
Revised: August 27, 2024
Accepted: September 12, 2024
Published: September 19, 2024

REFERENCES

1. Pous, N., Puig, S., Dolors Balaguer, M., and Colprim, J. (2015). Cathode potential and anode electron donor evaluation for a suitable treatment of nitrate-contaminated groundwater in bioelectrochemical systems. Chem. Eng. J. *263*, 151–159. https://doi.org/10.1016/j.cej.2014.11.002.
2. Puig, S., Coma, M., Desloover, J., Boon, N., Colprim, J., and Balaguer, M.D. (2012). Autotrophic denitrification in microbial fuel cells treating low ionic strength waters. Environ. Sci. Technol. *46*, 2309–2315. https://doi.org/10.1021/es2030609.
3. Nguyen, H.D., and Babel, S. (2022). Insights on microbial fuel cells for sustainable biological nitrogen removal from wastewater: A review. Environ. Res. *204*, 112095. https://doi.org/10.1016/j.envres.2021.112095.
4. Sun, H., Xu, S., Zhuang, G., and Zhuang, X. (2016). Performance and recent improvement in microbial fuel cells for simultaneous carbon and nitrogen removal: A review. J. Environ. Sci. *39*, 242–248. https://doi.org/10.1016/j.jes.2015.12.006.
5. Zhou, E., Lekbach, Y., Gu, T., and Xu, D. (2022). Bioenergetics and extracellular electron transfer in microbial fuel cells and microbial corrosion. Curr. Opin. Electrochem. *31*, 100830. https://doi.org/10.1016/j.coelec.2021.100830.
6. Shi, K., Cheng, W., Jiang, Q., Xue, J., Qiao, Y., and Cheng, D. (2022). Insight of the bio-cathode biofilm construction in microbial electrolysis cell dealing with sulfate-containing wastewater. Bioresour. Technol. *361*, 127695. https://doi.org/10.1016/j.biortech.2022.127695.
7. Ding, A., Zhao, D., Ding, F., Du, S., Lu, H., Zhang, M., and Zheng, P. (2018). Effect of inocula on performance of bio-cathode denitrification and its microbial mechanism. Chem. Eng. J. *343*, 399–407. https://doi.org/10.1016/j.cej.2018.02.119.
8. Yu, J., Widyaningsih, E., Park, Y., and Lee, T. (2021). Nitrogen removal and microbial community diversity in single-chamber electroactive biofilm reactors with different ratios of the cathode surface area to reactor volume. Sci. Total Environ. *758*, 143677. https://doi.org/10.1016/j.scitotenv.2020.143677.
9. Liu, W., and Wu, Y. (2021). Simultaneous nitrification, denitrification and electricity recovery of *Halomonas* strains in single chamber microbial fuel cells for seawater sewage treatment. J. Environ. Chem. Eng. *9*, 106761. https://doi.org/10.1016/j.jece.2021.106761.
10. Wu, Y., Du, Q., Wan, Y., Zhao, Q., Li, N., and Wang, X. (2022). Autotrophic nitrate reduction to ammonium via reverse electron transfer in *Geobacter* dominated biofilm. Biosens. Bioelectron. *215*, 114578. https://doi.org/10.1016/j.bios.2022.114578.
11. Blanchet, E., Desmond, E., Erable, B., Bridier, A., Bouchez, T., and Bergel, A. (2015). Comparison of synthetic medium and wastewater used as dilution medium to design scalable microbial anodes: Application to food waste treatment. Bioresour. Technol. *185*, 106–115. https://doi.org/10.1016/j.biortech.2015.02.097.
12. Vilajeliu-Pons, A., Puig, S., Pous, N., Salcedo-Dávila, I., Bañeras, L., Balaguer, M.D., and Colprim, J. (2015). Microbiome characterization of MFCs used for the treatment of swine manure. J. Hazard Mater.

288, 60–68. https://doi.org/10.1016/j.jhazmat.2015.02.014.

13. Tong, Y., and He, Z. (2013). Nitrate removal from groundwater driven by electricity generation and heterotrophic denitrification in a bioelectrochemical system. J. Hazard Mater. *262*, 614–619. https://doi.org/10.1016/j.jhazmat.2013.09.008.
14. Zhang, Y., and Angelidaki, I. (2013). A new method for *in situ* nitrate removal from groundwater using submerged microbial desalination-denitrification cell (SMDDC). Water Res. *47*, 1827–1836. https://doi.org/10.1016/j.watres.2013.01.005.
15. Ren, Y., Lv, Y., Wang, Y., and Li, X. (2020). Effect of heterotrophic anodic denitrification on anolyte pH control and bioelectricity generation enhancement of bufferless microbial fuel cells. Chemosphere *257*, 127251. https://doi.org/10.1016/j.chemosphere.2020.127251.
16. Jin, X., Guo, F., Ma, W., Liu, Y., and Liu, H. (2019). Heterotrophic anodic denitrification improves carbon removal and electricity recovery efficiency in microbial fuel cells. Chem. Eng. J. *370*, 527–535. https://doi.org/10.1016/j.cej.2019.03.023.
17. Venkata Mohan, S., Velvizhi, G., Annie Modestra, J., and Srikanth, S. (2014). Microbial fuel cell: Critical factors regulating bio-catalyzed electrochemical process and recent advancements. Renew. Sustain. Energy Rev. *40*, 779–797. https://doi.org/10.1016/j.rser.2014.07.109.
18. Xiao, Z., Awata, T., Zhang, D., Zhang, C., Li, Z., and Katayama, A. (2016). Enhanced denitrification of *Pseudomonas stutzeri* by a bioelectrochemical system assisted with solid-phase humin. J. Biosci. Bioeng. *122*, 85–91. https://doi.org/10.1016/j.jbiosc.2015.11.004.
19. Lovley, D.R. (2011). Powering microbes with electricity: direct electron transfer from electrodes to microbes. Environ. Microbiol. Rep. *3*, 27–35. https://doi.org/10.1111/j.1758-2229.2010.00211.x.
20. Pous, N., Carmona-Martínez, A.A., Vilajeliu-Pons, A., Fiset, E., Bañeras, L., Trably, E., Balaguer, M.D., Colprim, J., Bernet, N., and Puig, S. (2016). Bidirectional microbial electron transfer: Switching an acetate oxidizing biofilm to nitrate reducing conditions. Biosens. Bioelectron. *75*, 352–358. https://doi.org/10.1016/j.bios.2015.08.035.
21. Tian, T., and Yu, H.Q. (2020). Denitrification with non-organic electron donor for treating low C/N ratio wastewaters. Bioresour. Technol. *299*, 122686. https://doi.org/10.1016/j.biortech.2019.122686.
22. Gomila, M., Mulet, M., García-Valdés, E., and Lalucat, J. (2022). Genome-Based Taxonomy of the *Genus Stutzerimonas* and Proposal of *S. frequens* sp. nov. and *S. degradans* sp. nov. and Emended Descriptions of *S. perfectomarina* and *S. chloritidismutans*. Microorganisms *10*, 1363. https://doi.org/10.3390/microorganisms10071363.
23. Chen, S., Jing, X., Yan, Y., Huang, S., Liu, X., Chen, P., and Zhou, S. (2021). Bioelectrochemical Fixation of Nitrogen to Extracellular Ammonium by *Pseudomonas stutzeri*. Appl. Environ. Microbiol. *87*, 5. https://doi.org/10.1128/AEM.01998-20.
24. Yamada, T., Kawaichi, S., Matsuyama, A., Yoshida, M., Matsushita, N., and Nakamura, R. (2016). Extracellular Electron Transfer of Pseudomonas stutzeri Driven by Lithotrophic and Mixotrophic Denitrification. Electrochemistry *84*, 312–314. https://doi.org/10.5796/electrochemistry.84.312.
25. Li, R., Gao, S.-C., Fan, X., Ma, Y.-M., Ren, X.-P., Gao, T.-P., and Liu, Y. (2024). Enhanced nitrate removal through autotrophic denitrification using microbial fuel cells via bidirectional extracellular electron transfer. Microchem. J. *204*, 111026. https://doi.org/10.1016/j.microc.2024.111026.
26. Yang, N., Liu, H., Zhan, G.Q., and Li, D.-P. (2020). Sustainable ammonia-contaminated wastewater treatment in heterotrophic nitrifying/denitrifying microbial fuel cell. J. Clean. Prod. *245*, 118923. https://doi.org/10.1016/j.jclepro.2019.118923.
27. Li, Z., Zhang, Q., Jiang, Q., Zhan, G., and Li, D. (2019). The enhancement of iron fuel cell on bio-cathode denitrification and its mechanism as well as the microbial community analysis of bio-cathode. Bioresour. Technol. *274*, 1–8. https://doi.org/10.1016/j.biortech.2018.11.070.
28. Qiu, B., Hu, Y., Tang, C., Chen, Y., and Cheng, J. (2021). Simultaneous mineralization of 2-anilinophenylacetate and denitrification by Ru/Fe modified biocathode double-chamber microbial fuel cell. Sci. Total Environ. *792*, 148446. https://doi.org/10.1016/j.scitotenv.2021.148446.
29. Jin, X., Yang, N., Liu, H., and Wang, S. (2022). Membrane penetration of nitrogen and its effects on nitrogen removal in dual-chambered microbial fuel cells. Chemosphere *297*, 134038. https://doi.org/10.1016/j.chemosphere.2022.134038.
30. Tang, M., Guo, Z., Xu, X., Sun, L., Wang, X., Yang, Y., and Chen, J. (2023). Performance and microbial mechanism of eletrotrophic bio-cathode denitrification under low temperature. J. Environ. Manag. *328*, 116960. https://doi.org/10.1016/j.jenvman.2022.116960.
31. Yuan, J., Yuan, H., Huang, S., Liu, L., Fu, F., Zhang, Y., Cheng, F., and Li, J. (2021). Comprehensive performance, bacterial community structure of single-chamber microbial fuel cell affected by COD/N ratio and physiological stratifications in cathode biofilm. Bioresour. Technol. *320*, 124416. https://doi.org/10.1016/j.biortech.2020.124416.
32. Peng, X., Wang, Z., Huang, J., Pittendrigh, B.R., Liu, S., Jia, X., and Wong, P.K. (2017). Efficient degradation of tetrabromobisphenol A by synergistic integration of Fe/Ni bimetallic catalysis and microbial acclimation. Water Res. *122*, 471–480. https://doi.org/10.1016/j.watres.2017.06.019.
33. Miqueleto, A.P., Dolosic, C.C., Pozzi, E., Foresti, E., and Zaiat, M. (2010). Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment. Bioresour. Technol. *101*, 1324–1330. https://doi.org/10.1016/j.biortech.2009.09.026.
34. Ding, L., Han, B., and Zhou, J. (2022). Characterization of the facultative anaerobic *Pseudomonas stutzeri* strain HK13 to achieve efficient nitrate and nitrite removal. Process Biochem. *118*, 236–242. https://doi.org/10.1016/j.procbio.2022.04.021.
35. Huang, B., Feng, H., Wang, M., Li, N., Cong, Y., and Shen, D. (2013). The effect of C/N ratio on nitrogen removal in a bioelectrochemical system. Bioresour. Technol. *132*, 91–98. https://doi.org/10.1016/j.biortech.2012.12.192.
36. Palmer, K., Biasi, C., and Horn, M.A. (2012). Contrasting denitrifier communities relate to contrasting N2O emission patterns from acidic peat soils in arctic tundra. ISME J. *6*, 1058–1077. https://doi.org/10.1038/ismej.2011.172.
37. Li, Y., Williams, I., Xu, Z., Li, B., and Li, B. (2016). Energy-positive nitrogen removal using the integrated short-cut nitrification and autotrophic denitrification microbial fuel cells (MFCs). Appl. Energy *163*, 352–360. https://doi.org/10.1016/j.apenergy.2015.11.021.
38. Pei, H., Ji, Y., Hu, W., Meng, P., and Shao, Y. (2014). Denitrifying characterization and identification of a novel soil bacterium XP-2. Desalination Water Treat. *52*, 6996–7003. https://doi.org/10.1080/19443994.2013.822329.
39. Maag, M., and Vinther, F.P. (1996). Nitrous oxide emission by nitrification and denitrification in different soil types and at different soil moisture contents and temperatures. Appl. Soil Ecol. *4*, 5–14. https://doi.org/10.1016/0929-1393(96)00106-0.
40. Zhao, B., Cheng, D.Y., Tan, P., An, Q., and Guo, J.S. (2018). Characterization of an aerobic denitrifier Pseudomonas stutzeri strain XL-2 to achieve efficient nitrate removal. Bioresour. Technol. *250*, 564–573. https://doi.org/10.1016/j.biortech.2017.11.038.
41. Liu, Y., Kim, H., Franklin, R.R., and Bond, D.R. (2011). Linking Spectral and Electrochemical Analysis to Monitor c-type Cytochrome Redox Status in Living Geobacter sulfurreducens Biofilms. ChemPhysChem *12*, 2235–2241. https://doi.org/10.1002/cphc.201100246.
42. Cao, L., Sun, H., Ma, Y., Lu, M., Zhao, M., Li, E., and Liu, Y. (2023). Analysis and enhancement of the energy utilization efficiency of corn stover using strain Lsc-8 in a bioelectrochemical system. Microb. Cell Fact. *22*, 54. https://doi.org/10.1186/s12934-023-02058-6.
43. Cao, L., Ma, Y., Deng, D., Jiang, H., Wang, J., and Liu, Y. (2020). Electricity production of microbial fuel cells by degrading cellulose coupling with Cr(VI) removal. J. Hazard Mater. *391*, 122184. https://doi.org/10.1016/j.jhazmat.2020.122184.
44. Guo, J., Cheng, J., Li, B., Wang, J., and Chu, P. (2019). Performance and microbial community in the biocathode of microbial fuel cells under different dissolved oxygen concentrations. J. Electroanal. Chem. *833*, 433–440. https://doi.org/10.1016/j.jelechem.2018.12.015.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Strain Lyy	This paper	–
G. sulfurreducens PCA	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	GenBank: GCA_000007985.2
Chemicals, peptides, and recombinant proteins		
Carbon felt	Beijing Sanye Carbon	–
Carbon sheets	Beijing Sanye Carbon	–
Saturated calomel reference electrode	Shanghai Chenhua Instrument	CHI 150
Potassium nitrate (KNO_3)	Guangdong Guanghua Technology	CAS: 7757-79-1
Sodium bicarbonate ($NaHCO_3$)	Sinopharm Chemical Reagent	CAS: 144-55-8
Proton membrane	DuPont	Nafion™ N117
Sodium acetate (CH_3COONa)	Guangdong Guanghua Technology	CAS: 127-09-3
Critical commercial assays		
16S rDNA sequencing	Qingke Biotech	https://www.tsingke.com.cn/
Deposited data		
KEITHLEY 2700	Saifan Optoelectronic Instrument	www.tek.com.cn/
Software and algorithms		
Mega 11	Molecular Evolutionary Genetics Analysis	www.megasoftware.net/
Origin 2019b	Originlab	https://www.originlab.com/
Other		
Anaerobic operating box LAI-3-T	Longyue Instrument Equipment	https://longyuesh.com/
Cary 60 UV-Vis	Agilent Technologies Inc.	www.agilent.com.cn/
VMP-3 Multichannel Potentiostat	Bio-Logic Science Instruments	https://www.biologic.net/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study does not use experimental methods typical in the life sciences.

METHOD DETAILS

Culture mediums and enrichment of MBD

The initial inoculum was activated sludge obtained from the anoxic tank of a coking wastewater plant in Shanxi Province, China. The sludge (1 mL) was then transferred into 20 mL serum vials containing denitrification medium (DM) and incubated at 37°C in the incubator for 60 h. The DM medium used for enrichment and denitrification contained: 0.6 g/L KH_2PO_4, 1.64 g/L CH_3COONa, 1 g/L KNO_3, 1 g/L $NaHCO_3$, 0.1 g/L KCl, 0.1 g/L $CaCl_2$, 0.2 g/L $MgSO_4$, 5 mL vitamin solution (Table S1) and trace mineral solution (Table S2), respectively.[25,41]

Following three rounds of directional cultivations with acetate and KNO_3, 10 mL of the enriched culture was inoculated into the bio-cathode MFC, the medium was replaced whenever the voltage dropped below 20 mV until stabilization to further enrich the bio-cathode denitrification culture. The cathode denitrification medium (DECA) used in the MFC structure contained[25]: 6 g/L KH_2PO_4, 3 g/L Na_2HPO_4, 0.5 g/L NaCl, 1 g/L KNO_3, 1 g/L $NaHCO_3$, 0.1 g/L KCl, 0.1 g/L $CaCl_2$, 0.2 g/L $MgSO_4$ and 5 mL vitamin solution (Table S1). The anodic inoculum, *G. sulfurreducens* PCA, was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The growth NB medium for *G. sulfurreducens* PCA contained: 1.5 g/L NH_4Cl, 0.6 g/L KCl, 0.1 g/L $MgCl_2$, 0.3 g/L KH_2PO_4, 0.1 g/L $CaCl_2$, 5 mL vitamin solution (Table S1) and trace mineral solution (Table S2), as the previously reported,[42] with 1.64 g/L acetate and 4.64 g/L fumaric acid as the electron donor and acceptor, respectively. The cultures were maintained anaerobically at 37 ± 1°C after sparging with N_2 for 20 min and adjusting to pH 7.2. Concentrations of NO_3^--N and NO_2^--N were measured every 12 h to evaluate the denitrification ability of the mixed culture. After more than five batches, the MBD with power generation and denitrification capabilities was obtained. DNA extraction was performed using small

sections of cathode carbon felt upon reaching the maximum output voltage of MFC. Subsequently, the microbial community was characterized using 16S rDNA gene amplification sequencing technology (Tsingke Biotech, China).

System construction and operation

A dual-chamber MFC with a volume of 120 mL was constructed as described in previously reported.[25,42] A proton exchange membrane (Nafion 117; DuPont, USA) was used to separate the two chambers. Carbon rod (geometric area of 3.93 cm^2) and carbon felt (effective area of 10.5 cm^2) connected to a copper wire was used as the anode and cathode electrode, respectively. The anode chamber was filled with NBA medium (NB medium with 1.64 g/L acetate as electron donor) and inoculated with 10 mL of *G. sulfurreducens* PCA culture, while the cathode chamber was filled with 100 mL DECA medium and inoculated with 10 mL of the enriched culture. The output voltages of MFC were recorded using a Keithley instrument (model 2400) with a 1000 Ω resistance connected in the circuit.

A half-cell experiment using a three-electrode system (−0.3V vs. SCE) was performed on a 16-channel potentiostat (VMP-3, Bio-Logic Science Instruments, France) to investigated the electrotrophic denitrification performance of MBD.[25] The graphite plate (geometric area of 2.2 cm^2) was used as the work and counter electrode, respectively. A saturated calomel reference electrode (SCE, 0.244 V vs. SHE) was used to measure the potential. Differential pulse voltammetry (DPV) was carried out at a scan rate of 4 $mV \cdot s^{-1}$ from −0.7 V to 0.7 V. All experiments were performed anaerobically at 30 ± 1°C unless otherwise stated.

Isolation of single strain and 16S rRNA gene-based identification

Serial dilutions (10°, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of the MBD were anaerobic inoculated onto solid DM medium (1.2% agar) with 1.64 g/L acetate and 1.50 g/L KNO_3 at 37 ± 1°C.[34,43] Then single colonies were selected and analyzed multiple times to ensure the purity of the isolated strain. The isolated strain was then inoculated into DM medium and incubated at 37°C for 60 h to evaluate its denitrification capabilities.[34] For further characterization, a single colony was suspended in 10 μL of deionized water for PCR analysis. The amplification reaction was conducted using the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′).[43] The amplified 16S rDNA sequence was compared with existing sequences in the NCBI database.

Denitrification characteristics under different conditions

The denitrification characteristics of the isolated single strain were investigated under various culture conditions, including C/N ratio, pH, DO, and temperature. The C/N ratios were adjusted to 2, 4, 6, 8, 10, and 12 by different acetate feeding, respectively. The temperatures of the incubator were adjusted to 20, 25, 30, 35, 40, and 45°C. The initial pHs were respectively adjusted to 4, 5, 6, 7, 8 and 9; The KNO_3 concentration were respectively adjusted to 500, 1000, 1500, 2000, 2500, and 3000 mg/L.

All the above experiments were conducted triplicate in serum vials with 50 mL of DM medium and 5% (v/v) inoculation (with non-inoculation medium as controls), and all the incubated medium with a constant NO_3^--N concentration was cultivated at 35°C pH 7.2 for 60 h unless specified. Samples were periodically obtained to measure the $OD_{600\ nm}$, NO_3^--N, NO_2^--N, and total nitrogen (TN) removal.

Analysis of nitrogen removal

Sampled were taken periodically and centrifuged at 12,000 rpm with a 0.22 μm membrane filtration. NO_3^--N, and NO_2^--N concentrations were determined by the ultraviolet spectrophotometry and the diazotization-coupling reaction method[44] using a spectrophotometer (Cary 60 UV-Vis, Agilent, United States) respectively. Total nitrogen (TN) was defined as the sum of NO_3^--N and NO_2^--N. NO_3^--N removal efficiency (η,%) and TN removal rate (v, mg/L/h) were calculated as follows[34]:

$$\eta = (C_0 - C_t) / C_0 \times 100\% \quad \text{(Equation 1)}$$

$$v = (C_0 - C_t) / t \quad \text{(Equation 2)}$$

where t is the total time for the incubation (h), C_0 is the initial concentration (mg/L), and C_t is the final concentration at time t.

QUANTIFICATION AND STATISTICAL ANALYSIS

The output voltages of MFC in Figure 1A were recorded using a Keithley instrument (model 2400), the data of chronoamperometry in Figure 2A and DPV in Figure 2B were measured by a 16-channel potentiostat (VMP-3, Bio-Logic Science Instruments, France). Statistics of electrochemical properties in Figures 1A and 2, denitrification performance in Figures 1B and 5, and species relative abundance in Figure 3 were performed using Origin 2019b. Statistics of sequencing in Figure 4 were performed using Mega 11 via the Maximum Likelihood method. All data of denitrification performance were presented as the mean ± standard deviation.